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# STRATEGIC IMPROVEMENTS IN EFFICACY OF DNA DAMAGING CHEMOTHERAPEUTIC DRUGS THROUGH MODULATION OF SURVIVAL FACTORS.

VOLUME I

By

Vasudha Sharma

A DISSERTATION

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## ABSTRACT

STRATEGIC IMPROVEMENTS IN EFFICACY OF DNA DAMAGING CHEMOTHERAPEUTIC DRUGS THROUGH MODULATION OF SURVIVAL FACTORS.

By

Vasudha Sharma

This dissertation describes the development of various strategies that may be utilized to improve the efficacy of DNA damaging chemotherapeutic drugs *via* modulation of survival proteins. Unlike an ideal situation, where normal cells should undergo minimal "collateral damage", in most clinical scenarios, the effectiveness of chemotherapy suffers a range of complex factors including systemic toxicity due to a lack of specificity, rapid drug metabolism, and both intrinsic and acquired drug resistance resulting from activation of pro-survival factors such as NF-κB.

Antitumor drugs exert their antitumor activity *via* the initiation of apoptotic signaling pathways ultimately resulting in cell death. Apoptosis or programmed cell death is an ordered cellular mechanism used to regulate and control cell number and eliminate damaged or mutated cells. Modification of this normal process might result in the interference of the delicate balance between cell proliferation and cell death and lead to a variety of diseases. Chapter I describes the various problems and perspectives linked to cancer and current day chemotherapy and also suggests possible ways to make the cancer treatment more effective.

Conventional chemotherapy suffers a compromised clinical efficacy due to the induction of anti-apoptotic factors, such as NF-κB, activation of DNA repair etc. concurrent with the desired induction of programmed cell death. Inhibition of such anti-apoptotic survival

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factors might retards the cellular resistance induced by chemotherapeutic agents. Chapter II describes the use of 8-substituted theophyllines and caffeines in modulation of such survival or repair pathways upon camptothecin treatment. Chapter III describes the use of *trans*-imidazolines in modulation of NF-κB *via* inhibition of degradation of IκB-α resulting in enhancement of apoptosis in CEM leukemia cells. Studies revealed that only the apoptotic response induced by DNA damaging agents could be enhanced by the presence of imidazolines. No effect was seen for anti-tubulin agents such as Taxol. Mechanistic studies involving various time and concentration dependent effects and potentiation as a function of activation of NF-κB, effect of enantiomers of *trans*-imidazolines etc. have been described using a variety of assays.

The generation of diverse small molecular scaffolds with potential biological activity is an ongoing theme in our group. Previously, our lab described a mild method using lewis acids for highly stereoselective 1, 3 dipolar cycloadditions on münchnones, with different dipolarophiles. We describe in chapter IV, the substrate controlled reversal of diastereoselectivity to provide stereochemically diverse structures-- *cis*-imidazolines. These might be important in inhibition of p53-MDM2 binding, another survival event in most tumor cells. Further, chapter V describes a skeletally diverse indoleazepine, its synthesis and its biological effects in inhibition of cytokine production and specific inhibition of checkpoint chk2. These might find use in reducing side effects in normal cells. Finally, chapter VI describes a prodrug methology to selectively target tumor cells owing to their reducing environment.

To my
years
ho

To my grandma, Late Mrs. Urmila Sharma who died of cancer on 7th July 2005. In her 3 years long battle with cancer, paralleling my time in graduate school, she showed us all how to live with pain and not miss out on living each day, with love and giving.

To my loving family, for giving me the model of life—it is a beautiful one!

To Mahesh

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This dissertation would be incomplete without the mention of all those who contributed to my successful graduate career. Jetze has been an enthusiastic, sometimes "over-enthusiastic" advisor to work with. Since I am his first graduate student, both Jetze and I have gone through times of learning and teaching. Affirmations are a simple, powerful, free way to maintain/improve morale. During my first summer, I quote him, "I love your love for work" laid the foundation of the confidence he had in me. In my initial years, it helped me achieve goals that might have seemed out of reach at that point of time. Whether I was lucky for his career or he for mine is a topic that is feverishly discussed from time to time. Time probably will be a better judge.

I would like to thank Profs. Borhan, Wulff, Weliky and Blanchard for serving on my committee and their advice on several occasions. In fact, Babak (Dr. Borhan) has been a mentor and a friend in a true sense for his ability to listen. Sometimes, just the fact that you are being heard helps, and people who have been through graduate school would agree there is no count to such times. I admire his ability to be able to ask questions ranging from specific to the more philosophical ones. I have great respect for Prof. Wulff, especially his composure, wisdom, perspective and how he deals with people. Both Babak and Prof. Wulff gave me a perspective when I needed it the most. While Prof. Joan Broderick could not be a part of my final defense due to her recent move to Montana State, she was extremely kind and understanding throughout my graduate career. My association with Prof. Weliky and Blanchard as committee members was brief, but I found both of them very accommodating and understanding. I want to thank

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the graduate school for a dissertation completion fellowship. I also want to thank Prof. Maleczka for help with different things at different times and mainly for CEM 852.

All the past and present group members have been friendly. Kevin, Jin, Manasi and Jayakumar were good work colleagues. I enjoyed their company working late hours in the lab. They kept me in good humor through good and bad times.

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## KEY TO SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| ADMET | Administration, distribution, metabolism, elimination, toxicity |
| ALLN | Acetyl-L-leucinyl-L-leucinyl-L-norleucinal |
| Anhyd. | Anhydrous |
| ASM | Airway Smooth Muscle |
| ATP | Adenosine Tri Phosphate |
| ATR | Ataxia-telangiectasia-mutated |
| ATM | ATM-Rad3-related |
| BRCA1 | Breast Cancer 1 |
| CC95 | CYtotoxic Concentration at which 95% cell population dies |
| CDDP | *cis*- Diamminodichloro Platinum(II) (Cis-Platin) |
| CDK | Cyclin Dependent Kinase |
| CDC26 | Cell division cycle 26 |
| Chk1 | Checkpoint kinase 1 |
| Chk2 | Checkpoint kinase 2 |
| CK1 | Casein Kinase 1 (CSNA1K1) |
| CML | Chronic myelogenous leukemia |
| COX-2 | Cyclooxygenase-2 |
| CPT /CPT-11 | Camptothecin/Irinotecan |
| CSA | camphor sulfonic acid |
| dFdCTP | 2',2'-difluoro-2'-deoxycytidine |
| DNA-PK | DNA dependent-Protein Kinase |
| DCC | Dicyclohexylcarbodiimide |
| DMAP | 4-(dimethylamino)pyridine |
| DCM | Dichloromethane |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxy ribo nucleic acid |
| DOS | Diversity oriented synthesis |
| DTIC | Dimethyl triazeno imidazolecarboxamide (dacarbazine) |
| DTT | Dithiothreitol |
| $\delta$ | chemical shift (parts per million) |
| EDCI | Ethyl dimethylaminopropyl carbodiimide |
| EDTA | Ethylene Diamine Tetraacetic acid |
| EGFR | epidermal growth factor receptor |
| EMSA | Electro Mobility Shift Assay (gel shift assay) |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EIA | Enzyme Immuno Assay |
| eq. | Equivalent |
| EtOAc | Ethyl Acetate |
| FAB | Fast Atom Bombardment |
| FACS | Fluorescence aided cell sorter |
| g | gram |
| $GI_{50}$ | Growth Inhibition by 50% |
| GSK-3$\beta$ | Glycogen synthase kinase-3$\beta$ |

| | |
|---|---|
| GPCR | G-Protein Coupled Receptor |
| HEPES | 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid |
| HIF | Hypoxia Inducible factor |
| HTS | High throughput screen |
| HRMS | High resolution mass spectrometry |
| Hsp | Heat Shock Protein |
| h | hour |
| $IC_{50}$ | Inhibitory concentration for 50% inhibition of activity probed |
| IR | Infra Red |
| I-κB | Inhibitor of κB |
| IKK | I-κB kinase |
| IL-8 | Interleukin-8 (cytokine) |
| *J* | coupling constant |
| KHMDS | Potassium HexamethylDisilazamide |
| LAH | Lithium aluminium hydride |
| LPS | Lipopolysaccharaide |
| M | Molar(concentration) |
| MHz | Mega Hertz |
| *m/z* | mass to charge ratio |
| mA | milli Amperes |
| MAPK | Mitogen-Activated Protein Kinase |
| MDM2 | Murine Double Minute2 |
| MDR | Multi-Drug Resistance |
| MGMT | *O*6-methyl guanine-DNA methyl transferase |
| MEKK-1 | MAPKinase Kinase |
| MMP | Matric metalloproteinase |
| MS | Mass Spectrometry |
| nM | nano molar |
| NBS | *N*-bromosuccinamide |
| NMR | nuclear magnetic resonance |
| NF-κB | Nuclear factor-κB |
| NSAIDs | Non-Steroidal Anti-Inflammatory Drugs |
| PCR | Polymerase chain reaction |
| PDTC | pyrrolinedithiocarbamate |
| PDE | Phosphodiesterase |
| P-gp | P-glycoprotein |
| PI-3K | PhosphoInositol 3-kinase |
| PMA | Phorbol Myristal Acetate |
| PHA | Phytohemagglutinin. |
| PPA | Pyrophosphoric acid |
| Proteasome | multi-catalytic protease unit in cells |
| Rb | Retinoblastoma protein |
| RNA | Ribonucleic acid |
| *R* | rectus(Cahn-Ingold-Prelog system) |
| *S* | sinister(Cahn-Ingold-Prelog system) |
| SEMCl | 2-(trimethylsilylethoxy)methyl chloride |

| | |
|---|---|
| SMPPI | Small molecule inhibitors of protein-protein interactions |
| ST | Sulphotransferases |
| TEA | Triethyl amine |
| TMSCl | Trimethylsilyl chloride |
| TNF-α | Tumor Necrosis Factor-α |
| μM | micro molar |
| V | Volts |

# CHAPTER I

## PROBLEMS, PERSPECTIVES AND PROMISES IN CURRENT CANCER CHEMOTHERAPY: FINDING ANSWERS TO QUESTIONS PERTAINING LIFE OR CELL DEATH??

*The cell, over the billions of years of her life, has covered the earth many times with her substance, found ways to control herself and her environment, and insure her survival. It is this cell which plans and composes all organisms, and which transmits to them its defects and potentialities.....Albert Claude*

### A. Introduction

Apoptosis or programmed cell death is a cellular mechanism used to regulate cell number and eliminate damaged or mutated cells. Such an ordered control mechanism is important for organ formation during development and overall homeostasis in multi-cellular organisms. It is an important biological process to eliminate unwanted cells with potentially harmful genomic mutations or cells that did not develop normally. Modification of this normal process might result in the interference of the delicate balance between cell proliferation and cell death and lead to a variety of diseases. [1, 2] Just recently understood, this process has intrinsic weaknesses, which when compromised, often lead to inappropriate cell death and disease. Most cancers, through various mechanistic strategies manifest capabilities to cause alterations in cell growth and physiology leading to a malignant growth: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, unlimited potential to replicate, sustained angiogenesis, and tissue invasion and metastasis.[3] Ideally, chemotherapeutic drugs should specifically target only tumor cells and should decrease tumor burden by inducing

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cytotoxic and/or cytostatic effects with minimal "collateral damage" to normal cells. However, in reality, the effectiveness of chemotherapy suffers a range of complex factors including systemic toxicity due to a lack of specificity, rapid drug metabolism, and both intrinsic and acquired drug resistance.

**i) DOUBLE JEOPARDY: Why are cancer cells inherently resistant to the chemotherapeutic drugs?** Most conventional anti-cancer agents cause cell damage directly or indirectly by damaging the DNA. Cell death in response to DNA damage has mostly been shown to occur via apoptotic pathways. When the anti-cancer agents have a therapeutic response, they often show selection of pre-malignant cells by *eliminating* the drug-sensitive cells. Hence, on one hand, therapy –induced killing of cancer cells might lead to a therapeutic response; preferential killing of drug-sensitive cells often selects for resistance and might lead to tumor progression.[4]

Resistance to cancer therapeutic agents could be non-oncogenic or oncogenic. While the former pertains to the drug-metabolism, transport or mutation of targets causing prevention or repair of the target. For example, the *O*6-methyl guanine-DNA methyl transferase MGMT repairs the alkylation caused on DNA by DNA alkylating agents.[5] Over expression of MGMT prevents carcinogenesis[6]. The latter refers to the alterations in cell cycle or apoptosis causing pathway (e.g. overexpression of oncoproteins ErbB and lacking tumor suppressors such as p53 and Rb protein might cause the cancer cells to 'ignore' the damage).

Multi-drug resistance (MDR) has been associated with the *in vivo* and *in vitro* over expression of *mdr1* gene product, P-glycoprotein, which confers resistance to a variety of lipophilic drugs including vinca alkaloids, daunamycin etc. This sets up an efflux pump

to decrease the intracellular concentration of the drug thus leading to resistance to a particular drug. However, activation of P-glycoprotein mediated efflux may be just one of the possible mechanisms that induce resistance in tumor cells.[7]

Cytotoxicity of chemotherapeutic drugs is dependent on the *in vivo* availability of drugs and their metabolites. Changes in cellular protein levels employed in detoxification processes as well as those employed in activation of chemotherapeutic drugs predictably may allow an escape for tumor cells and select for a new type of adaptive resistance. For e.g. anti-tumor agent, mitomycin C requires metabolite activation by reductases such as DT diaphorase and cytochrome p450 reductase; however, the chemosensitivity to the drug depends on the levels of the enzyme in the cell.[8]

Since oncogenes drive important biological processes such as cell division and growth, their over expression might lead to alterations in the maintenance of the delicate balance between cell growth and cell death. By virtue of these properties, oncogenes or their products become prime candidates for inherent or acquired resistance to anti-tumor therapy.

**ii) Sensitizing cancer cells to anti-tumor therapy**

One of the major changes in cancer cells that limit the activity of a drug is the initiation of anti-apoptotic pathways (Figure I-1) in tumor cells either through mutation or down-regulation of a pro-apoptosis protein such as p53[9] or up-regulation of anti-apoptosis proteins such as NF-κB, [10] which regulates apoptosis or MDM2 (murine double minute), [11] a natural inhibitor of p53.

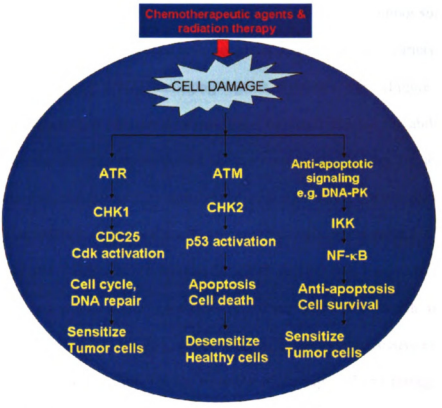


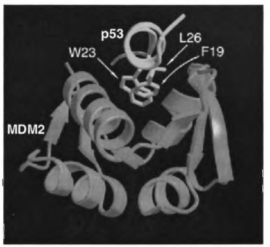

**Figure I-1.** Activation of various pathways involved in cell cycle, survival and apoptosis upon exposure to chemotherapy or radiation therapy.

**B. p53: the guardian of the genome.**

The p53 tumor suppressor protein plays a key role in the cell's response to stress. This stress can lead to loss of the p53 function as a 'guardian of the genome'. Loss of p53 activity occurs in more than 50% of the cancer types.[9] While it is of utmost importance to understand the etiology of malignancy to reduce cancer incidence, elucidation through studies involving gene damage can be particularly challenging. Mutations in p53, which arise both due to exogenous carcinogens and endogenous biological processes, have provided some clues about the nature of genetic alterations. The balance between mutations caused by the exogenous and endogenous factors may vary for different cancers of the same population or vice versa. A disruption in the activity of p53 occurs either by a lesion to prevent activation of p53, a mutation of the p53 gene or via

inhibition of the downstream mediators of its function. p53 acts as a tumor suppressor by inducing growth arrest, cell senescence or apoptosis in response to a variety of cellular stress stimuli including DNA damaging agents, hypoxia etc. (Figure I-1), thus maintaining the control of cell proliferation. Since activated p53 has the ability to cause arrest or apoptosis, the cells have evolved various mechanisms to keep the levels of p53 low under normal conditions. The stability of p53 primarily depends on its physical interaction with MDM2, which inhibits the transactivational activity of p53.[12] This causes degradation of p53.[13] MDM2 is amplified in ~ 40% of sarcomas leading to inactivation of p53. Activated p53 leads to induction of mdm2 expression which inhibits p53 stabilization thus setting up an auto-regulatory loop that prevents excessive expression of p53 under unstressed conditions. Hence, targeting reactivation of p53 through inhibition of MDM2 via disruption of p53-MDM2 binding may cause significant anti-cancer effects. In the past, several studies including microinjection of anti-bodies to the p53 binding site, [14] inhibition of MDM2 using anti-sense oligonucleotides,[15a] etc. have been used to clearly illustrate that disruption of the p53 regulatory circuit, leads to activation of p53 pathway. Ideally, from a clinical perspective, small drug –like molecules that selectively bind MDM2 at the p53 pocket without physically affecting p53 or its activity are most desirable. For a long time, disruption of protein-protein interactions was thought to be a difficult task for therapeutic intervention. This is a result of the large and flat interacting faces between the participating proteins. As an exception to this idea, the crystal structure of MDM2 bound to a peptide from the transactivation domain of p53 has revealed that MDM2 possesses a relatively deep hydrophobic pocket that is filled primarily by three side chains from the helical region of the peptide.[15b] This well-defined

pocket on the MDM2 highlights the fact that small molecules might block the interaction of MDM2 with p53. There have been a few successful approaches that have targeted the surfaces of one or both the partners of the complex.[16]



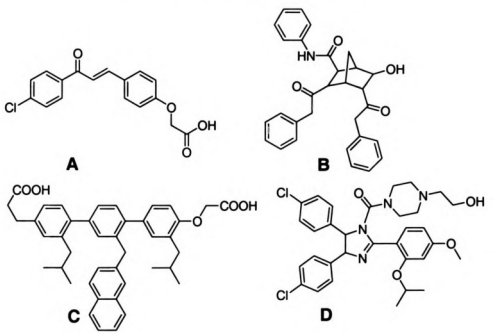




**Figure I-2A (Top).** p53-MDM2 binding pocket. **2B (Bottom).** Inhibitors of p53-MDM2 interaction. A: Chalcone derivative B-1[17] B:Syc-7[18] C: Terphenyl derivative [19] D: Nutlin-3[22]

The potency of the chalcone derivative B-1 and Syc-7 (Figure I-2, A and B) in inhibiting the MDM2-p53 binding is low in the micromolar range.[17] Recently, Hamilton and

coworkers reported the synthesis of a terphenyl scaffold (C, Figure I-2), which proved to be a potent MDM2 inhibitor.[19] Another natural product, chlorofusin a fungal metabolite is an inhibitor of p53-MDM2 interaction with and $IC_{50}$ of 5$\mu$M, though the exact binding site for it is not known.[20] Appella and co-workers showed a series of α-aminoketones which reactivated p53 in SaOS-2 cells (a mutant cell line R175H, R281G) and induced selective cellular cytotoxicity.[21] While Appella *et al* suggest a formal possibility of selectively targeting cells expressing mutant p53, they did not see restoration of wild type properties of p53, such as the expression of its endogenous target HDM2 (human double minute).

Developed by Hoffman LaRoche, nutlins, are *cis*-imidazolines that are potent inhibitors of MDM2 in the nanomolar range.[22] These molecules interact with MDM2, mimicking the interaction of three p53 amino acid residues within the hydrophobic cavity of MDM2. In cell culture, these imidazolines activated the p53 pathway and transcription of its genes (p21, MDM2). Further, the nutlins did not effect the phosphorylation of Ser15 of p53, which is normally seen in genotoxic stress, indicating that the activity of these molecules is very specific.

The activity of p53 doesn't just involve its activation and accumulation but also post-translational modification[23] in response to various stimuli, which can direct its functional role in cells.[24] It has been accepted widely that tumor types that have a wild type p53 usually suffer aberrations in the p53 pathway (such as high levels of MDM2), which can partially or completely abrogate the tumor suppressor activity of p53.[25] Tumors with wild type p53 and high levels of MDM2 are expected to benefit the most from therapy targeting the p53-MDM2 interactions, since inhibition of this interaction would imply

restoration of wild type characteristics of p53. Consistent with these predictions, nutlins were found to cause cytotoxicity in SJSA-1 osteosarcoma cells which have an amplified *mdm2* gene.[9] However, the question pertaining the number of human tumors under this category (wild type p53 and high levels of MDM2) in a clinical setting remains unanswered. Since nutlins do not directly affect the p53, the biological pathway for activation and transcription of p53 can be dissected both upstream and downstream in a variety of cell lines and conclusions for the biological activity of nutlins can be drawn based on their p53 status. Although MDM2 antagonists such as nutlins do not cause DNA damage, yet they activate the p53 pathway. This is particularly considered advantageous over traditional chemotherapy since it would relieve the patients of genotoxic stress.

**C. Repair mechanisms: Checkpoints.**

One of the important functions of p53 after it gets activated is to impose G1/S and G2/M checkpoints in the cell cycle.[26] Eukaryotic cells undergo continuous assault by environmental agents (such as UV light and reactive chemicals) and normal metabolic byproducts (such as reactive oxygen intermediates). Irrespective of the source, genetic damage threatens cell survival. The inability to carry out repairs and/or failure in maintaining this balance often leads to the transformation of normal cells into malignancies and other pathological complications. [27] Cells respond to DNA damage by activation of signaling pathways that control DNA repair and cell cycle delays.[32d] To ensure that cells pass accurate copies of their genomes onto their progeny, cell-cycle machinery is monitored by certain phyllogenetically conserved surveillance pathways termed 'cell-cycle checkpoints'. Overall, checkpoints detect damaged or abnormally structured DNA, and coordinate cell-cycle progression with DNA repair. Thus, the

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maintenance of integrity of DNA after chromosomal insults depends on the coordination between DNA repair assembly and cell cycle checkpoint controls.[28] Normally, cell-cycle checkpoint activation retards or arrests cell-cycle progression, allowing time for appropriate repair mechanisms to correct genetic lesions before they are passed on to the next generation of daughter cells. DNA damage checkpoints were initially defined as non-essential regulatory pathways that control the cells to arrest the cell cycle in response to DNA damage, allowing time for repair. However, in addition to controlling cell-cycle arrest, recent studies have illustrated the role of these pathways in controlling the activation of repair pathways,[29] the composition of telomeric chromatin and the movement of DNA repair proteins to damaged genomic sites,[30] activation of transcriptional machinery,[27] telomere length[31] and, in certain cell types (such as thymocytes) for reasons not completely understood, induction of cell death by apoptosis via induction of p53.[32] Thus, it is now clear that a multifaceted response is generated by a sub-routine of the checkpoint once the DNA damage response pathway is triggered (Figure I-3).

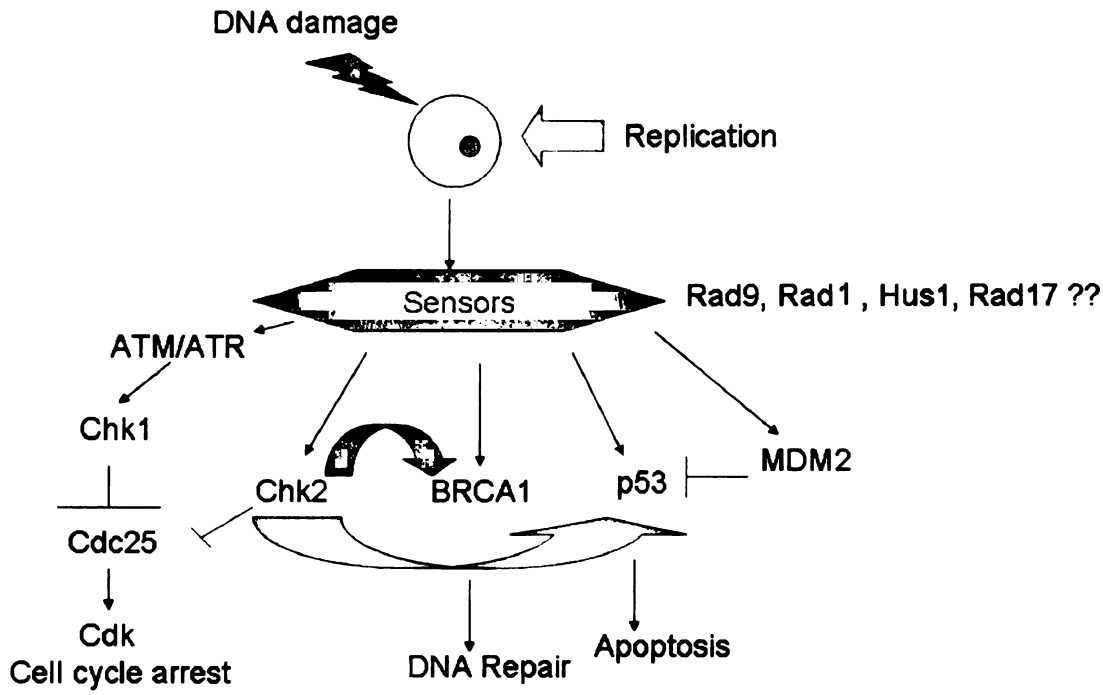



**Figure I-3.** DNA damage response pathway. Arrowheads indicate positive regulation, T indicates negative regulation. The pathway has not been completely elucidated here.

The initial DNA damage sensors are currently unknown. For a long time, poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) were proposed as DNA damage sensors since they could bind to DNA directly and be activated upon DNA strand breaks.[32e] However, Jimenez and co-workers provided genetic evidence according to which these proteins are not activators of the global DNA damage response.[33] Studies suggest a group of four conserved proteins (Rad1, Rad9, Rad17 and Hus1 which form a DNA responsive complex[34]) as candidates for sensors because they share some of the properties expected for sensors, including an essential genetic role in the activation of the DNA damage response pathway and the potential to interact with nucleic acids. In the DNA damage response, Rad17 homologues, can mediate interactions with the Rad1–Rad9–Hus1 complex, [35] and are possibly involved in maintaining the damage signal until repair is completed. Simplistically, in S. *pombe*, Rad17 complexes

bind to damaged DNA directly, and then load the Rad1 complexes onto DNA, which leads to activation of the main damage response kinase Rad3.[36] In response to DNA damage, Rad-3 dependent phosphorylation of checkpoint are dependent on Rad1 and Rad17, however, there is evidence that these proteins that are reputed as sensors may not always be required for other phosphorylation events such as Rad3-dependent phosphorylation of Rad26 in response to ionizing radiation (IR).[36] This latter observation does not preclude the possibility that the Rad1 and Rad17 complexes can act as sensors. The phosphorylation of Rad26-Rad3 might be an initial response to double stranded breaks which requires the presence of Rad1 and Rad17 at a later time for the damage signal to amplify and translate. This suggests a possibility of different sensory pathways to function in order to completely activate the damage response. This situation is further complicated by the fact that a given DNA damaging agent generates several types of DNA damage, possibly activating several sensors. At present, it is impossible to distinguish between a sensory role and an effector role for any of these proteins, and it is plausible that the activity of an entire complex must be intact to properly sense and respond to damage.

**i) DNA damage transduction and regulation of DNA repair.** Several checkpoint genes participate in the cell cycle surveillance program, and are also essential for cell and organism survival,[27, 37] suggesting that these pathways are not only surveyors of occasional damage, but are firmly integrated components of cellular physiology.

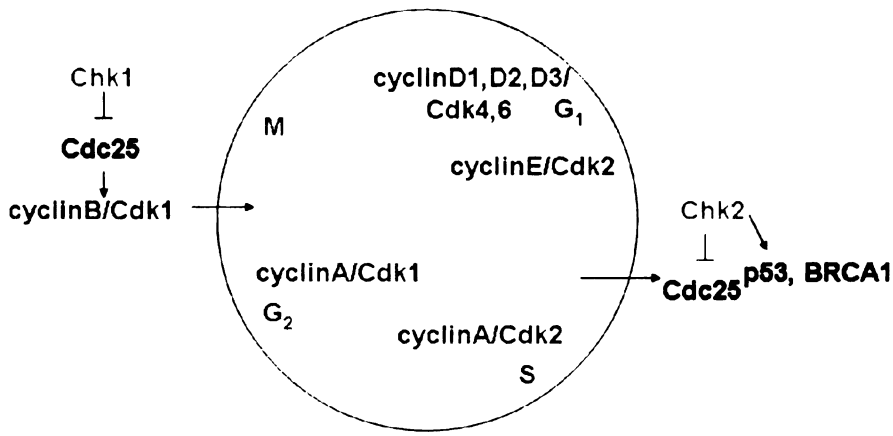



**Figure I-4.** Cell cycle and control of checkpoints at G1/S, G2/M phases Arrowheads indicate positive regulation, T indicates negative regulation. The pathway has not been completely elucidated here.

The DNA damage checkpoints act at mainly three stages of the cell cycle, G1/S transition, progression through S, G2/M boundary.[38] According to the current understanding; the G2 checkpoint DNA damage activates ATM and ATR. [27] A signal is then transmitted to the downstream kinases Chk1 and Chk2 which phosphorylate Cdc25 at Ser-216.[39] This phosphorylation inactivates the Cdc25 and facilitates its association with 14-3-3 proteins[40, 41] by creating a binding site for 14-3-3 proteins. In the 14-3-3-bound form, Cdc25C is either catalytically inhibited or sequestered in the cytoplasm (or both). In any case, the phosphorylated, 14-3-3-bound form of Cdc25C is prohibited from dephosphorylating and activating the mitotic cyclin B. Hence, Cdc2 kinase, and the damaged cells are effectively blocked from entering mitosis.

**ii) Recovery, adaptation or apoptosis: a matter of life and death?**

Once DNA repair is complete, a cell arrested at a checkpoint must then resume cell-cycle progression. This process, termed 'recovery', is under genetic control. Esashi et *al* have

suggested a role for Cdc2 kinase in this process.[42] They provide evidence for phosphorylation of T215 as a requirement for recovery from checkpoint arrest. Cdc2 has been shown previously to be a target for repression during checkpoint arrest.[27] Therefore, the roles of Cdc2 in checkpoint regulation are obviously complex and future studies will be required to unravel this complexity fully. Unicellular cells arrested in $G_2$/M by the DNA-damage checkpoint will eventually proliferate, even if the damage is not repaired.[43] This option, termed 'adaptation', comes from evolution to make an attempt for survival. However, for multi-cellular organisms this is not the best alternative as cells that adapt to the presence of irreparable DNA damage will do so by fixing mutations in that cell's lineage.

Apoptosis is a third alternative outcome of activation of checkpoint pathways in metazoan cells. The link between apoptosis and checkpoints has been well characterized in mammalian cells.[44] In certain cell types, DNA damage dependent apoptosis is mediated by the tumor suppressor protein p53. ATM is also actively involved in some apoptotic responses.[45] A common connection between various death stimuli seems to be the accumulation of reactive oxygen species which are necessary and sufficient for the apoptotic phenotype.

**iii) When checkpoints fail:** Similar to other signal transduction processes, checkpoints could exhibit adaptation,[46] or have an internal error rate. Usually, if the DNA damage checkpoint fails, the DNA repair will be compromised. Under this situation, the cells would experience the consequences of a replication of a damaged template. For instance, a covalent modification of a N-base on the DNA template might affect the stalling of the

fork during replication, which would then resume downstream of the damage leading to the formation of a lesion[38]

**iv) CDK and checkpoint kinase inhibitors- Targeting cell division cycle in cancer:** Since progression through the cell cycle is regulated by CDK/cyclin complexes which are monitored by Chk1 and Chk2 checkpoints, these can eliminate hazardous, damaged cells through induction of cell death and prevent the organism against cancer. A common goal in cancer chemotherapy is to kill cancer cells while sparing the normal tissue. Taking advantage of physiological differences between normal cells and cancer cells could facilitate localized delivery of cytotoxic agents and application of agents that effect frequently dividing cells more than the slowly or non-dividing cells. The common occurrence of checkpoint defects distinguishes cancer cells from normal cells, thereby providing a potential target for therapeutic intervention. Unlike normal cells, which are completely armored with checkpoint responses, tumor cells are defective in some checkpoint pathways and this ineffectiveness eventually is driven to cell death due to accumulation of DNA damage. One strategy for anti-cancer therapy has been to inhibit the G2 checkpoint signaling in p53-deficient cancers that lack G1 checkpoint to enhance the impact of concomitant treatment with DNA-damaging drugs or radiation.[47] Several reports have identified novel compounds capable of inhibiting G2/M arrest in cells lacking p53 [48] (e.g. Staurosporine and its 7-hydroxy derivative UCN-01, caffeine) . These studies have demonstrated a lower dose of radiation or chemotherapeutic agent to effectively kill the cancer cells. Normal cells with functional p53 would not be affected since they retain their ability to undergo G1 arrest.

**v) Cyclin dependent kinase inhibitors**

Meijer and coworkers reported the utility of adenine-based heterocycles as CDK inhibitors, which bind in the ATP-binding pocket with H-bonds.[49] Markwalder *et al* synthesized a series of pyrazolopyrimidinones[50] that have shown activity against CDK1, CDK2, CDK4. This inhibition profile was found to be fairly non-selective over a panel of cell lines independent of their p53 or Rb status. BMS-387032 (1, Table I-1) showed some selectivity for CDK2 vs. CDK1 and CDK4.[51] Animal models indicated rapid and significant tumor regression in >50% test cases when dosed up to the maximal tolerated dose.[51] The pyralazopyridines SQ-67563 and BMS-265246[52] have been reported to be selective inhibitors of CDK1 and CDK2 (2, Table I-1). Both of these classes of compounds showed similar potency towards CDK1 and CDK2. SQ-67563 induced a G2/M arrest indicating a CDK1 inhibition, which correlated with inhibition of phosphorylation of Rb protein, H1 etc. in A2780 cells. The oxindole compounds (3 and 4, Table I-1), these compounds showed very specific activity towards CDK2. Additional cellular profiling was suggestive of both G1 and G2 arrests in a time and concentration dependent manner.[53, 54] Aloisines (5, Table I-1) are synthetic pyrrolopyrazines that inhibited CDKs along with GSK3. The anti-proliferative effect of aloisines was reversible indicating that they showed more of a cytostatic effect than a cytotoxic profile. What was intriguing was the fact that the biochemical effect on kinase inhibition was 100-400 nM range while cytotoxic effects were seen at ~10 $\mu$M.[55] BMI-1026 (6, Table I-6), a bis-aminopyrimidine exhibits equal activity against CDKs 1, 2, and 5 but is highly selective over other mitotic regulators.

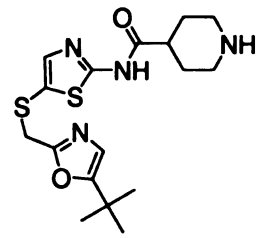
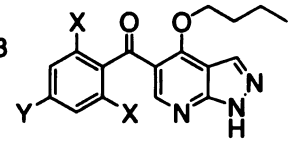
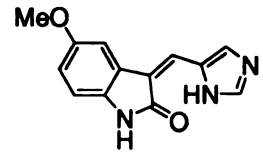
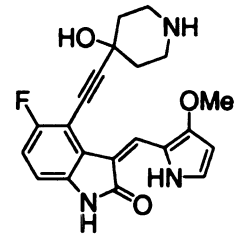
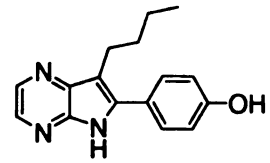
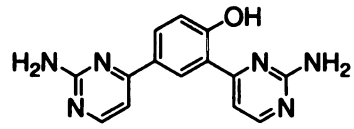
| Compound | Structure | Identified *in vitro* target ($IC_{50}$ in nM) | Reference |
|---|---|---|---|
| **1**<br>BMS-387032 |  | CDK2/cyclinE(48)<br>CDK1/cyclinB(480)<br>CDK4/cyclinD(925) | 51 |
| **2**<br>X=Y=H, SQ-67563 |  | CDK1/cyclinB(150)<br>CDK2/cyclinE(110) | 52a |
| X=F, Y=Me<br>BMS265246 | | CDK1/cyclinB(6)<br>CDK2/cyclinE(9)<br>CDK4/cyclinD(230) | 52b |
| **3**<br>SU9516 |  | CDK2/cyclinA(31)<br>CDK2/cyclinE(82)<br>CDK1/cyclinB(230) | 53 |
| **4** |  | CDK2/cyclinE(6) | 54 |
| **5** |  | CDK2/cyclinA(120)<br>CDK2/cyclinE(400)<br>CDK2/cyclinB(150)<br>CDK5/p25(200)<br>CDL5/p35(160)<br>GSK3α (500) | 55 |
| **6**<br>BMI-1026 |  | CDK1/cyclinB(8)<br>CDK2/cyclinA(2)<br>CDK5/p25(1) | 56 |

**Table I-1.** Structures and their *in vitro* activity against CDKs.

Pharmacologically, it distinguishes between CDK1 and 2 in a way that synchronous cells released from G1 upon treatment led to G2/M arrest at low concentration and upon

increase of concentration, an increase in sub-G1 population was seen. This suggests that CDK1 inhibition occurs more potently than CDK2 for this small molecule and further, CDK1 inhibition can lead to mitotic arrest and lead to an increase in the apoptotic population.[56]

Given the wide range of mutations that occur in tumors at an individual level, investigation of alternative strategies to modulate checkpoint responses in cancer therapy is clearly warranted. Eventually, there is a possibility for the requirement of tailor-made outcomes based on the tumor–profile and defects in the pertinent pathways. Activated checkpoint therapy (ACT) is a theoretically different approach to selectively enhance cell death in cancer cells. For example, β-lapachone stabilizes a pro-apoptotic factor E2F1 that is usually expressed in higher levels in cancer cells.[57]

Between Chk1 and Chk2, there is some overlapping specificity for the substrate.[58a] However, there are differential roles pertaining oncogenesis and embryogenesis. While there is success in targeting Chk1 using UCN-01 upto clinical trials, the Chk2 kinase has been found to be nonessential in embryo development and maintains the genomic integrity only conditionally –under circumstances that cause double strand breaks. In the Chk2-deficient mice model, the tumor suppressor role of Chk2 is evident mainly when external carcinogens were utilized to increase the genotoxic burden on the cells.[58b] Since, multiple cell types lacking Chk2 often show resistance to radiation induced apoptosis, usage of small molecule inhibitors targeting Chk2 might protect sensitive tissues as lymphocytes from the side effects of the therapy. The most important issue would be to identify inhibitors specific to Chk2 and test if this type of combinational strategy would

be able to reduce the incidence of tumors. We have shown the synthesis and efficacy of an indoleazepine (2, Table I-2) which shows such specificity for Chk2 kinase.[59] Debromohymenialdisine (DBH, 1 Table I-2) on the other hand did not show specificity for either Chk1 or Chk2.

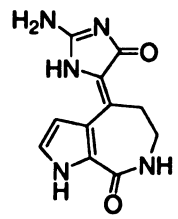
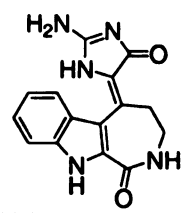
| Compound | Structure | Identified *in vitro* target ($IC_{50}$ in nM) | Reference |
|---|---|---|---|
| 1<br>DBH |  | Chk1 (3000)<br>Chk2 (3500) | 48b |
| 2 |  | Chk1 (237)<br>Chk2 (8)<br>CDK1 (800)<br>CDK2 (177)<br>GSK3β (64)<br>MEKK1 (89) | 59 |

**Table I-2.** Structure and kinase activities of debromohymenialdisine and indoleazipine.

Bartek and coworkers[60] have shown the coordination involvement of Chk1 and Chk2 in regulation of the proto-oncogenic human Cdc25A phosphatase in response to ionizing radiation. In absence of genotoxic burden to the cells, Chk1 targets the same four residues of Cdc25A as in presence of genotoxic burden. This function is vital for the regulation of physiological turnover of Cdc25A, and thereby normal cell cycle transitions. This phosphorylation event is also required for the DNA damage-induced degradation of Cdc25A[60] via the ubiquitin/proteosome assembly, which silences Cdc25A. As a result of inhibition of the CDKs, this event translates in a promptly deployed cell cycle delays in G1,[61] in S,[62] as well as in G2[63] phases of the cell cycle. Conceptually, the requirement of

Chk1 for the maintenance the S phase events explains why *Chk1* is an essential gene and cannot be substituted even by a functionally overlapping kinase such as Chk2. Typically, there is scarcity of cancer-related mutations of Chk1, due to the lethal consequences of Chk1 deficiency in animal models.

The current knowledge about Chk1 and Chk2 is still insufficient to be applied for the benefit of cancer chemotherapy or radiation therapy. Researchers all over the world are searching for additional substrates of these kinases, and integrate these with the checkpoint signaling. A complete assessment of the scope and importance of cancer-related abnormalities (in particular Chk2) would help in improving the current treatments for cancer. Finally, synthesis and isolation of new small molecule inhibitors of Chk1 and Chk2 and design and rationalizing novel strategies of checkpoint modulation, combined with the traditional radiation and chemotherapy models, hold promise for improved treatment of cancer.

**D. p53 and NF-κB crosstalk**

Inactivation of the apoptotic pathway is a significant contributor in the development of cancer. Two important transcription factors that regulate this event are p53 (the 'guardian' of the cell) and NF-κB (the 'mastermind' for cell survival). As a general consensus, it is believed that NF-κB is a cell-death suppressor. The mammalian nuclear transcription factor κB (NF-κB) is a pivotal regulator of the inducible activation of anti-apoptotic signaling pathways.[64] NF-κB, is a multisubunit complex involved in the regulation of gene transcription, including the regulation of apoptosis.[64, 65] Five distinct subunits of NF-κB are found in mammalian cells, which include NF-κB1 (p105/p50),

NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel.[65] These subunits can compose a variety of homo-or heterodimers, which are used to control the specificity and selectivity of specific DNA control elements.[66, 67] In most unstimulated mammalian cells NF-κB exists mainly as a homodimer (p50/p50) or heterodimer (p50/p65) in the cytoplasm in the form of an inactive complex with the inhibitory protein I-κB (Figure I-5). Many cellular stimuli including: antineoplastic agents, [68] viruses (e.g. HIV), cytokines, phorbol esters and oxidative stress, result in the IKK mediated phosphorylation of I-κB on serines 32 and 36, followed by ubiquitinylation and subsequent degradation by the 26 S proteosome. [64]

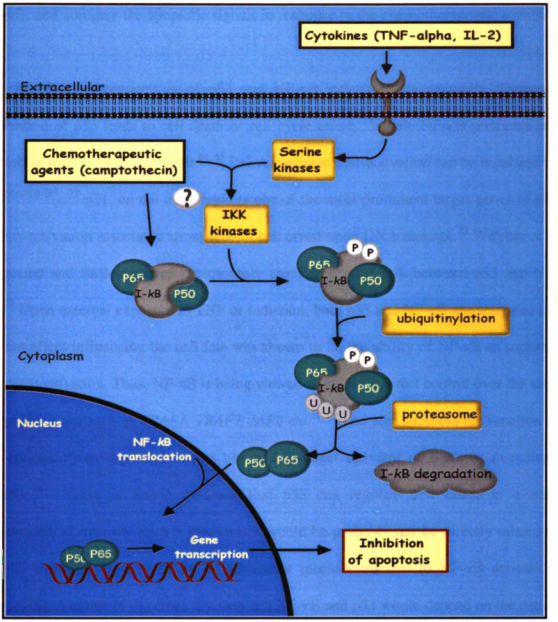



**Figure I-5.** NF-κB signaling cascade

Degradation of IκB ensures the release of NF-κB.[69] Upon release, NF-κB translocates into the nucleus where the subunits bind with specific DNA control elements and initiate gene transcription (Figure I-5). Prior to DNA binding, additional protein phosphorylation events are required for optimal and specific gene transcription.[70] Antiapoptotic genes such as, *TRAF1*, *TRAF2*, *c-IAP1*, *c-IAP2*, *IXAP*, and *IEX-1L*, are directly regulated by

NF-κB, and abrogate the apoptotic signals in response to the chemotherapeutic agents.[10, 71]

The functional consequences of p53 and NF-κB induction are generally divergent. Activation of p53 leads to cell death or cell cycle arrest, [26] while NF-κB promotes cell growth or resistance to cell death.[64, 65] Rel-A mediated transactivation can be regulated by $p21^{WAF1/CIP1}$. [72] p21, on the other hand is one of the most prominent target genes of p53 and its activation associated strongly with cell arrest upon DNA damage.[73] Webster *et al* suggested and further proved a previously unrealized cross talk between p53 and NF-κB.[67] Upon external exposure to TNF or radiation, both p53 and NF-κB are activated but the net effect influencing the cell fate was shown to be the ability of NF-κB to suppress p53 transactivation. Thus, NF-κB is being viewed as having a direct control over the anti-apoptotic genes such as *TRAF1, TRAF2, IAP2* etc.[10, 71] and also suppress the function of pro-apoptotic transcriptional factors like p53 (a direct activator of Bax), in an indirect manner.[74] There is ample evidence that p53 can regulate cell death in a non-transcriptional manner. Such a mechanism would be probably not be directly related to NF-κB and this might override the resistance mechanisms due to NF-κB activation. Hence, the outcome of the cross talk between NF-κB and p53 would depend on the nature of stimuli and also the cell type. Mutant p53 does not inhibit NF-κB regulation, in fact, a lot of research on NF-κB regulation has been performed on cells with mutant p53 or inactivated p53.[67] Activation of NF-κB is usually transient in most cell types.[75a] This observation is consistent with a modulatory effect of NF-κB on p53 and due to a fluctuation in the levels of p53 and NF-κB, the various genes that are induced by these two transcription factors might be expected to alter. In some diseases, however, such as

Hodgkinson's disease and breast cancer, the activation of NF- κB is constitutive, and its inhibition can reverse the phenotype even in clinical samples.[75b] It is logical to speculate, that inhibition of p53 function might contribute to NF-κB's role in tumorigenesis and other diseases. Further, it is possible that novel cancer therapies involving re-activation of *wt* p53 function might benefit from coordinate suppression of NF-κB to promote a favorable outcome. Hietanen *et al* showed activation of p53 in cervical carcinoma cells using small molecule leptomycin C and actinomycin C.[76] Maurici and coworkers used amifostine to restore transcriptional activity of p53 of mutant proteins in yeast systems.[77]

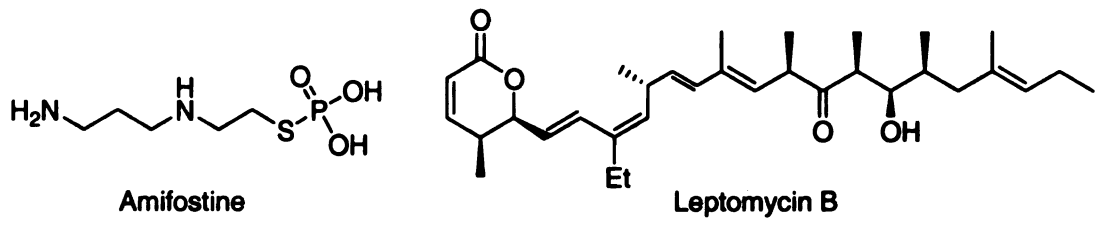



**Figure I-6.** Structures of Aminofostine and Leptomycin B, reactivators of p53

Bcr-Abl, a chimeric oncoprotein important in cellular transformation as well as protection of cells from cell death in response to cytokines and DNA damaging agents, activates NF-κB via multiple pathways. Bcr-Abl facilitates the translocation of NF-κB across the nuclear membrane in a Ras dependent manner.[78] Researchers at Novartis developed a small molecule imatinib (Gleevec®) which specifically inhibits the activity of Bcr-Abl in cells producing this protein. Now approved by FDA, this molecule showed dose-dependent inhibition and even eradication of Bcr-Abl containing tumors when administered orally in animal models.[79a]

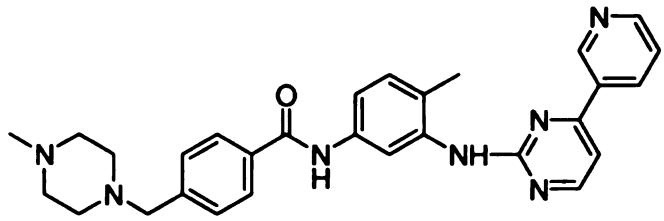


**Figure I-7.** Imatinib, a specific inhibitor of Bcr-Abl

Although imatinib mesylate produces high rates of responses in a clinical setting there is some universal resistance and clinical relapse that seen in advanced phases of chronic myelogenous leukemia (CML).[79b] Usually, this resistance is caused by specific ATP binding site mutations or amplification of Bcr-Abl gene, resulting in a Bcr-Abl tyrosine kinase that is resistant to further inhibition by imatinib.[80] Alternative mechanisms that are Bcr-Abl-independent but drive the growth and survival may also be responsible for imatinib resistance. Novel tyrosine kinase inhibitors that target Bcr-Abl tyrosine kinase, or small molecules that downregulate Bcr-Abl levels regardless of its *wt* or mutant status are required for future therapy of imatinib-resistant chronic myelogenous leukemia.

**E. Resistance pathways: activation of NF-κB and its role in cell survival**

As mentioned in Section C, NF- κB plays a central role in regulating the cell survival pathways and inhibition of apoptotis. It can be activated under the influence of cytokines or DNA damaging agents. The NF-κB signaling cascade is unique in terms of the rapidity of the activation, its mechanism and and regulation. The proteolytic degradation of the phosphorylated I-κBα is an irreversible step required for the NF-κB translocation and in essence is a commitment to transcriptional activation. Only the Rel proteins of all the various kinds of subunits are transcriptionally active. While p50/p65 causes transcriptional activation, a homodimer, p50/p50 is transcriptionally repressive.[81] Butterfly –like crystal structure of NF-κB of various forms bind to DNA through the N-

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terminal region.[82] Upon, DNA binding, NF-κB regulates the transcription of an exceptionally large number of genes especially those involved in immune and inflammatory responses.[83] In most cells, NF-κB circumvents apoptosis via induction of survival genes. However, under some special conditions it can induce apoptosis.[84] A significant indication that NF-κB contributes in cell survival comes from the fact that mice with Rel-/- do not survive and die before birth with extensive degeneration of liver indicating that the role of NF-κB in early liver development. [85]

**i) NF-κB: a major culprit or an innocent bystander?**

Baldwin *et al* [86] have demonstrated that cells with inactivated NF-*κ*B are more prone to undergo apoptosis in response to pro-apoptotic stimuli other than the inflammatory cytokine TNF-α. DNA-damaging agents, such as ionizing radiation and daunorubicin, activate NF-κB, and are more toxic to cells when NF-κB activation is blocked by expression of a dominant-negative IκB-*α* mutant. We have shown enhancement of the apoptotic signal of camptothecin using *trans*-imidazolines via modulation of NF-κB pathway.[87a] A therapeutic sense of these observations is that it may be possible to enhance the efficacy of anti-cancer drugs by inhibiting NF-κB activation. Several anti-cancer compounds are cytotoxic and kill tumor cells by inducing apoptosis. Most of these also activate NF-κB in a variety of cell lines.[87] By combining cancer chemotherapeutics with a compound that suppresses NF-κB activation, their anti-tumor activity may be enhanced. (Figure I-8)

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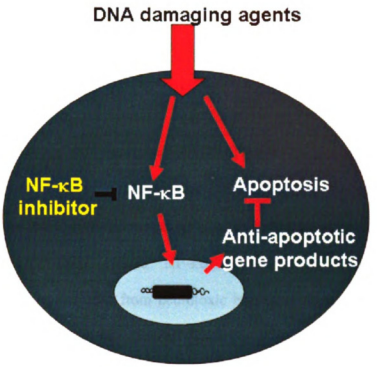



**Figure I-8.** Upon DNA damage apoptosis and activation of NF-κB are triggered.

The anti-apoptotic genes activated by NF-κB in response to endogenous TNF-α or exogenous genotoxic stress remain to be identified.

Multi-cellular organisms seem to have evolved a link between NF-κB activation with protection from apoptosis mainly to avoid unnecessary cell suicide in response to certain pro-apoptotic signals, such as TNF-α. Hence, a cell would commit to death only if the damage or genotoxic burden is so high that it hinders the replication, transcription or protein synthesis. In such cases, the cell may not be able to mount and alleviate the NF-κB-dependent genetic response and anti-apoptotic signals would not be activated. Hence, the organism can monitor itself for damaged or infected cells and eliminate them while the transcriptionally and translationally functional cells would make it to the next

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progeny. NF-κB is thus a key regulator connecting the execution of apoptosis with an analysis of functional cellular machinery.

**ii) NF-κB and apoptosis**

There is some evidence that induction of NF-κB might ***induce*** apoptosis. Such an opposite effect was observed by Grill *et al.* While studying the glutamate-induced toxicity in neuronal cells they also observed significant induction of NF-κB. [88] Aspirin (methyl salicylate), which blocks NF-κB activation via inhibition of IκB phosphorylation[89] protected cells from neurotoxic effects of glutamate, thus indicating that NF-κB activation appears to cause cell death. Nerve growth factor (NGF) activates NF-κB in Schwann cells through the neurotrophin receptor p75.[90] The receptor, p75 mediates cell death as well as survival depending on the developmental state of neurons. Whether NF-κB performs a pro-apoptotic or anti-apoptotic role in this glial cell type is a question unanswered yet. Studies of this type with NF-κB-activating neurotoxins (even $\beta$ amyloid peptide), indicate, that in neurons, unlike several other cell types, NF-κB maybe involved in triggering cell death.

Apoptotic cell death was also seen in human embryonic kidney cell line (293 cells) by serum withdrawal with NF-κB activation.[91] In another study, Sindbis-Virus-induced apoptosis in a carcinoma cell line was shown to require NF-κB activation.[92] Thus, there are clear situations in which NF-κB activation seems to be required for apoptosis to occur. Interestingly, the list of target genes for NF-κB include both anti-apoptotic (see above) as well as pro-apoptotic genes, such as c-*myc* and *p53.* [64] Ultimately, the answer to the question whether NF-κB acts as a promoter or attenuator of cell death may depends

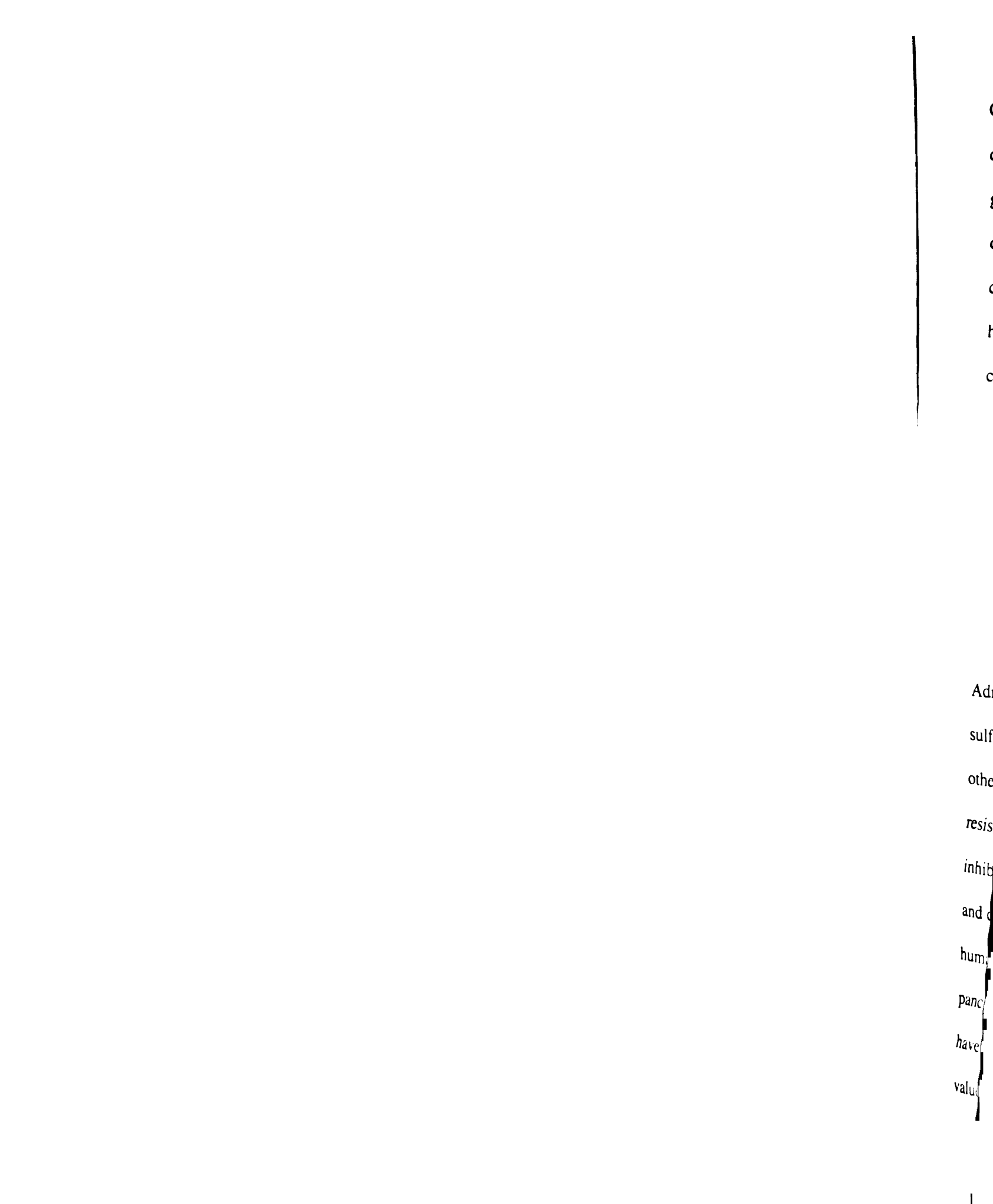
on both the cell type and the nature of the apoptosis-inducing stimulus. In different cell types, NF-κB could perform opposing functions by activating distinct patterns of genes in conjunction with cell-type-specific transcription factors. The effect of various apoptosis-inducing stimuli may vary with respect to the kind of pathways they can activate, in addition to those related to apoptosis and NF-κB. Additional activated transcription factors, such as AP-1, may be influential in determining whether NF-κB can oppose or promote apoptosis via the induction of a wide spectrum of genes. Hence, NF-κB appears to be a genetic switch whose biological effect would depend on the set of genes that can be accessed in a given cell type. Recently, it has been unleashed that blocking NF-κB pathways also increases the cell's vulnerability to cytotoxic stimuli in a pharmacological sense.[68c, 87a] While this may be a desirable outcome when using cancer chemotherapeutics for tumors, it may not be advantageous when treating inflammatory diseases with NF-κB inhibitors. Most anti-inflammatory drugs would have to rely on partial inhibition of NF-κB which protects from apoptosis and is also sufficient to reduce the production of cytokines. Since inflammation and tumorigenesis conditions overlap to some extent, the future development of small molecules that target NF-κB for improved chemotherapeutic treatments will certainly benefit from the recent findings.

**iii) Kinase inhibition and Modulation of NF-κB**

The NF-κB signaling cascade involves a variety of kinases. There have been numerous studies involving kinases that inhibit the NF-κB signaling cascade. Kinases play significant role in phosphorylation in biological processes involved in tumorigenesis and invasion. GSK-3β, a serine/threonine kinase pays an important role in regulation of glycogen synthesis.[93] Several reports suggest the pro-apoptotic role of GSK-3β in HIV-

tat, staurosporine induced cell death, disruption of *gsk-3β* causes lethality in mouse embryonic fibroblasts (MEFs). In GSK-3β deficient MEFs, there is an intrinsic defect in the activation of NF- κB.[94] Intriguingly, some mechanisms of chemoresistance in pancreatic cancer are due to overactive NF- κB signaling pathways.[95] Thus, dissecting and elucidating the mechanisms controlling NF- κB -mediated cell survival pathways in pancreatic cancer may aid in the identification of novel anticancer targets. Thus, there is conflicting information about the role of GSK-3β in cell survival and death. On one hand, GSK-3β inactivation leads to sustained β-catenin levels, which ultimately culminate in enhanced cellular proliferation. This however, does not resolve well with the observation that low β-catenin expression is associated with more aggressive pancreatic cancer and poor survival.[96] It has also been suggested that GSK-3β plays a key role in the regulation of NF-κB mediated gene transcription. This would predict that GSK-3β inactivation would decrease cell proliferation and survival.

Aggarwal *et al* showed that in *gsk-3β* -/- MEFs, TNF-α can't induce phosphorylation and subsequent degradation of I-κB,[94b] while Woodgett *et al* indicate that the NF-κB inactivity lies downstream of IκB phosphorylation in *gsk-3β-/-* MEFs.[94a] Billadeau *et al* showed that the regulation of cell survival by GSK-3β occurs through an NFκB-dependent pathway in MEFs post phosphorylation step.[97] They emphasize that overexpression of p50/p65 unit, rescued cells of cell survival effects of GSK-3β. Such an effect was not seen with a constitutively active mutant of IKKβ. Schwabe *et al* suggested the role of GSK-3β in hepatocytes in phosphorylating p65 subunit between 354 and 551, though its mechanistic effect in pancreatic cancer cells has not been determined yet.[98]



Gemcitabine (trade name Gemzar) is a clinically used drug for treatment of pancreatic cancer[99]and also used as a radiation-sensitizing agent.[101] The cytotoxic effects of gemcitabine are exerted through incorporation of dFdCTP into DNA with the assistance of dFdCDP, resulting in inhibition of DNA synthesis and induction of apoptosis.[100] It is cell-cycle phase specific and is active in S and $G_1$/S-phases.[101] Pancreatic cancer cells have elevated levels of NF-κB which may further increase due to the genotoxic burden on cells, thus, leading to induced chemoresistance. [95]

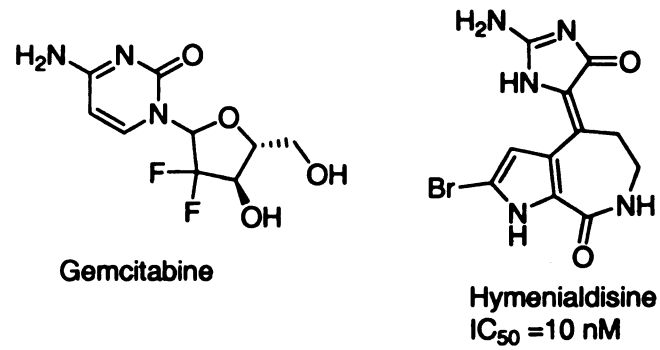



**Figure I-9.** Structures of gemcitabine and hymenialdisine.

Administration of agents that inhibit NF-κB activation like gliotoxin, MG-132 or sulfasalazine in pancreatic cancer cells can make them more sensitive to gemcitabine and other chemotherapeutic agents such as VP-16 or doxorubicin, whose mechanism of resistance is due to increased NF-κB activity. Indeed, the combination of NF-κB inhibitors with other chemotherapeutic agents may be more effective in cancer treatment and clinical trials using such treatment strategies are currently being employed in several human cancers.[102] Thus, inhibition of GSK-3β may sensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine, but this remains to be addressed. Wan *et al* [103] have shown the kinase inhibition profile of hymenialdisine and analogs with an $IC_{50}$ value of 10 nM for the natural product hymenialdisine. PDTC is a non-selective inhibitor

of NF-κB, though itself is cytotoxic probably due to its non-selective nature.[104] Thus, GSK-3β is uniquely and importantly involved in pancreatic cancer cell proliferation and survival, partially through its regulation of NF-κB activity. It is probable that GSK-3β regulates other molecular pathways as well that might contribute to pancreatic cancer. To summarize, the fact that GSK-3β regulates several proteins involved in controlling cell cycle, survival and proliferation, growth and metabolism, suggests its broader role in cancer.

**iv) IκB-kinase and NF-κB: IKK complex –an integrator of all signals to activate NFκB.**

For most cases, the activation of NF-κB requires IκB-α phosphorylation and subsequent degradation after polyubiquitination. Hence, inhibition of phosphorylation of IκB-α can ultimately lead to the inhibition of transcriptional activity of NF-κB. This would include both the anti-apoptotic genes as well as those responsible for the immune and inflammatory responses.

Thalidomide, which was used as a sedative in the 1950s,[105] is an agent in clinical use that inhibits NF-κB partly by inhibiting the IκB kinase activity leading to inhibition of the phosphorylation of IκBα.[106]

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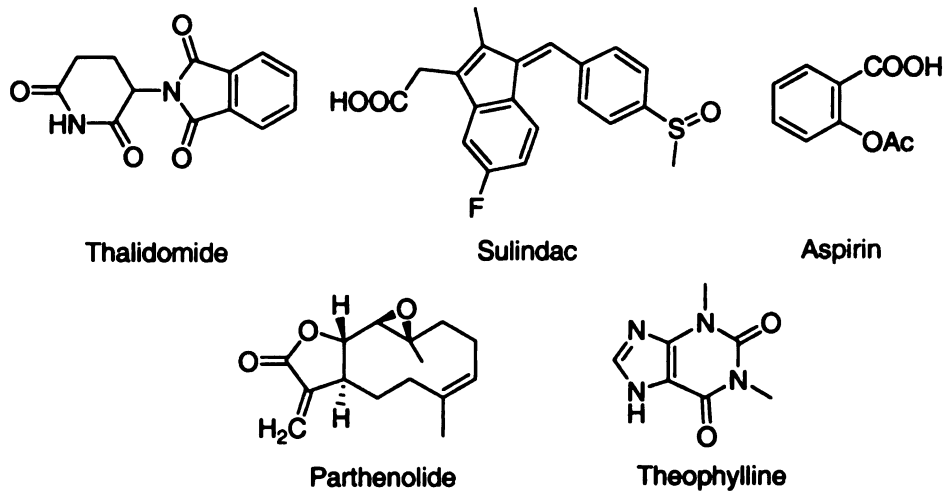



**Figure I-10.** IκB-Kinase inhibitors

NF-κB regulates *IL-8* which controls the development of angiogenesis in certain models.[107, 108] Since thalidomide inhibits NF-κB, thalidomide may function as an anti-angiogenic factor, and suppression of NF-κB-regulated expression of *IL-8* might prove to be important in anti- angiogenic therapy in cancer systems. The ability of thalidomide to inhibit IKK activity causes suppression of downstream events such as inhibition of NF-κB-DNA binding and transcriptional activity. NF-κB regulates genes involved in inflammatory responses as well as those in cell survival and inhibition of apoptosis. Hence, it is likely that inhibition of NF-κB by thalidomide might explain its anti-inflammatory and anti-oncogenic properties. Baldwin *et al* [106] suggest a possibility of modulation of other factors or signaling pathways with thalidomide that may be involved in inflammatory or oncogenic potential. Hence, inhibition of NF-κB can be considered a unifying model to suppress both inflammation and angiogenesis.

Other IKK-inhibitors are $As_2O_3$, [109] parthenolide, [110] sulindac, [111] theophylline, [112] aspirin[88, 113] etc. which block the enzymatic activity of the kinase. This effect ultimately

culminates in inhibition of NF-κB and its transcriptional machinery. Both sulindac and aspirin are non-steroidal anti-inflammatory agents. In addition to anti-inflammatory properties, sulindac has been demonstrated to have a role in the prevention of colon cancer.[114] Sulindac and its derivatives, aspirin and sodium salicylate inhibit the activity of an IκB kinaseβ (IKKβ) which is required to activate the NF-κB pathway. [111, 113] There is evidence provided by Yamamoto and coworkers[111] that the growth inhibitory and anti-inflammatory properties are due inhibition of the activity of cyclooxygenases, a gene product of NF-κB transcription. These results suggest that the growth inhibitory and anti-inflammatory properties of sulindac may be regulated in part by inhibition of kinases that regulate the NF-κB pathway.

**v) Ubiquitination and Proteosome inhibitors**

IKK-mediated phosphorylation at Ser 32 and Ser 36 includes rapid polyubiquitination. The most important sites of ubiquitinylation are Lys 21 and 22 of the N-terminal region. Protein ubiquitinylation is a three step process. [115] First, the 76 amino- acid polypeptide, ubiquitin, is activated through an ATP-dependent process by E1-a ubiquitin activating enzyme. The ubiquitin is then transferred to cysteine in the active site of an E2 enzyme-a ubiquitin carrier protein and finally, the transfer of the activated ubiquitin to the target protein is catalyzed by an E3 enzyme-the ubiquitin ligase. The E3 enzyme provides the specificity to recognize the substrate. Although the ubiquitinylation is constitutively active, it offers the possibility of inhibition by factors that can be regulated. Such a regulation for NF-κB signaling transduction is not known. Ubiquitination allows the proteosome to degrade the protein. Once IκB-α is degraded, the p50/p65 unit translocates

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to the nucleus and starts its transcriptional machinery. There are several proteasomal inhibitors known to inhibit this degradation. In such cases, IκB-α undergoes phosphorylation but it is not degraded. Hence, NF-κB is prevented from translocating into the nucleus and thus, its transcription is prevented.

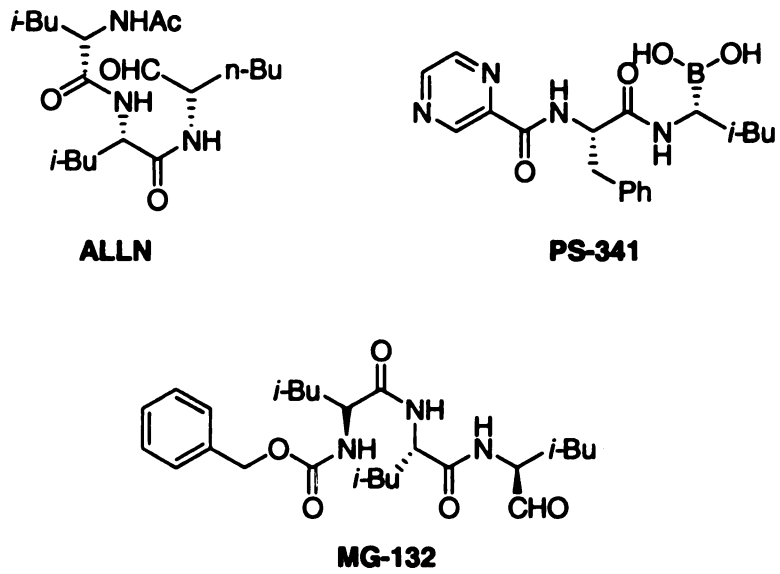



**Figure I-11.** Proteasome Inhibitors

Multiple myeloma –a B-cell malignancy is a resistance disease making it incurable or with a significant rate of mortality. [116] NF-κB levels are elevated in relapsing multiple myeloma suggesting that this could be used as a prognostic marker as well as a target in therapy to prevent progression of the disease.[117, 118] Bortezomib® (previously Velcade or PS-341) causes inhibition of degradation of IκB-α, and markedly reduces nuclear translocation of NF-κB. [118]

By combining a non-cytotoxic dose of PS-341 with chemotherapeutic agents such as melphalan or doxorubicin, it was found that the cell lines became sensitive by $10^5$ - $10^6$ fold than were necessary to kill these cells without PS-341.

We have shown the use of novel imidazolines which sensitize leukemia cells towards chemotherapy with topoisomerase-I inhibitor, camptothecin.[87]

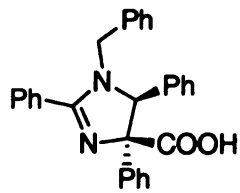
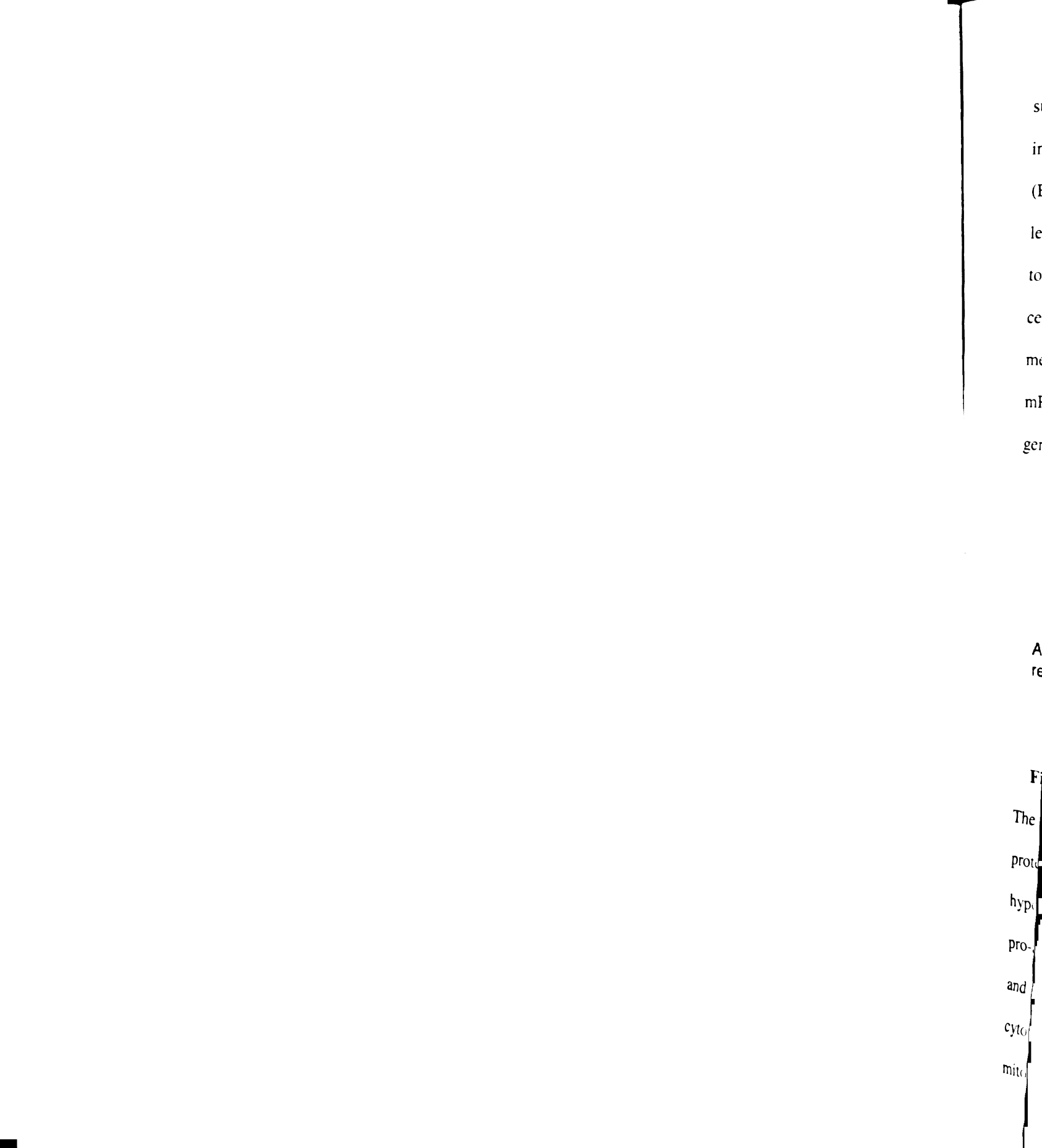



**Figure I-12.** Structure of imidazoline

We found that the chemosensitizing agent (Figure I-12) was found to sensitize leukemia cells at low nanomolar concentrations to camptothecin and drastically enhance the level of camptothecin mediated apoptosis. Combination therapy of CEM cells with camptothecin and 10 nM imidazoline provided ~44 fold enhancement of efficacy after 48 hours. The ability of the imidazoline to enhance the apoptotic events initiated by camptothecin is postulated to be *via* the inhibition of the NF-κB signaling pathway. Inhibition of the NF-κB pathway was found to occur via the inhibition of degradation of the inhibitory protein I-κBα. These studies indicate that combination therapy of classical anticancer agents with small molecule NF-κB inhibitors has the potential to target this chemoresistance and provides a new strategy to treat chemoresistant tumors.

**F. $Bcl_2$ proteins: life or death switch**

Apoptosis is an orderly and synchronized process that has distinct signaling pathways[1, 2] which converge at effector caspase activation. Proteins of the Bcl-2 family share the Bcl-2 homology (BH) domains and can behave as pro- or anti- apoptotic proteins. Pro-



survival members such as Bcl-2 and Bcl-$X_L$ preserve the mitochondrial outer membrane integrity to prevent the release of apoptogenic molecules.[119a] Pro-apoptotic members (Bax-like) act as sensors of cellular damage and initiate the death process.[119b] Perturbed levels of either of these proteins may contribute to oncogenesis and tumor-cell resistance to drug-induced cell death. Hence, pro-survival proteins are attractive targets for inducing cell death or sensitizing cells to death induced by chemotherapeutic drugs. Use of an 18-mer antisense nucleotide sequence (Genasense or G3139) targeting the transcription of mRNA, entered phase II trials.[119c] Mitochondria are important for the cell as they generate ATP and also actively contribute in Bcl2 regulated apoptotic cell death.[120]

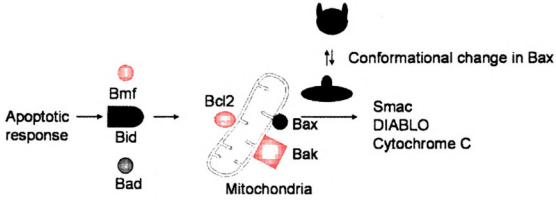



**Figure I-13.** Control of mitochondrial membrane permeabilization by Bcl-2 proteins

The molecular mechanisms in response to the apoptotic stimuli and the role of Bcl-2 proteins in regulating these are a matter of intense controversy. Among them, is a hypothesis by Thompson *et al* indicating the formation of outer membrane channels by pro-apoptotic factors such as Bax and Bak. Knock-out studies in MEFs indicated that Bax and Bak are required for permeabilization in response to the apoptotic stimuli from cytotoxic drugs.[121] Bax, which normally resides in the cytosol could translocate to the mitochondrial outer membrane upon induction of apoptosis.[122] Bak on the other hand is

associated at the outer membrane of the mitochondria and undergoes conformational changes upon apoptosis.[123] In such as situation, Bad and Bim can bind to Bcl-2 to cause conformational changes such that it doesn't have inhibitory effects on Bax or Bak. Finally, pro-apoptotic Bax and Bak would form channels to release cytochrome C into the cell.

**i) Overexpression of *bcl2* : targets or tools ??**

Overexpression of Bcl-2 was described as a consequence of t(14;18) translocation and has [124] been considered as a prognostic marker of acute myeloid leukemia[125] and non-Hodgkin's lymphomas. [126] Other changes like mutations in the *bax* gene[127] and changes in pro and anti-apoptotic ratios have also been identified. [128] Based on the important role of Bcl-2 proteins in regulating the mitochondrial outer membrane permeabilization and their frequently modified expressions, the anti-apoptotic proteins as well as the pro-apoptotic proteins could be used as targets or tools in cancer chemotherapy. Strategic, temporal control of proteins may not cause a burden on the cell and can be exploited for therapy.

**ii) Antisense oligonucleotide targeting Bcl2**

Antisense oligonucleotides therapy utilizes short, synthetic sequences which bind to the RNA so that the translation into proteins is prevented. Specific DNA:RNA duplexes prevent the conversion into proteins (the functional equivalent of a gene) and hence, block the target of the gene.[129] Mechanistically, this involves a variety of processes such as inhibition of splicing and cleavage of RNA by RNAse H found in the cytoplasm as well as the nucleus.[130] De Casare *et al* have shown the effects of oblimersen on the anti-tumor activity of gimatecan, a topo-1 inhibitor from the camptothecin family currently undergoing phase II clinical trials.[131] Oblimersen is an antisense oligonucleotide that

binds the bcl-2 mRNA sequence and results in the degradation of bcl-2 mRNA and down-regulation of Bcl-2 protein.[132] Jansen *et al* and Millward *et al* independently suggested the therapeutic potential of oblimersen combined with several cytotoxic agents against a variety of solid and hematologic malignancies through preclinical and clinical studies.[133, 134] In particular, a clinical study of the dacarbazine/oblimersen combination supported beneficial outcomes in melanoma patients receiving the combination in terms of progression- free survival.[134]

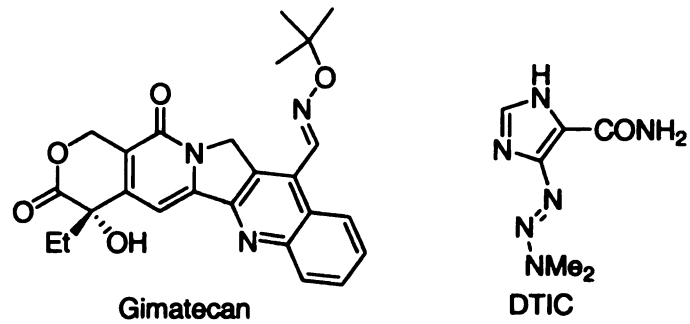



**Figure I-14.** Structures of topo-1 inhibitor, gimatecan and DNA alkylator, DTIC (dacarbazine).

Both cell models as well as animal models showed an increased sensitivity to gimatecan, in presence of oblimersen. Several clinical trials for oblimersen are on going with chemotherapeutic agents such as dexamethasone, mitotrexone etc.

**iii) Small molecule inhibitors of Bcl-2 family.**

Antimycin A and its methoxy derivative bind to the BH3 binding groove of Bcl-2 and Bcl-$X_L$; thus, inducing membrane permeability and apoptosis.[135] Two classes of small molecules that disrupt the binding of Bak to Bcl-$X_L$[136]

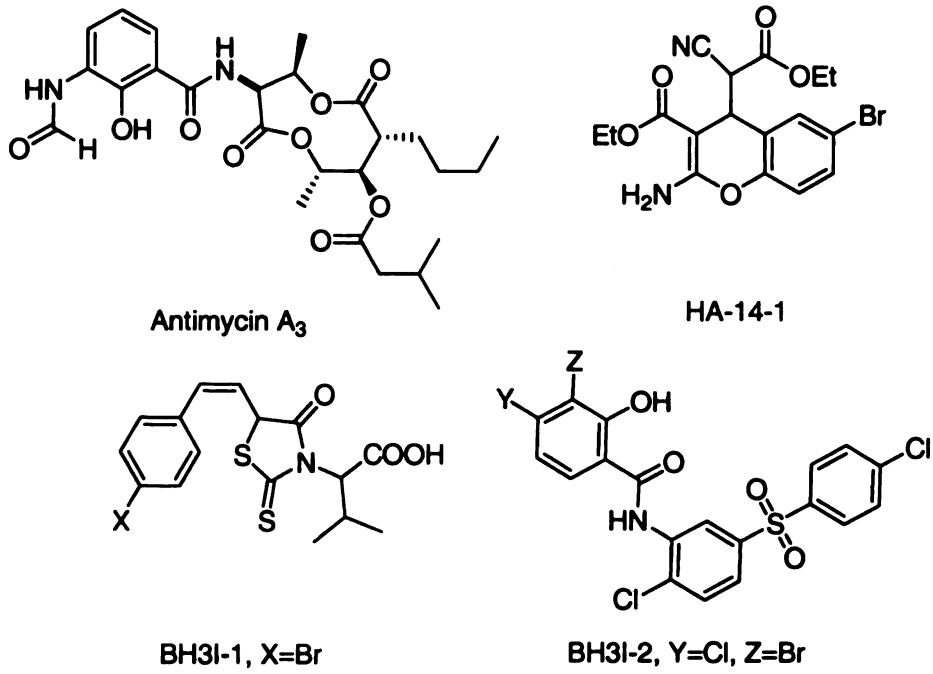



**Figure I-15.** Structures of Antimycin $A_3$, HA-14-1 and Degeterev's BH3-Bcl-$X_L$ binding inhibitors

HA14-1 directly binds to Bcl-2 and reduces the membrane potential of mitochondira to induce apoptosis via activation of caspase-3.[137] Mitochondria in general, and Bcl-2 proteins in particular, are promising new targets in cancer therapy owing to their central role in the regulation of apoptosis as well as their importance in the development of resistance to chemotherapy. The chemical inhibitors developed in this study could circumvent the upstream anti-apoptotic barriers in transformed cells *in vitro* and might be used to explore the feasibility of anti-apoptotic agents as novel anti-cancer therapies

## G. Summary

Resistance to chemotherapy can arise due to a wide variety of non-oncogenic and oncogenic reasons. Activation of anti-apoptotic signaling pathways have compromised the efficacy of antitumor agents in the clinic. Antitumor drugs exert their antitumor activity *via* the initiation of apoptotic signaling pathways such as activation of p53, ultimately resulting in cell death. Mutations in the pro-apopotic factors such as p53 contribute to this inherent resistance. Small molecules have provided temporal control over the levels of both anti-apoptotic and pro-apoptotic factors. These would not only offer a handle to understand biological pathways, but also help in identifying targets and modulate their functions for potential therapeutic use in future. More strategically designed novel inhibitors are desired to address the specificity issues. It is probable that a 'tailor-made' therapy to target specific pathways in certain cancers proves most beneficial.

## H. References


1) Green, D. R. *Cell* **1998**, *94*, 695–698.

2) Hengartner, M.O. *Nature* **2000**, *407*, 770–776.

3) Hanahan, D.; Weinberg, R. A. *Cell* **2000**, *100*, 57–70.

4) Blagosklonny, M. V. *Cell Death Differ.* **2005**, *12*, 592-602.

5) Middleton M. R., Margison, G. P. *Lancet Oncol.* **2003**, *4*, 37-44.

6) Zaidi, N. H.; Allay, E.; Ayi, T.C.; Li, B. F.; Dumenco, L. L.; Sy, M. S.; Gerson, S. L. *Carcinogenesis* **1995**, *16*, 1047-1053.

7) (a) Giaccone G.; Van Ark-otte J.; Rubio, G. J.; Gazdar, A. F.; Broxterman, H. J.; Dingemans, A. – M. C.; Flens, M. J.; Scheper, R. J.; Pinedo, H. M. *Int. J. Cancer* **1996**, *66*, 760-767. (b) Murase, M.; Kodera, Y.; Kondo, K.; Sekiguchi, H.; Fujiwara, M.; Kasai, Y.; Akiyama, S.; Ito, K.; Takagi, H. *J. Surg. Oncol.* **1996**, *61*, 223-229.

8) Fitzsimmons, S. A.; Workman, P.; Grever, M.; Pauli, K.; Camalier, R.; Lewis, A. D. *J. Natl. Cancer Inst.* **1996**, *88*, 259-269.

9) (a) Hollstein, M.; Sidransky, D.; Vogelstein, B.; Harris, C. C. *Science* **1991**, *253*, 49-53. (b) Burns, T.F.; El-Deiry, W. S. *J. Cell. Physiol.* **1999**, *181*, 231-239.

10) Wang, C. Y.; Mayo, M. W.; Korneluk, R. G.; Goeddel, D. V.; Baldwin A. S., Jr. *Science* **1998**, *281*, 1680-1683.

11) Schon, O.; Friedler, A.; Bycroft, M.; Freund, S. M.; Fersht, A. R. *J. Mol. Biol.* **2002**, *323*, 491-501.

12) (a) Momand, J.; Zambetti, G. P.; Olson, D.C.; George, D. L.; Levine, A. J. *Cell*, **1992**, *69*, 1237-1245. (b) Thut, C. J.; Goodrich, J. A.; Tjian, R. *Genes Dev.* **1997**, *11*, 1974-1986. (c) Freedman, D. A.; Wu, L.; Levine, A. J., *Cell Mol. Life Sci.* **1999**, 55, 96-107.

13) (a) Haupt, Y.; Maya, R.; Kazaz, A.; Oren, M. *Nature*, **1997**, *387*, 296-299. (b) Honda, R.; Tanaka, H.; Yasuda, H. *FEBS Lett.* **1997**, *420*, 25-27. (c) Kubbutat, M. H.; Jones, S. N.; Vousden, K. H. *Nature*, **1997**, *387*, 299-303. (d) Midgeley, C. A.; Lane, D. P. *Oncogene*, **1997**, *15*, 1179-1189.

14) Blaydes, J. P.; Gyre, V.; Rowson, J. M.; Wynford-Thomas, D. *Oncogene*, **1997**, *14*, 1859-1868.

15) (a) Chen, L.; Lu, W.; Agarwal, S.; Zhou, W.; Zhang, R.; Chen, J. *Mol. Med.* **1999**, *5*, 21-34. (b) Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. *Science*, **1996**, *274*, 948-953.

16) Chene, P. *Mol. Cancer Res.* **2004**, *2*, 20-28.

17) Stoll, R.; Renner, C.; Hansen, S.; Palme, S.; Klein, C.; Belling, A.; Zeslawski, W.; Kamionka, M.; Rehm, T.; Muhlhahn, P.; Schumacher, R.; Hesse, F.; Kaluza, B.; Voelter, W.; Engh, R. A.; Holak, T. A., *Biochemistry* **2001**, *40*, 336-44.

18) Zhao, J.; Wang, M.; Chen, J.; Luo, A.; Wang, X.; Wu, M.; Yin, D.; Liu, Z. *Cancer Lett.* **2002**, *183*, 69-77.

19) Yin, H.; Lee, G.-Y.; Park, H. S.; Payne, G. A.; Rodriguez, J. M.; Sebti, S. M.; Hamilton, A. D. *Angew. Chem. Int. Ed.* **2005**, *44*, 2704-2707.

20) Duncan, S. J.; Gruschow, S.; Williams, D. H.; McNicholas, C.; Purewal, R.; Hajek, M.; Gerlitz, M.; Martin, S.; Wrigley, S. K.; Moore, M. *J. Am. Chem. Soc.* **2001**, *2*, 554-560.

21) Myers, M. C.; Wang, J.; Iera, J. A.; Bang, J. -K.; Hara, T.; Saito, S.; Zambetti, G. R.; Appella, D. H. *J. Am. Chem. Soc.* **2005**, *127*, 6152.

22) Vassilev, L. T.; Vu, B. T.; Graves, B.; Carvajal, D.; Podlaski, F.; Filipovic, Z.; Kong, N.; Kammlott, U.; Lukacs, C.; Klein, C.; Fotouhi, N.; Liu, E. A. *Science* **2004**, *303*, 844-8.

23) (a) Appella, E.; Anderson, C. W. *Eur. J. Biochem.* **2001**, *268*, 2764-72. (b) Kapoor, M.; Lozano, G. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2834-2837.

24) (a) Ashcroft, M.; Kubbutat, M. H.; Vousden, K. H. *Mol. Cell. Biol.* **1999**, *19*, 1751-1759. (b) Wu, Z.; Earle, J.; Saito, S.; Anderson, C. W.; Appella, E. *Mol. Cell. Biol.* **2002**, *22*, 2441-2449. (c) Thompson, T.; Tovar, C.; Yang, H.; Carvajal, D.; Vu, B. T.; Xu, Q.; Wahl, G. M.; Heimbrook, D. C.; Vassilev, L. T., *J. Biol. Chem.* **2004**, *279*, 53015-22.

25) (a) Vogelstein, B.; Lane, D.; Levine, A. J. *Nature* **2000**, *408*, 307-310. (b) Vousden, K. H.; Lou, X. *Nat. Rev. Cancer* **2002**, *2*, 594-604.

26) Levine, A. J. *Cell* **1997**, *88*, 323-331.

27) (a) Elledge, S. J. *Science*, **1996**, *274*, 1664. (b) Martin, N. M.B. *J. Photochem. Photobiol. B: Biology* **2001**, *63*, 162-170. (c) Hartwell, L. H.; Kastan, M. B. *Science* **1994**, *266*, 1821–1828.

28) (a) Zhou, B. -B.; Elledge, S. J. *Nature*, **2000**, *408*, 433-439. (b) Abraham, R. T. *Genes Dev.* **2001**, *15*, 2177-2196.

29) (a) Cortez, D.; Wang, Y.; Qin, J.; Elledge, S. J. *Science* **1999**, *286*, 1162-1166. (b) Lim, D.- S.; Kim, S.-T.; Xu, B.; Maser, R. S.; Lin, J. Y.; Petrini, J. H. J.; Kastan, M. B. *Nature* **2000**, *404*, 613-617. (c) Zhao, S.; Weng, Y.- C.; Yuan, S. -S. F. ; Lin, Y.- T.; Hsu, H. -C.; Lin, S.-C. J.; Gerbino, E.; Song, M.- H.; Zdzienicka, M. Z.; Gatti, R. A.; Shay, J. W., Ziv, Y. S.; Lee, E. Y.-H. P. *Nature* **2000**, *405*, 473. (d) Gatei, M.; Young, D.; Cerosaletti, K. M.; Desai-Mehta, A.; Spring, K.; Kozlov, S.; Lavin, M. F.; Gatti, R. A.; Concannon, P.; Khanna, K. *Nature Genet.* **2000**, *25*, 115-119.

30) (a) Martin, S. G.; Laroche, T.; Suka, N.; Grunstein, M.; Gasser, S. M. *Cell* **1999**, *97*, 621-633. (b) Mills, K. D., Sinclair, D. A.; Guarente, L. *Cell* **1999**, *97*, 609-620.

31) Ritchie, K. B.; Mallory, J. C.; Petes, T. D. *Mol. Cell. Biol.* **1999**, *19*, 6065-6075.

32) (a) Lowe, S. W.; Schmitt, E. M.; Smith, S. W.; Osborne, B. A.; Jacks, T. Nature **1993**, *362*, 847-849. (b) Clarke, A. R.; Purdie, C. A.; Harrison, D. J. ; Morris, R. G.; Bird, C. C.; Hooper M. L.; Wyllie, A. H. *Nature* **1993**, *362*, 849-852. (c) Xu, Y. Baltimore, D. *Genes Dev.* **1996**, *10*, 2401-2410. (d) Hirao, A.; Kong, Y. Y.; Matsuoka, S.; Wakeham, A.; Ruland, J.; Yoshida, H.; Liu, D.; Elledge, S. J.; Mak, T. W. *Science* **2000**, *287*, 1824-1827. (e) Woo, R. A.; McLure, K. G.; Lees-Miller, S. P.; Rancourt, D. E.; Lee, P. W. *Nature* **1998**, *394*, 700-704.

33) Jimenez, G.S.; Bryntesson, F.; Torres-Arzayus, M.I.; Priestley, A.; Beeche, M.; Saito, S.; Sakaguchi, K.; Appella, E.; Jeggo, P. A.; Taccioli, G.E.; Wahl, G.M.; Hubank, M. *Nature* **1999**, 400 81-3.

34) Volkmer, E.; Karnitz, L. M. *J. Biol. Chem.* **1999**, *274*, 567-570.

35) Green, C. M.; Erdjument-Bromage, H.; Tempst, P.; Lowndes, N. F. *Curr. Biol.* **2000**, *10*, 39-42.

36) Edwards, R. J.; Bentley, N. J.; Carr, A. M. *Nature Cell Biol.* **1999**, *1*, 393-398.

37) (a) Brown, E. J.; Baltimore, D. *Genes Dev.* **2000**, *14*, 397-402.(b) de Klein, A.; Muijtjens, M.; van Os, R.; Verhoeven, Y.; Smit, B.; Carr, A.; Lehmann, A.; Hoeijmakers, J. *Curr. Biol.* **2000**, *10*, 479-482. (c) Liu, Q.; Guntuku, S.; Cui, X. S.; Matsuoka, S.; Cortez, D.; Tamai, K.; Luo, G.; Carattini-Rivera, S.; DeMayo, F.; Bradley, A.; Donehower, L. A.; Elledge, S. J. *Genes Dev.* **2000**, *14*, 1448-1459.

38) (a) Paulovich, A. G.; Toczyski, D. P.; Hartwell, L. H. *Cell* **1997**, 88, 315-21. (b) Paulovich, A. G.; Hartwell, L. H., *Cell* **1995**, 82, 841-7.

39) (a)Cliby, W. A.; Roberts, C. J.; Cimprich, K. A.; Stringer, C. M.; Lamb, J. R.; Schreiber, S. L.; Friend, S. H. *EMBO J.* **1998**, *17*, 159-169.(b) Wright, J. A.; Keegan, K. S.; Herendeen, D. R.;Bentley, N. J.; Carr, A. M.; Hoekstra, M. F.; Concannon, P. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 7445-7450 (c) Savitsky, K.; Bar-Shira, A.; Gilad, S.; Rotman, G.; Ziv, Y.; Vanagaite, L.; Tagle, D. A.; Smith, S.; Uziel, T.; Sfez, S.; Ashkenazi, M.; Pecker, I.; Frydman, M.; Harnik, R.; Patanjali, S. R.; Simmons, A.; Clines, G. A.; Sartiel, A.; Gatti, R. A.; Chessa, L.; Sanal, O.; Lavin, M. F.; Jaspers, N. G. J.; Taylor, A. M. R.; Arlett, C. F.; Miki, T.; Weissman, S. M.; Lovett, M.; Collins, F. S.; Shiloh, Y. *Science* **1995**, *268*, 1749-53. (d) Matsuoka, S.; Huang, M.; Elledge, S. J. *Science* **1998**, *282*, 1893-1897. (e) Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. *Science* **1997**, *277*, 1497-1501. (f) Chaturvedi, P.;Eng, W. K.; Zhu, Y.;Mattern, M. R.; Mishra, R.; Hurle, M. R.; Zhang, X.; Annan, R. S.; Lu, Q.; Faucette, L. F.; Scott, G. F.; Li, X.; Carr, S. A.; Johnson, R. K.; Winkler, J. D.; Zhou, B. B. *Oncogene* **1999**, *18*, 4047-4054.

40) Blasina, A.; Van de Weyer, I.; Laus, M. C.; Luyten, W. H. M. L.; Parker, A. E.; McGowan, C. *Curr. Biol.* **1999**, *9*, 1-10.

41) (a) Peng, C.-Y.; Graves, P. R.; Thoma, R. S.; Wu, Z.; Shaw, A. S.; Piwnica-Worms, H. *Science* **1997**, *277*, 1501-1505. (b) Yang, J.; Winkler, K.; Yoshida, M.; Kornbluth, S. *EMBO J.* **1999**, *18*, 2174-2183. (c) Dalal, S. N.; Schweitzer, C. M.; Gan, J.; DeCaprio, J. *Mol. Cell. Biol.* **1999**, *19*, 4465-4479. (d) Zeng, Y.; Piwnica-Worms, H. *Mol. Cell. Biol.* **1999**, *19*, 7410-7419.

42) Esashi, F.; Yanagida, M. *Mol. Cell.* **1999**, *4*, 167–174.

43) Toczyski, D. P.; Galgoczy, D. J. ; Hartwell, L. H. *Cell* **1997**, 90, 1097–1106.

44) Evan, G.; Littlegood, T. *Science*, **1998**, *281*, 1317-1322.

45) Meyn, M. S. *Clin Genet* **1999**, *55*, 289–304.

46) Sandall, L. L; Zakian, V. A. *Cell* **1993**, *75*, 729-739.

47) Dixon, H.; Norbury, C. J. *Cell cycle* **2002**, *1*, 362-368.

48) (a)Sørensen, C. S.; Syljuåsen, R.G.; Falck, J.; Schroeder, T.; Rönnstrand, L.; Zhou, B.-B.; Bartek, J.; Lukas, J. *Cancer Cell* **2003**, *3*, 247–258. (b) Curman, D.; Cinel, B.; Williams, D. E.; Rundle, N.; Block, W. D.; Goodarzi, A. A.; Hutchins, J. R.; Clarke, P. R.; Zhou, B. B.; Lees-Miller, S. P.; Andersen, R. J.; Roberge, M. *J. Biol. Chem.* **2001**, 276, 17914-9. (c) Jackson, J. R.; Gilmartin, A.; Imburgia, C.; Winkler, J. D.; Marshall, L. A.; Roshak, A. *Cancer Res.* **2000**, 60, 566-72. (d) Roberge, M.; Berlinck, R. G.; Xu, L.; Anderson, H. J.; Lim, L. Y.; Curman, D.; Stringer, C. M.; Friend, S. H.; Davies, P.; Vincent, I.; Haggarty, S. J.; Kelly, M.

T.; Britton, R.; Piers, E.; Andersen, R. J. *Cancer Res.* **1998**, 58, 5701-6.

49) Meijer, L.; Raymond, E. *Acc. Chem. Res.* **2003**, *36*, 417-425.

50) Markwalder, J. A.; Arnone, M. R.; Benfield, P. A.; Boisclair, M.; Burton, C. R.; Chang, C. H.; Cox, S. S.; Czerniak, P. M.; Dean, C. L.; Doleniak, D.; Grafstrom, R.; Harrison, B. A.; Kaltenbach, R. F., 3rd; Nugiel, D. A.; Rossi, K. A.; Sherk, S. R.; Sisk, L. M.; Stouten, P.; Trainor, G. L.; Worland, P.; Seitz, S. P. *J. Med. Chem.* **2004**, *47*, 5894-5911.

51) Misra, R. N.; Xiao, H. Y.; Kim, K. S.; Lu, S.; Han, W. C.; Barbosa, S. A.; Hunt, J. T.; Rawlins, D. B.; Shan, W.; Ahmed, S. Z.; Qian, L.; Chen, B. C.; Zhao, R.; Bednarz, M. S.; Kellar, K. A.; Mulheron, J. G.; Batorsky, R.; Roongta, U.; Kamath, A.; Marathe, P.; Ranadive, S. A.; Sack, J. S.; Tokarski, J. S.; Pavletich, N. P.; Lee, F. Y.; Webster, K. R.; Kimball, S. D., *J. Med. Chem.* **2004**, *47*, 1719-1728.

52) (a) Misra, R. N.; Rawlins, D. B.; Xiao, H. Y.; Shan, W.; Bursuker, I.; Kellar, K. A.; Mulheron, J. G.; Sack, J. S.; Tokarski, J. S.; Kimball, S. D.; Webster, K. R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1133-6. (b) Misra, R. N.; Xiao, H.; Rawlins, D. B.; Shan, W.; Kellar, K. A.; Mulheron, J. G.; Sack, J. S.; Tokarski, J. S.; Kimball, S. D.; Webster, K. R. *Bioorg. Med. Chem. Lett.* **2003**, 13, 2405-8.

53) Moshinsky, D. J.; Bellamacina, C. R.; Boisvert, D. C.; Huang, P.; Hui, T.; Jancarik, J.; Kim, S. H.; Rice, A. G. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 1026-31.

54) Luk, K. C.; Simcox, M. E.; Schutt, A.; Rowan, K.; Thompson, T.; Chen, Y.; Kammlott, U.; DePinto, W.; Dunten, P.; Dermatakis, A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 913-917.

55) Mettey, Y.; Gompel, M.; Thomas, V.; Garnier, M.; Leost, M.; Ceballos-Picot, I.; Noble, M.; Endicott, J.; Vierfond, J. M.; Meijer, L. *J. Med. Chem.* **2003**, *46*, 222-36.

56) Seong, Y.; Min, C; Li, L.; Yang, J. Y.; Kim, S.; Cao, X.; Kim, K.; Yuspa, S. H.; Chung, H.; Lee, K. S. *Cancer Res.* **2003**, *63*, 7384-7391.

57) Li, Y.; Sun, X.; LaMont, J.T.; Pardee, A. B.; Li, C. J. *Proc. Natl. Acad. Sci. USA* **2003** *100*, 2674–2678.

58) Bartek, J.; Lukas, J. *Cancer cell* **2003**, *3*, 421-429.(b)Takai, H.; Naka, K.; Okada, Y.; Watanabe, M.; Harada, N.; Saito, S.; Anderson, C. W.; Appella, E.; Nakanishi, M.; Suzuki, H.; Nagashima, K.; Sawa, H.; Ikeda, K.; Motoyama, N. *EMBO J.* **2002**, *21*, 5195-5205.

59) Sharma, V.; Tepe, J. J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4319-4321.

60) Sørenson, C. S.; Syljuåsen, R. G.; Falck, J.; Schroeder, T.; Rönnstrand, L. ; Khanna, K. K.; Zhou, B.-B.; Bartek, J. ; Lukas, J. *Cancer cell*, **2003**, *3*, 247-258.

61) Mailand, N.; Falck, J.; Lukas, C.; Syljuasen, R.G.; Welcker, M.; Bartek, J. ; Lukas, J. *Science* , **2000**, *288*, 1425–1429.

62) Falck, J.; Petrini, J. H.; Williams, B. R.; Lukas, J.; Bartek, J. *Nat. Genet.* **2002**, *30*, 290–294.

63) Mailand, N.; Podtelejnikov, A.V.; Groth, A.; Mann, M.; Bartek, J.; Lukas, J. *EMBO J.* **2002**, *21*, 5921–5929.

64) Baeuerle, P. A.; Henkel, T. *Annu. Rev. Immunol.* **1994**, *12*, 141-179.

65) Baldwin, A. S., Jr. *Annu. Rev. Immunol.* **1996**, *14*, 649-683.

66) Perkins, N. D. *Int. J. Biochem. Cell Biol.* **1997**, *29*, 1433-1448.

67) Webster, G.A.; Perkins, N. D. *Mol. Cell Biol.* **1999**, *19*, 3485-3495.

68) (a) Das, K.C.; White, C. W. *J. Biol. Chem.* **1997**, *272*, 14914-14920.(b) Baldwin, A. S. *J. Clin. Invest.* **2001**, *107*, 241-246. (c) Hideshima, T.; Chauhan, D.; Richardson, P., Mitsiades, C.; Mitsiades, N.; Hayashi, T.; Munshi, N.; Dang, L.; Castro, A.; Palombella, V.; Adams, J.; Anderson, K. C. *J. Biol. Chem.* **2002**, *277*, 16639-16647. (d) Bottero, V.; Busuttil, V.; Loubat, A.; Magne, N.; Fischel, J. L.; Milano, G.; Peyron, J. F. *Cancer Res.* **2001**, *61*, 7785-7791. (e) Um, J. H.; Kang, C. D.; Lee, B. G.; Kim, D. W.; Chung, B. S.; Kim, S. H. *Oncogene* **2001**, *20*, 6048-6056. (f) Weldon, C. B.; Burow, M. E.; Rolfe, K. W.; Clayton, J. L.; Jaffe, B. M.; Beckman, B. S. *Surgery* **2001**, *130*, 143-150. (g) Arlt, A.; Vorndamm, J.; Breitenbroich, M.; Folsch, U. R.; Kalthoff, H.; Schmidt, W. E.; Schafer, H. *Oncogene* **2001**, *20*, 859-868. (h) Liu, S.; Yu, Y.; Zhang, M.; Wang, W.; Cao, X. *J. Immunol.* **2001**, *166*, 5407-5415. (i) Kim, J.Y.; Lee, S.; Hwangbo, B.; Lee, C.T.; Kim, Y. W.; Han, S. K.; Shim, Y. S.; Yoo, C. G. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 140-146.

69) Lin, Y. C.; Brown, K.; Siebenlist, U. *Proc. Natl. Acad. Sci. U S A* **1995**, *92*, 552-556.

70) (a) Karin, M.; Lin, A. *Nat. Immunol.* **2002**, *3*, 221-227. (b) Zhong, H.; SuYang, H.; Erdjument-Bromage, H.; Tempst, P.; Ghosh, S. *Cell* 1997, *89*, 413-424. (c) Sizemore, N.; Leung, S.; Stark, G. R. *Mol. Cell Biol.* **1999**, *19*, 4798-4805. (d) Madrid, L.V.; Wang, C.Y.; Guttridge, D.C.; Schottelius, A. J.; Baldwin, A. S., Jr.; Mayo, M. W. *Mol. Cell Biol.* **2000**, *20*, 1626-1638.

71) Wu, M. X.; Ao, Z.; Prasad, K. V.; Wu, R.; Schlossman, S. F. *Science* **1998**, *281*, 998-1001.

72) Perkins, N. D.; Felzien, L. K.; Betts, J. C.; Leung, K.; Beach, D. H.; Nabel, G. J., *Science* **1997**, *275*, 523-7.

73) (a) Deng, C.; Zhang, P.; Harper, J. W.; Elledge, S. J.; Leder, P. *Cell* **1995**, 82, 675-84. (b) el-Deiry, W. S.; Harper, J. W.; O'Connor, P. M.; Velculescu, V. E.; Canman, C. E.; Jackman, J.; Pietenpol, J. A.; Burrell, M.; Hill, D. E.; Wang, Y.; Wiman, K. G.; Mercer, W. E.; Kastan , M. B.; Kohn, K. W.; Elledge, S. J.; Kinzler, K. W.; Vogelstein, B. *Cancer Res.* **1994**, 54, 1169-74.

74) Miyashita, T.; Reed, J. C. *Cell* **1995**, *83*, 293-299.

75) (a)Perkins, N. D. *Int. J. Biochem. Cell Biol.* **1997**, *29*, 1433-1448.(b) Nakshatri, H.; Bhat-Nakshatri, P.; Martin, D. A.; Goulet, R. J., Jr.; Sledge, G. W., Jr. *Mol. Cell. Biol.* **1997**, *17*, 3629–3639.

76) Hietanen, S.; Lain, S.; Krausz, E; Blattner, C.; Lane, D. P. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8501-8506.

77) Maurici, D.; Monti, P.; Campomenosi, P.; North, S.; Frebourg, T.; Fronza, G.; Hainaut, P. *Oncogene* **2001**, *20*, 3533-3540.

78) Reuther, J. Y.; Reuther, G. W.; Cortez, D.; Pendergast, A. M.; Baldwin, A. S., Jr. *Genes Dev.* **1998**, *12*, 968-81.

79) (a) Madhusudan, S.; Ganesan, T. S. *Clinical Biochem.* **2004**, *37*, 618-635.(b) Druker, B. J.; Sawyers, C. L.; Kantarjian, H.; Resta, D.J.; Reese, S. F.; Ford, J. M.; Capdeville, R.; Talpaz, M. *N. Engl. J. Med.* **2001**; *344*, 1038–42.

80) Nimmanapalli, R.; Bhalla, K. *Curr. Opin. Oncol.* **2002**, 14, 616-620.

81) Karin, M.; Ben-Neriah, Y. *Ann. Rev. of Immunol.* **2000**, *18*, 621-663.

82) (a) Chen, F.E.; Huang, D.-B.; Chen, Y.- Q.; Ghosh, G. *Nature*, **1998**, *391*, 410-413.(b) Chen, Y. Q.; Ghosh, S.; Ghosh, G. *Nat. Struct. Biol.*, **1998**, *5*, 67-73.

83) (a) Baeuerle, P. A.; Baichwal, V. R. *Adv. Immunol.* **1997**, *65*, 111-137. (b) Barnes, P. J.; Karin, M. *N. Engl. J. Med.* **1997**, 336, 1066-1071. (c) Siebenlist, U.; Franzoso, G.; Brown, K. *Annu. Rev. Cell Biol.* **1994**, *10*, 405-455.

84) Baichwal, V. R.; Baeuerle, P. A. *Curr. Biol.* **1997**, *7*, R94-96.

85) Beg, A. A.; Sha, W. C.; Bronson, R. T.; Ghosh, S.; Baltimore, D. *Nature* **1995**, *376*, 167–170.

86) Wang, C. –Y.; Mayo, M. W.; Baldwin, A. S. Jr, *Science* **1996**, *274*, 784–787.

87) (a) Sharma, V.; Lansdell, T. A.; Peddibhotla, S.; Tepe, J. J. *Chem. Biol.* **2004**, 11, 1689-99. (b) Huang, T. T.; Wuerzberger-Davis, S. M.; Seufzer, B. J.; Shumway, S. D.; Kurama, T.; Boothman, D. A.; Miyamoto,S. *J. Biol. Chem.* **2000**, 275, *13*, 9501-9509. (c) Yeh, P. Y.; Chuang, S.-E.; Yeh, K. H.; Song, Y.C.; Cheng, A.-L. *Biochem. Pharmacol.* **2003**, *66*, 25-33. (d) Sodhi, A.; Singh, R. A. K. *Immunology Lett.* **1998**, *63*, *9-17.*

88) Grilli, M; Pizzi, M; Memo, M; Spano, P. F. *Science* **1996**, *274*, 1383–1385.

89) Amann, R; Peskar, B. A. *Eur. J. Pharmacol.* **2002**, *447*, 1-9.

90) Carter, B. D.; Kaltschimdt, C.; Kaltschimdt, B.; Offenhauser, N.; Bohm-Matthaei, R.; Baeuerle, P. A.; Barde, Y. –A. *Science* **1996**, *272*, 542–545.

91) Grimm, S.; Bauer, M. K. A.; Baeuerle, P. A.; Schulze-Osthoff, K. *J. Cell. Biol.* **1996**, *134*, 13–23.

92) Lin, K. –I. ; Lee, S. –H.; Narayanan, R.; Baraban, J. M.; Hardwick, J. M. Ratan, R. R. *J Cell Biol* **1995**, *131*, 1149–1161.

93) Doble, B. W.; Woodgett, J. R. *J. Cell. Sci.* **2003**, 116, (Pt 7), 1175-86.

94) (a) Hoeflich K. P.; Luo J.; Rubie E. A.; Tsao M. S.; Jin O.; Woodgett J. R. *Nature* **2000**, *406*, 86–90. (b) Takada Y.; Fang X.; Jamaluddin M. S.; Boyd D. D.; Aggarwal, B. B. *J. Biol. Chem.* **2004**, *279*, 39541–54.

95) Arlt, A.; Gehrz, A.; Muerkoster, S.; Vorndamm, J.; Kruse, M. L.; Folsch, U. R.; Schafer, H. *Oncogene* **2003**, *22*, 3243-51.

96) Watanabe, I.; Hasebe, T.; Sasaki, S.; Konishi, M.; Inoue, K.; Nakagohri, T.; Oda, T.; Mukai, K.; Kinoshita, T. *Pancreas* **2003**, *26*, 326-33.

97) Ougolkov, A.V.; Fernandes-Zapico, M. E.; Savoy, D. N.; Urrutia, R. A.; Billadeau, D. D. *Cancer Res.* **2005**, *65*, 2076-2081.

98) Schwabe, R. F.; Brenner, D. A. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2002**, *283*, G204-11.

99) Heinemann V. *Oncology* **2001**, *60*, 8-18.

100) Tsai, C. -M.; Chang, K. -T.; Chen, J. -Y.; Chen, Y. -M.; Chen, M. -H.; Perng, R.-P. *Cancer Res.* **1996**, *56*, 794-801.

101) http://www.cancerbacup.org.uk/Treatments/Chemotherapy/Individualdrugs/Gemcitabine. (accessed 09/10/05) (b) Robinson, B. W.; Shewach, D. S. *Clin. Cancer Res.* **2001**, *7*, 2581-2589.

102) Orlowski, R. Z.; Baldwin, A. S., Jr., *Trends in Mol. Med.* **2002**, *8*, 385-389.

103) Wan, Y.; Hur, W.; Cho, C. Y.; Liu, Y.; Adrian, F. J.; Lozach, O.; Bach, S.; Mayer, T.; Fabbro, D.; Meijer, L.; Gray, N. S. *Chem. Biol.* **2004**, *11*, 247-59.

104) Cuzzocrea, S.; Chatterjee, P. K.; Mazzon, E.; Dugo, L.; Serraino, I.; Britti, D.; Mazzullo, G.; Caputi, A. P.; Thiemermann, C. *Br. J. Pharmacol.* **2002**, *135*, 496-510.

105) Lenz, W. *Teratology* **1988**, *38*, 203-215.

106) Keifer, J. A.; Guttridge, D. C.; Ashburner, B. P.; Baldwin, A. S., Jr., *J. Biol. Chem.* **2001**, *276*, 22382-7.

107) Yoshida, S.; Ono, M.; Shono, T.;Izumi, H.; Ishibashi, T.; Suzuki, H.; Kuwano, M. *Mol. Cell. Biol.* **1997**, *17*, 4015-4023.

108) Koch, A. E.; Polverini, P. J.; Kunkel, S. L.; Harlow, L. A.; DiPietro, L. A.; Elner, V. M.; Elner, S. G.; Strieter, R. M. *Science* **1992**, *258*, 1798-1801.

109) Kapahi, P.; Takahashi, T.; Natoli, G.; Adams, S. R.; Chen, Y.; Tsien, R. Y.; Karin, M. *J. Biol. Chem.* **2000**, 275, 36062-6.

110) Kwok, B. H.; Koh, B.; Ndubuisi, M. I.; Elofsson, M.; Crews, C. M. *Chem. Biol.* **2001**, 8, 759-66.

111) Yamamoto, Y.; Yin, M. –J.; Lin, K.-M.; Gaynor, R. B. *J. Biol. Chem.* **1999**, *274*, 38, 27307-27314.

112) Sagara, H.; Makino, S.; Chibana, N.; Ota, M.; Holgate, S. T.; Church, M. K.; Fukuda, T. *Int. Arch. Allergy Immunol.* **2001**, *124*, 371-376.

113) (a) Kopp, E.; Ghosh, S. *Science* **1994**, *265*, 956-959.(b) Yin, M.-J.;Yamamoto, Y.; Gaynor, R. B. *Nature* **1998**, *396*, 77-80.

114) (a) Thun, M. J.; Namboodiri, M. M.; Heath, C. W., Jr. *N. Engl. J. Med.* **1991**, *325*, 1593-1596. (b) Suh, O., Mettlin, C., and Petrelli, N. J. *Cancer (Phila.)* **1993**, *72*, 1171-1177 (c) Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., and Willett, W. C. *Ann. Intern. Med.* **1994**, *121*, 241-246. (d) Giovannucci, E., Egan, K. M., Hunter, D. J., Stampfer, M. J., Colditz, G. A., Willett, W. C., and Speizer, F. E. *N. Engl. J. Med.* **1991**, *333*, 609-614.

115) Pickart, C. M.; Eddins, M. J. *Biochem. Biophy. Acta* **2004**, *1695*, 55-72.

116) Vescio, R. A.; Berenson, J. R. *In*: J. S. Berek (ed.), Cancer Treatment, 5th ed., **2001**, 1503-1539.

117) Feinman, R.; Koury, J.; Thames, M.; Barlogie, B.; Epstein, J.; Siegel, D. S. *Blood* **1999**, 93, 3044-52.

118) Ma, M. H.; Yang, H. H.; Parker, K.; Manyak, S.; Friedman, J. M.; Altamirano, C.; Wu, Z. Q.; Borad, M. J.; Frantzen, M.; Roussos, E.; Neeser, J.; Mikail, A.; Adams, J.; Sjak-Shie, N.; Vescio, R. A.; Berenson, J. R. *Clin. Cancer Res.* **2003**, 9, 1136-44.

119) (a) Schmitt, E.; Cimoli, G.; Steyaert, A.; Bertrand, R. *Exp. Cell Res.* **1998**, 240, 107-121. (b) Letai, A.; Bassik, M. C.; Walensky, L. D.; Sorcinelli, M. D.; Weiler, S.; Korsmeyer, S. J. *Cancer Cell* **2002**, *2*, 183-192. (c) Webb, A.; Cunningham, D.; Cotter, F.; Clark, P. A.; di Stephano, F.; Ross, P.; Corbo, M.; Dziewanowska, Z. *Lancet* **1997**, *349*, 1137.

120) Vayssiere, J. L.; Petit, P. X.; Risler, Y.; Mignotte, B. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11752.

121) (a) Lindsten, T.; Ross, A. J.; King, A.; Zong, W. X.; Rathmell, J. C.; Shiels, H. A.; Ulrich, E.; Waymire, K. G.; Mahar, P.; Frauwirth, K.; Chen, Y.; Wei, M.; Eng, V. M.; Adelman, D. M.; Simon, M. C.; Ma, A.; Golden, J. A.; Evan, G.; Korsmeyer, S. J.; MacGregor, G. R.; Thompson, C. B. *Mol. Cell* **2000**, *6*, 1389. (b) Wei, M. C.; Zong, W. X.; Cheng, E. H.; Lindsten, T.; Panoutsakopoulou, V.; Ross, A. J.; Roth, K. A.; MacGregor, G. R.; Thompson, C. B.; Korsmeyer, S. J. *Science*, **2001**, *292*, 727.

122) (a) Antonsson, B.; Montessuit, S.; Sanchez, B.; Martinou, J. C. *J. Biol. Chem.* **2001**, *276*, 11615.(b) Eskes, R.; Desagher, S.; Antonsson, B.; Martinou, J. C. *Mol. Cell. Biol.* **2000**, *20*, 929.

123) (a) Nechushtan, A.; Smith, C. L.; Lamensdorf, I.; Yoon, S. H.; Youle, R. J. *J. Cell Biol.* **2001**, *153*, 1265.(b) Griffiths, G. J.; Dubrez, L.;Morgan, C. P.; Jones, N. A.; Whitehouse, J.; Corfe, B. M.; Dive, C.; Hickman, J. A. *J. Cell Biol.* **1999**, *144*, 903.

124) Tsujimoto, Y.; Cosmann, J.; Jaffe,E.; Croce, C. *Science*, **1985**, *228*, 1440.

125) Campos, L.; Rouault, J. P.; Sabido, O.; Oriol, P.; Roubi, N.; Vasselon, C.; Archimbaud, E.; Magaud, J. P.; Guyotat, D. *Blood* **1993**, *81*, 3091.

126) Hermine, O.; Haioun,C.; Lepage, E.; d'Agay, M. F.; Briere, J.; Lavignac, C.; Fillet, G.; Salles, G.; Marolleau, J. P.; Diebold, J.; Reyas, F.; Gaulard, P. Blood,

**1996**, *87*, 265.

127) Sturm, I.; Kohne, C. H.; Wolff, G.; Petrowsky, H.; Hillebrand, T.; Hauptmann, S.; Lorenz, M.; Dorken, B.; Daniel, P. T. *J. Clin. Oncol.* **1999**, *17*, 1364.

128) Schiller, A.B.; Clark, W. S.; Cotsonis, G.; Lawson, D.; DeRose, P. B.; Cohen, C. *Cytometry*, **2002**, *50*, 203.

129) Jansen, B.; Zangemeister-Wittke, U. *Lancet Oncol.* **2002**, *3*, 672.

130) Donis-Keller, H. *Nucleic Acids Res.* **1979**, *7*, 179.

131) De Cesare, M.; Perego, P.; Righetti,S. C.; Pratesi , G.; Carenini ,N.; Rivoltini ,L.; Zupi,G.; Del Bufalo, D.; Balsari, A.; Zunino, F. *Eur. J. Cancer* **2005**, *41*, 1213-1222.

132) (a) Klasa, R. J.; Gillum, A. M.; Klem, R. E.; Frankel, S. R. *Antisense Nucleic Acid Drug Dev* **2002**, *12*, 193-213.(b) Kim, R.; Emi, M.; Tanabe, K.; Toge, T. *Cancer* **2004**, *101*, 2491-2502.

133) Jansen, B.; Wacheck, V.; Heere-Ress, E.; Schlagbauer-Wadl, H.; Hoeller, C.; Lucas, T.;Hoermann, M.;Hollenstein, U.;Wolff, K.;Pehamberger, H. *Lancet* **2000**, *356*, 1728-1733.

134) Millward, M. J.; Bedikian, A. Y.; Conry, R. M.; Gore, H. E.; Pehamberger, W. Sterry; Pavlick, A. C.; De Conti, R. C.; Gordon, D.; Itri L. M. *Proc. Am. Soc. Clin. Oncol.* **2004**, *708*, 7505.

135) Kim, K. M.; Giedt, C. D.; Basanez, G.; O'Neil, J. W.; Hill, J. J.; Han, Y. H.; Tzung, S. P.;Zimmerberg, J.; Hockenbery, D. M.; Zhang, K. Y. *Biochemistry* **2001**, *40*, 4911.

136) Degeterev, A.; Lugovskoy, A.; Cardone, M.; Mulley, B.; Wagner, G.; Mitchison, T.; Yuan. J. *Nat. Cell Biol.* **2001**, *3*, 173-182.

137) Wang J. L.; Zhang, Z. J.; Chokski, S.; Shan, S.; Lu, Z.; Croce, C. m.; Alnemri, E. S.; Korngold, R.; Huang, Z. *Cancer Res.* **2000**, *60*, 1498.

# CHAPTER II

## 8-SUBSTITUTED XANTHINE DERIVATIVES: POTENTIATION OF CHEMOTHERAPY BY NF-κB INHIBITION

*Absence of evidence is not evidence for absence.....*

### A. Biological importance of xanthine scaffold

Extra cellular adenosine is involved in a variety of cytoprotective cellular responses like taming the heart against ischemia, counteracting the damaging effects of excitotoxicity and seizure activity and suppressing excessive inflammation and immune responses.[1] There are numerous possibilities for therapeutic intervention via modulation of adenosine receptors. Adenosine has a wide range of physiologocal activities arising from its interaction with the P1 purinoceptor, which is a class of G-protein-coupled receptor (GPCR) and has been further subclassified into four types - $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$.[2] Usually, the therapeutic interests pertaining adenosine receptors are related to anti-inflammatory effects, [3] cardio-protective, [4] cerebro-protective, [5] anti-cancer, [6] differentiation and embryogenesis[7] and anti-glaucoma [8] effects.

### B. Xanthine derivatives and inflammation related disorders.

Xanthine derivatives relax adult airway smooth muscle (ASM). Shaffer *et al* have shown a significant decrease in the active tension due to acetyl choline.[9] Several other reports show the bronchodilatory effect of caffeine and theophylline in asthmatic patients between 8-10 years of age.[10] Caffeine is also known to reduce the bronchoconstriction effect in mild asthma due to histamine.[11] Koc and coworkers performed animal models to show that pentoxyphylline usage may

reduce the risks of ischemic-reperfusion injury during and following cardiopulmonary bypass and aortic cross-clamping indicating that pentoxyphylline was shown to have preservative effects for myocardial functions in heart after trauma.[12a] Apart from adenosine itself and long-established antagonists such as theophylline and caffeine there are no new adenosine receptor agonist or antagonist drug molecules currently in clinical use. In view of the widespread actions of adenosine in the cardiovascular system and the possible role of adenosine in the pathogenesis of many diseases affecting the cardiovascular system, new agents that modulate adenosine actions have considerable therapeutic potential. The main diseases under consideration are hypertension, myocardial ischemia (infarction), cerebral ischemia (stroke), supraventricular tachycardia, renal failure, heart failure and peripheral ischemia (intermittent claudication). There is growing interest in the role of adenosine in inflammatory disorders as they relate to cerebrovascular and myocardial ischemia and actions of adenosine receptor agonists and antagonists on neutrophil and mast cell function. Applications in the treatment of renal dysfunction and as diuretics are claimed for novel adenosine $A_1$ receptor antagonists,[12b, 12c] treatment of depression,[12d] and treatment of supraventricular arrythmias[13a] dictate the potential of xanthine and related molecules in therapy.

Adenine derivatives such as reversine (Figure II-1) have been shown to be selective $A_3$ antagonists.[13a] Xanthines such as caffeine and theophylline are classical adenosine antagonists but of much low affinity for $A_3$ than for $A_1$, $A_{2A}$ or $A_{2B}$ receptors. PSB-10 and PSB-11 (Figure II-1) are water soluble derivatives of

xanthines and highly potent antagonists of adenosine. *In vivo* studies suggest that $A_3$ receptors are involved in a number of disorders of the central nervous and cardiovascular systems ($A_1$ and $A_{2A}$), pain ($A_1$) and wound healing ($A_{2A}$) as well as those in inflammation (diabetic foot ulcers ($A_{2A}$), colorectal cancer ($A_3$) and rheumatoid arthritis ($A_3$).[13b]

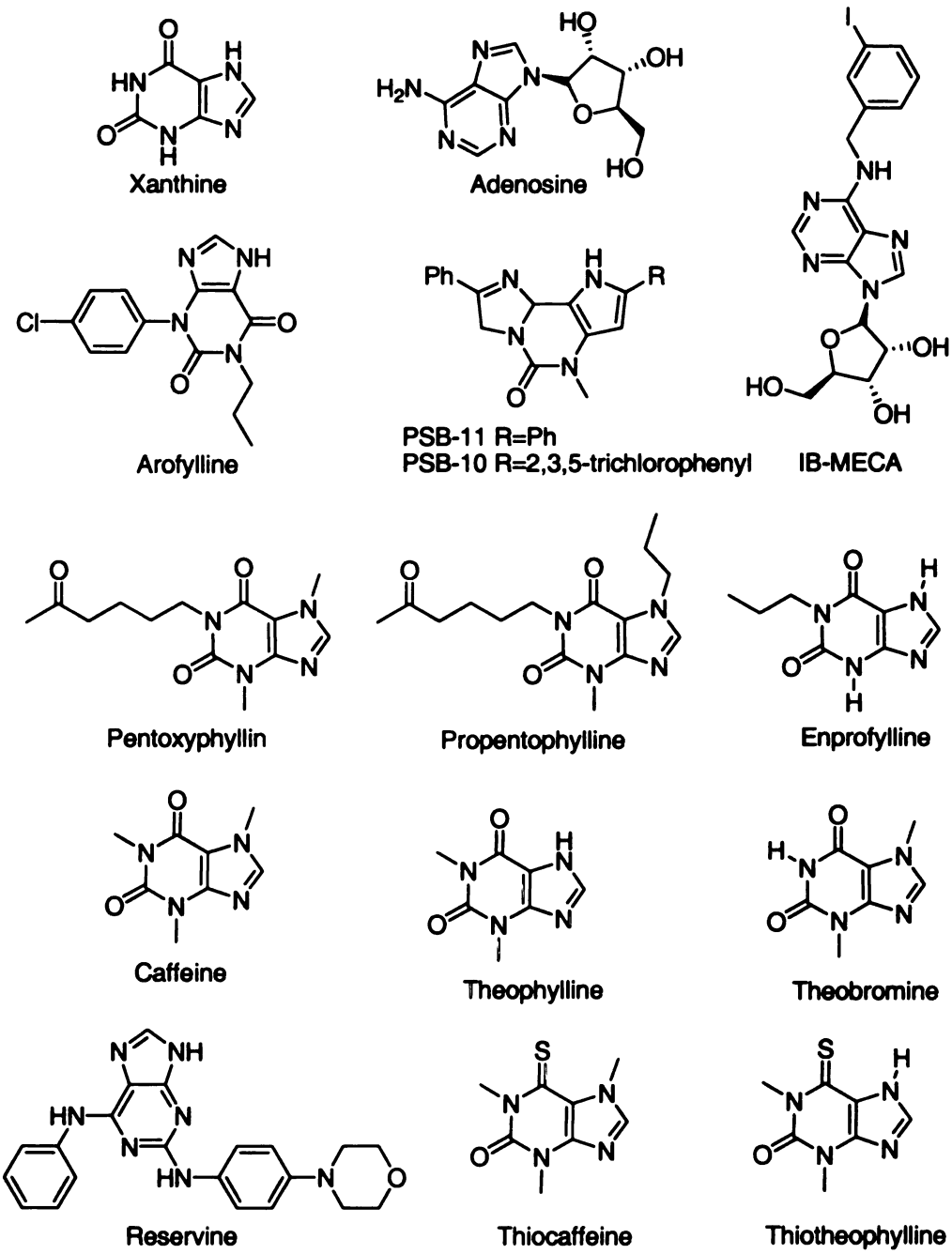



**Figure II-1.** Xanthine derivatives

The selective $A_3$ receptor agonist IB-MECA (Figure II-1) has been shown to induce behavioral depressant effects.[14a] Chronic treatment with IB-MECA improved the post-ischemic cerebral blood circulation while acute treatments showed opposite effects.[14b] A decrease in the intra cellular cAMP levels after

stimulation with CD28 causes T-cell activation. Phosphodiesterase (PDE4 or PDE7) appear to participate predominantly in this activation and inhibition of the PDE4 or PDE7 activity reduces T-cell activation and production of pro-inflammatory cytokines.[15]

**C. Controlling kinases: xanthines in cancer therapy**

An ideal target for small molecule drug discovery is one that is essential for the diseased state and non-essential for normal tissues. A recent emerging class of such as target is Bcr-Abl, which is targeted by the well known agent, Gleevec.[16] A common drug-like feature of the kinase family is their ability to bind to ATP. The ATP binds in a hydrophobic environment where it is stabilized by hydrogen-bonds.[17] Thus, planar, aromatic heterocyclic structures such as purines are amenable as kinase inhibitors and have made kinases eminent drug targets.

The cell cycle plays a vital role in the growth, development and central regulation of all life.[18] Deregulation of this control via accumulation of defects is one of the initial events in the development of cancer.[19] Cell cycle and cell division are is tightly regulated and organized by biochemical signals involving kinases. When the cell commits to divide, it prepares for division (late G1), duplicates DNA (S-phase), and separates the chromosomes into the daughter nuclei (M-phase). These transitions are mediated by CDKs, cyclins and also checkpoint kinases.[20] Cyclins and CDKs are regulatory partners and their association increases the catalytic activity of CDKs.[21] Mutants of CDKs either arrest cells at G2/M or could even block cell cycle at G1 stage.[22] CDK activity is deregulated in cancer.

Under normal conditions, CDKs (comprising of a catalytic CDK subunit and an activating cyclin), control various stages of cell cycle by acting as on/off switches. When a CDK is on, the cell cycle progresses through the stage that the particular CDK controls. When a CDK is off, the cell cycle stops when it reaches the stage controlled by that CDK.

**i) Regulation of CDKs:** When first synthesized the CDKs are not active, however, binding to cyclins imparts them partial activity (Figure II-2a).

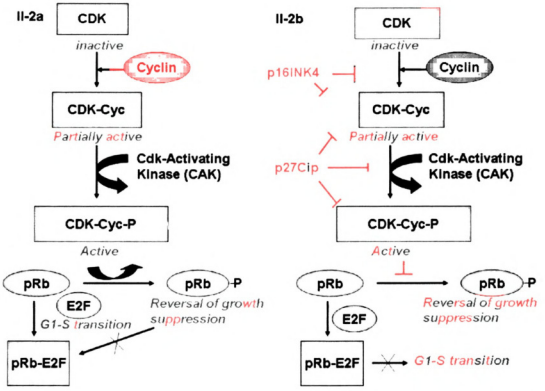
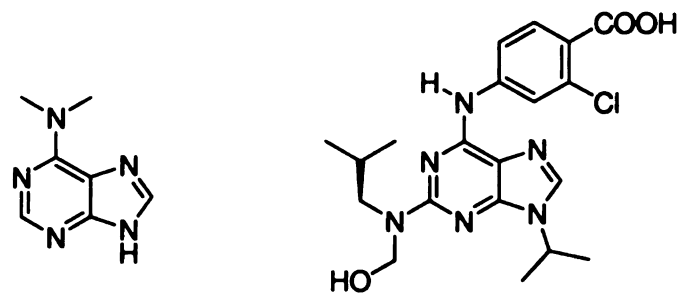



**Figure II-2a**. Schematic representation of regulation of CDK activity. **II-2b.** Schematic representation of regulation of CDK activity in presence of p16 or p27(inhibitors of CDK activity).

This is a key step since levels of cyclins are controlled by transcription and ubiquitin-mediated degradation.[21b] Cyclin binding activates the kinase by causing

conformational changes.[21a] Further, the regulation is controlled by a phosphorylation event on Thr by Cdk-Activating Kinase (CAK) which increases the activity of the complex >100 folds.[21c] p16INK and p27Cip cause inhibition of formation/accumulation of the active phosphorylated CDK:cyclin complex. p16INK causes allosteric changes in Cdk to propogate and alter the cyclin binding site. These allosteric changes by p16INK distort the adjacent ATP binding site leading to a reduction in the ATP affinity.[21d] p27 on the other hand, mimics ATP and alters the shape of the catalytic cleft.[21a] Hence, decrease in downregulators of CDK (such as p16, p21 or p27) or an increase in cyclin and CDKs would ultimately culminate in the same effect, an increase in cell proliferation.[23] Almost all cancers can thus be seen to have mutations or dysfunctional genes which can lead to increased CDK activity. [20] For example, decrease in p27 levels in breast cancer cells contributes to cell proliferation and is a prognostic marker of the disease. Over expression of cdc25A[24] and frequent mutations in tumor suppressor genes such as p16, p21, Rb e.g. hypermethylation, [25] exemplify a large variety of cancer mutations arising due to deregulation of CDKs. A large percentage of cancers also get an increased CDK activity due to alterations in signal transduction including Ras, cytoplasmic kinases etc.

Given the importance of CDKs in cell cycle control and the altered expression in cancers, there is a considerable effort focused on the development of small molecule inhibitors of CDKs or the enzymatic activity of the cyclin:CDK complex. 6-dimethyl aminopurine was the first inhibitor for CDK1, though it was found to be fairly non-specific and weak ($IC_{50}$=120 $\mu$M) (Figure II-3)




6-dimethylaminopurine
$IC_{50}$ (CDK1)=120 μM

Purvalanol-B
$IC_{50}$ (CDK1)=0.006 μM

**Figure II-3**. Structure of purine based kinase inhibitors

Caffeine and theophylline (Figure II-1) are naturally occurring methyl xanthine compounds often found in micromolar quantities in circulation as a result of dietary intake. Shepherd *et al* [26a] showed that caffeine and theophylline directly inhibit p110δ and other phosphoinositol kinases (PI3K). This inhibition of PI3K by methylxanthines could have beneficial therapeutic effects in at least three situations. Firstly, theophylline has been described to promote apoptosis while caffeine can sensitize cells to apoptosis[26b-d] indicating that these agents might prove to be useful in cancer chemotherapy directly or in combination with other clinically used drugs. The pro-apoptotic events may occur through inhibition of ATM – and Rad3 related kinase.[27] Class IA PI3Ks inhibit apoptosis, hence methylxanthines that are inhibitors of class IA PI3Ks would promote apoptosis.[28] This inhibition affecting PI3K, ATM and Rad-3 related kinase might be useful in treatment of cancer.[29] Class IB PI3K activity reduces ADP-induced platelet aggregation,[30] indicating that inhibition of PI3K may be useful in treating thrombosis. Finally, the p110δ isoform of class IA PI3Ks is expressed in a limited subpopulation of cells such as leukocytes and melanoma cells,[31] and a specific inhibitor of this isoform might prevent inflammation or cell migration.[32] However, long-term inhibition of p110δ is

likely to be undesirable because animals in which the p110δ gene is deleted show reduced B- and T-cell function and also develop inflammatory bowel disease.[33]

Effective anti-cancer therapy causes genotoxic agents to damage DNA and kill the dividing cells. However, in addition to causing apoptosis, genomic insults also trigger cell cycle checkpoint activation leading to checkpoint arrest augmenting the ability of the cells to repair the damaged DNA.[34, 35] Zhou *et al* showed that the alkaloid caffeine abolishes the mammalian G2/ATM DNA damaging checkpoint by inhibiting the ATM-kinase activity.[36a]

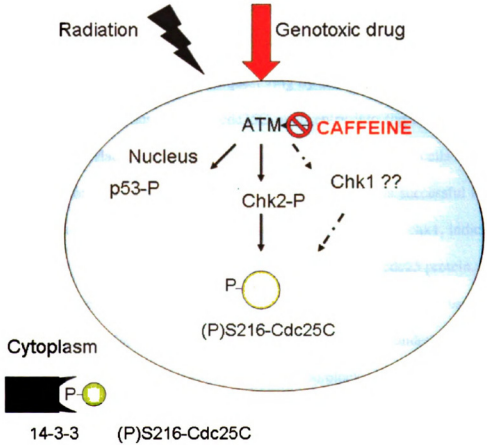



**Figure II-4**. Schematic diagram showing how caffeine overrides the activation of G2/M checkpoint by inhibiting ATM.

As described previously, an active Cdc2 (Cdk1) promotes entry into mitosis (Figure II-2b). This however can be inhibited via phosphorylation of cdc2 at Thr14 and Tyr15.[36b] Hence, entry into mitosis could be considered as dependent on the dephosphorylation events at Thr14 and Tyr15. The dual specificity phosphatase cdc25C, dephosphorylates cdc2 and catalyses entry into mitosis.[36c, d] Hyperphosphorylation of cdc25C activates its phosphatase activity.[36d] Phosphorylation of S216 can occur in a variety of situations such as chk1, chk2 and C-TAK1, which are specific for S216 phosphorylation in cdc25C resulting in the formation of a binding site for 14-3-3 family of proteins. While C-TAK1 is a cytoplasmic protein, suggesting a cytoplasmic retention of cdc25C upon phosphorylation and subsequent sequestering by 14-3-3, chk1 and chk2 are nuclear proteins and may phosphorylate cdc25C upon entry into the nucleus.[36e] Whether this phosphorylation prompts nuclear export in mammalian cells is unknown. Rad24, a homolog of 14-3-3 in S. *pombe* (a yeast strain) is successful in carrying out this export.[36f] This export was not seen in strains lacking chk1, indicating that upon DNA damage, chk1 may promote a nuclear export of cdc25 protein.

Zhou *et al* emphasized that although caffeine proved to be a useful tool in perceiving the existence of DNA damage checkpoints, the underlying mechanism still remained unclear.[36] The ability of caffeine to block radiation induced G2 arrest was correlated to a decrease in radiation induced phosphorylation of Cdc25C at Ser216, suggesting that it targets one of the proteins (probably ATM Chk2/Cds1) involved in the activation of the G2/M checkpoint. Since ATM-deficient cells are extremely sensitive to radiation, these results strongly suggest that inhibition of

ATM by caffeine is responsible for the radiosensitizing effects of this compound. While most drug discovery efforts focus on DNA-damaging agents to kill tumor cells preferentially, an alternative approach to make the current chemotherapy better is to find ways to increase tumor-specific killing by modifying tumor-specific responses to that damage. Powell *et al* [37] questioned whether p53 null cells which lack the G1-S arrest are more sensitive to X-ray damage when treated with agents that override G2-M arrest (Chk1). They used MEFs genetically matched to be (+) or (-) p53 and rat embryonic fibroblasts (+) or (-) for wild-type p53 function, irradiated with and without caffeine (a known checkpoint inhibitor). They observed that low doses of caffeine caused a 50% reduction in cell population undergoing the arrest in p53 (-) cells. However, at higher doses (2 mM caffeine), where sensitization was seen in both p53 (+) and p53 (-) cells, the radiosensitization and the G2-M override were more pronounced in the p53 (-) cells. The greater caffeine-induced radiosensitization in p53 (-) cells suggests that p53, in addition to the G1-S checkpoint control, may also influence aspects of G2-M arrest. These results indicate an opportunity for a profitable therapeutic intervention by combining DNA-damaging agents with compounds that disrupt G2-M arrest in tumors lacking functional p53.

Parallel to the synthesis of a library of compounds with a xanthine based structure, Daly *et al* showed a structure based activity profiling for G2 checkpoint inhibition by caffeine analogs.[39] Since caffeine inhibits the G2 checkpoint, it can override G2 arrest. Tumor cells which are p53 -/-, do not arrest at G1; however, their G2 checkpoint although impaired still provides them with an opportunity to repair

DNA damage and continue to divide. Since caffeine is not suitable for clinical use due to its other pharmacological activities like competitive antagonism at adenosine receptors, [39] inhibition of phosphodiesterases[40] and also toxicity at concentrations required to cause inhibition of the checkpoint, [41] Daly *et al* analyzed 56 analogs of caffeine and found that methyl substitutions are important at position $R_1$ and $R_3$ and no bulky substitutions at N-7 were tolerated (Figure II-5).[39] While 8-bromo and 8-$CF_3$ showed enhanced activity, the 8-phenyl analog showed limited activity due to solubility issues. In addition, the structure activity profile does not correlate with the activity profile of other targets indicating that caffeine might have a specific mechanism for overriding the G2 arrest.

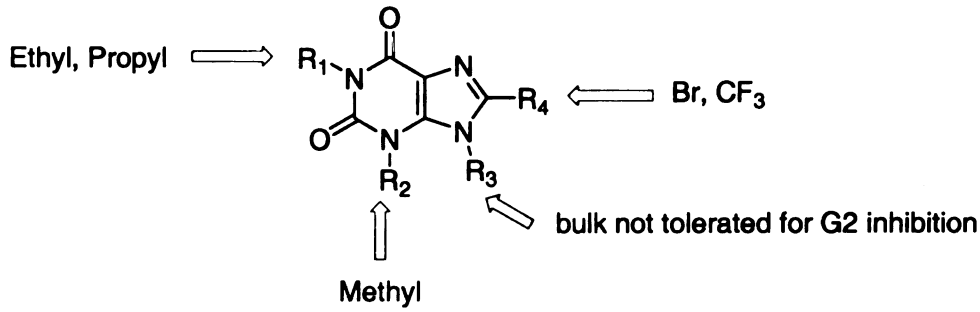



**Figure II-5.** Structurally important sites for Chk1 inhibition activity of Caffeine

Entry into mitosis is controlled by Cdc2 kinase. This kinase is indirectly controlled by Chk2 kinase, an enzyme that gets phosphorylated and gets activated after DNA damage in an ATM- dependent manner.[35, 42] Though solubility was a major problem in most caffeine derivatives, Daly *et al* showed a good correlation of G2 checkpoint inhibition and their synergistic cytotoxic effects with ionizing radiation in p53 -/- cells.[39]

**ii) Beyond kinases: purine binding targets in cancer therapy**

Kinases are an important class of enzymes that purines bind to due to their drug-

like characteristics. However, kinases are not the only enzymes that purines can bind; some other enzymes important in oncology are ATPases, GTPases, sulphotransferases etc.

**a) ATPases (Hsp90)**: Heat Shock Protein 90 (Hsp90) is an ATP dependent molecular chaperone proteins which are over-expressed upto 10 folds in cancer cells.[43] Many of the clients of Hsp90 are well-known oncoproteins which are frequently mutated and/or over-expressed such as p53, Bcl-Abr etc. Association with Hsp90 stabilizes these otherwise unstable proteins to function well in oncogenic signaling pathways essential to maintain the uncontrolled proliferation of tumor cells.[44] Occupancy of the ATP binding site causes destabilization and eventual ubiquitin dependent degradation.[45] Hence, inhibition of Hsp90 may be considered vital to inhibit activation of signaling pathways which might efficiently develop resistance to a specific drug treatment.[46] Depending on cellular context, such inhibitors might be effective in causing growth arrest, differentiation or apoptosis.[47] Inhibitors of Hsp90 could provide concurrent disruption of oncogenic pathways to provide treatment to obstinate tumors. 17AAG, a derivative of geldanamycin, was the first Hsp90 inhibitor to enter the clinical trials.[48a] While it provided extensive stabilization of the disease in animal models as well as phase I trials, and a reduction in the expression levels of various markers such as Raf-1,[48b] 17 AAG would not become a drug due to its poor solubility, lack of an easier synthetic route, hepatotoxicity associated with benzoquinone moiety, no selectivity for the type of Hsp90s.[49]

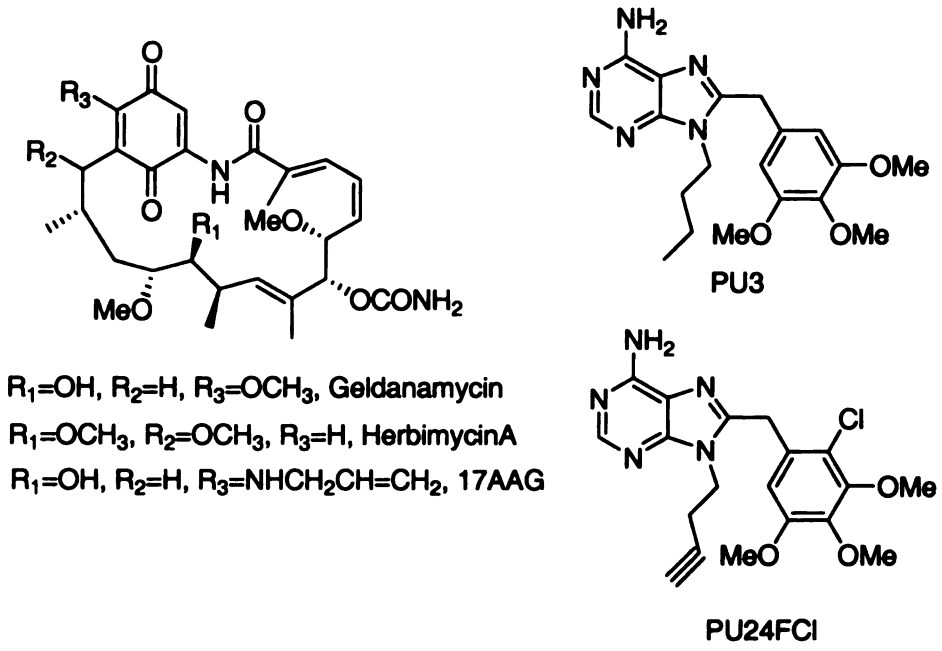



**Figure II-6.** Structures of some Hsp90 inhibitors

The water-soluble analogs of 17AAG have been synthesized though their clinical use is yet to be determined.[50] Computer-modeling studies have helped in designing the first purine based small molecule Hsp90 inhibitor, PU3 (Figure II-6), which showed inhibition at ~15 $\mu$M.[49] Subsequent structure optimization revealed that PU24FCl displays activity similar to 17AAG.[51] Further, while current drug development efforts target the N-terminal ATP binding site, recent studies suggest the existence of a hidden C-terminal ATP binding pocket for Hsp90.[52] Novobiocin, a gyrase inhibitor occupies this site and has been shown to inhibit Hsp90 activity also. Thus, inhibition of both N-terminal and C-terminal sites could harness the anti-cancer potential of Hsp90 inhibitors.

**b) Sulphotransferases** (ST) are enzymes that catalyze the transfer of a sulfate from the donor molecule PAPS (3'phosphoadenosine5'phosphosulfate) to a variety of intracellular substrates. ST enzymes are being considered as a

therapeutic target for inflammation as well as cancer.[53] Since these enzymes have important role in detoxification mechanisms, it can be hypothesized that they might potentiate the effect of cytotoxic chemotherapeutics. Among various classes of inhibitors, the purine based inhibitor (Figure II-7) shows maximum potency ($IC_{50}$=20 $\mu$M) and selectivity for N-acetylglucosamine-6-O-ST.[54]

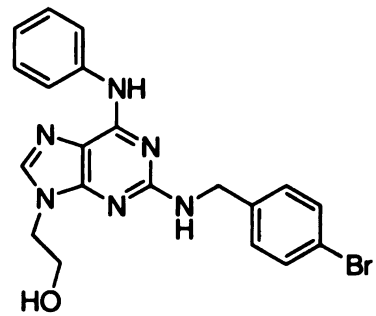



**Figure II-7**. Purine-based ST inhibitor

**D. Xanthines in signal transduction**

Xanthine based drugs have long been known as bronchodialators, anti-inflammatory agents in bowel disease etc.[55] Theophylline in particular is widely used as an anti asthma drug and it not only dilates bronchi, but also modulates the production of proinflammatory cytokines and inhibits inflammation.[56] Figure II-8 illustrates the sub-division of the anti-inflammatory effect of theophylline into immunomodulation, anti-neoplasic and hypercholestremic effect.

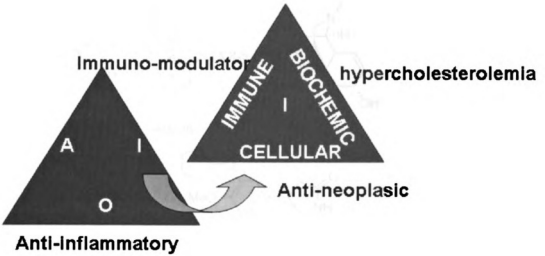



**Figure II-8.** Anti-inflammatory activity (A: Analgesic adjuvant, I: inflammatory, O:oropharyngeal) of theophylline correlates to its immunomodulatory, anti-neoplasic and hypercholestric effects.

Theophylline inhibits NF-κB activation, a factor that is essential for the expression of proinflammatory cytokines.[57] Finally, Ichiyama *et al* showed the inhibition of NF-κB being effected via inhibition of I-κB phosphorylation.[58] Their results showed that therapeutic theophylline (plasma theophylline is 5–15 μg/ml, 28-83 μM ) administration has an anti-inflammatory effect through inhibition of NF-κB activation (20 μM). Their western blot analysis revealed inhibition of phosphorylation of IκB-α similar to compounds like PDTC, aspirin, morphine[59] etc.

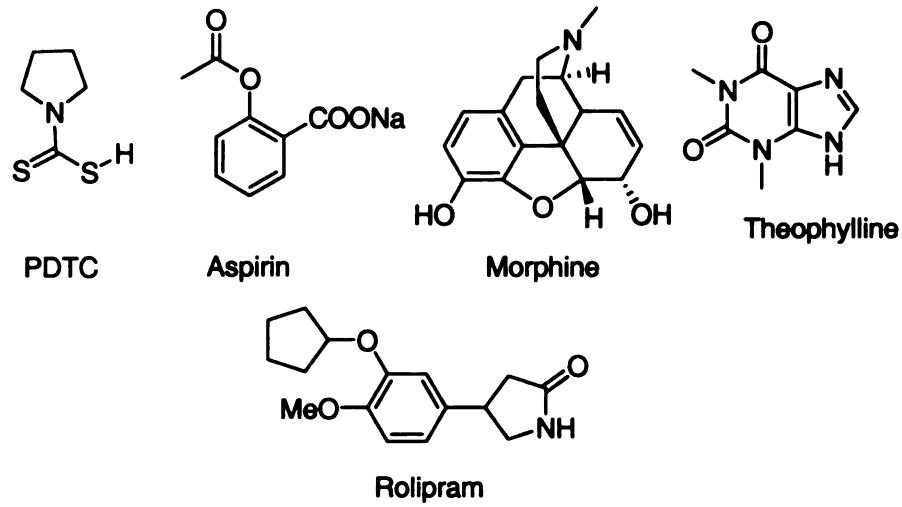



**Figure II-9.** Structures of some IκBα inhibitors.

Rolipram is a phosphodiesterase inhibitor and inhibited IκBα at concentrations comparable to theophylline indicating a possibility of the inhibition of IκBα via PDE inhibition.[58]

In the light of the important biological activity of the xanthines scaffold, we synthesized 8-subsituted theophyllines and caffeines. These were then screened to modulate the NF-κB signal transduction.

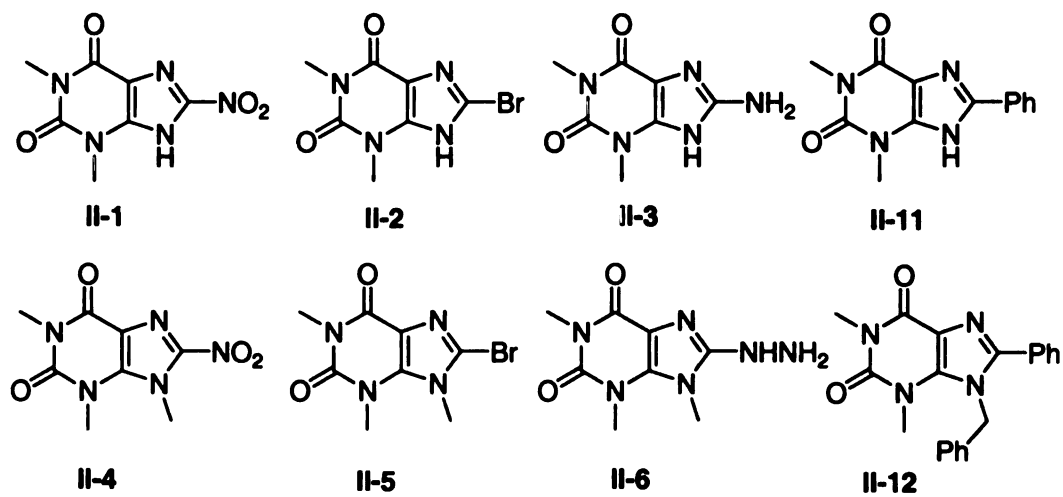
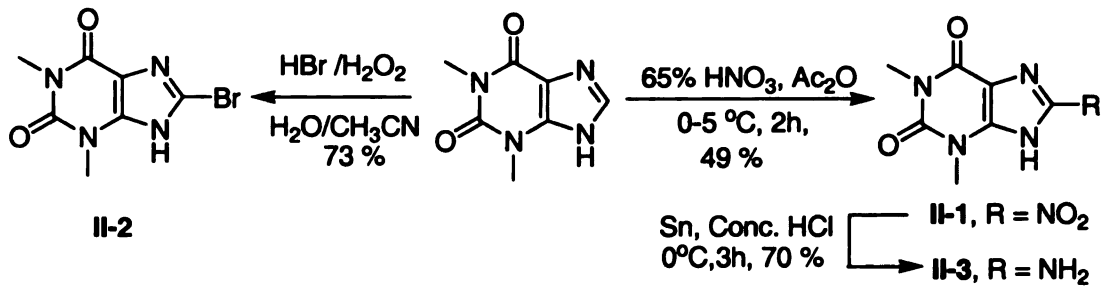


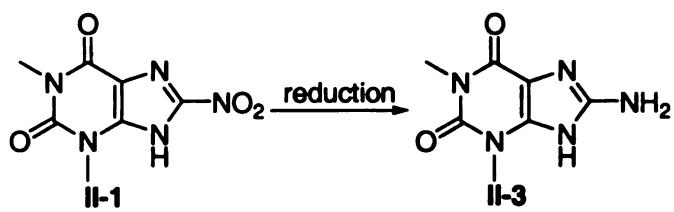

**Figure II-10.** Structures of theophyllines and caffeines.

The synthesis of the 8-substituted theophyllines was performed by starting with the commercially available starting materials, following routes reported earlier.




**Scheme II-1.** Synthesis of theophylline derivatives

Theophylline was treated with conc. $HNO_3$ in acetic anhydride at low temperature to form 8-nitrotheophylline(**II-1**) in modest yield,[60] which was reduced further with Sn and conc. HCl to form 8-aminotheophylline (**II-3**).[61] Theophylline was subjected to oxidative bromination using HBr/ $H_2O_2$ (**II-2**).[62] Table II-1 illustrates the other routes that were tried to convert **II-1** to **II-3**.[63]




| Entry | Reaction Conditions | Yield |
|---|---|---|
| 1 | Zn/conc. HCl | 0% |
| 2 | HI/Heat | decomposed |
| 3 | cyclohexene/Pd/C reflux | traces |
| 4 | $Na_2S_2O_4/NH_4OH$, $Na_2SO_4$ | traces |
| 5* | $Na_2S_2O_4$,$H_2O$/EtOH 1:1 | 0% |
| 6 | $Na_2S_2O_4/H_2O$/1,4-dioxane(1:1) | 0% |

*ethoxytheophylline was obtained

**Table II-1.** Various attempts to reduce **II-1** to **II-3**.

Various research groups have shown the conversion of 8-bromo into the 8-substituted amines (Figure II-11) via a nucleophilic displacement reaction.[64]

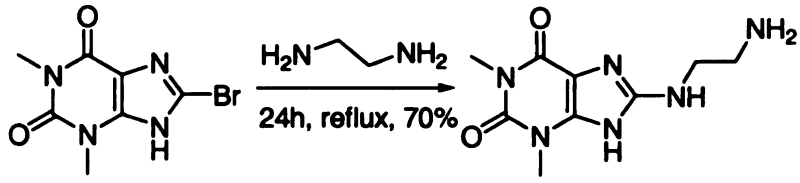
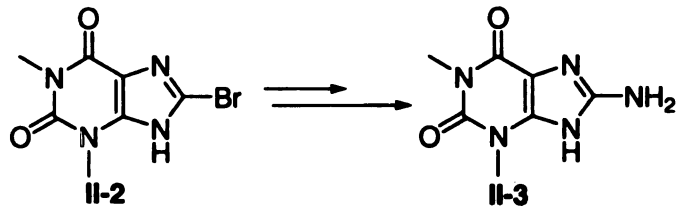



**Figure II-11.** Nucleophillic displacement reaction of bromotheophylline.

These were shown to have an important hypotensive and spasmolytic activity. 8-aminotheophylline has also found application in the synthesis of fused purines.[65]

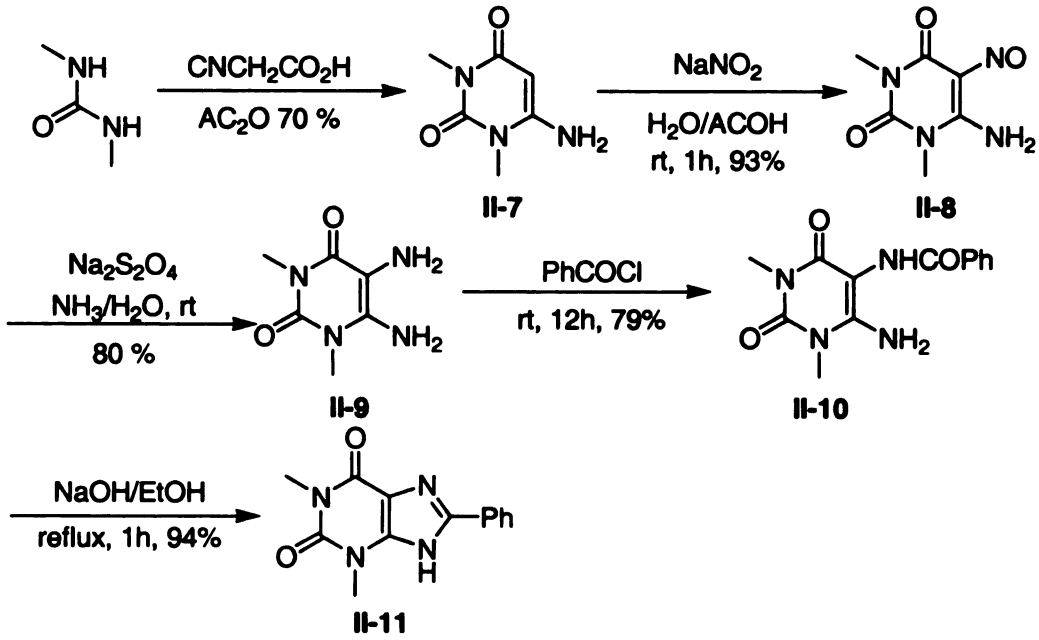



II-2 II-3

| Entry | Reaction Conditions | Yield |
|---|---|---|
| 1 | $NH_4OH$, MeOH, reflux, 3h | 0% |
| 2 | $BnNH_2$, MeOH, $H_2O$, reflux | 0% |
| 3 | $BnNH_2$, MeOH, sealed tube,150°C | traces |
| 4 | $BnNH_2$, $Pd_2(dba)_3$, BINAP, NaOtBu,toluene | 0% |

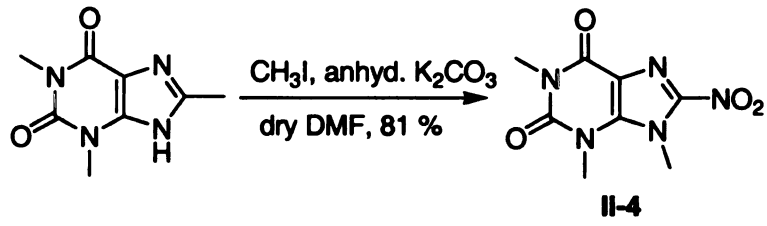
**Table II-2.** Attempts towards nucleophilic displacement on bromotheophylline **II-2.**[66]

8-Phenyltheophylline was synthesized starting with 5-aminouracil (Scheme II-2) which can be synthesized by methods described previously by other groups.[67]



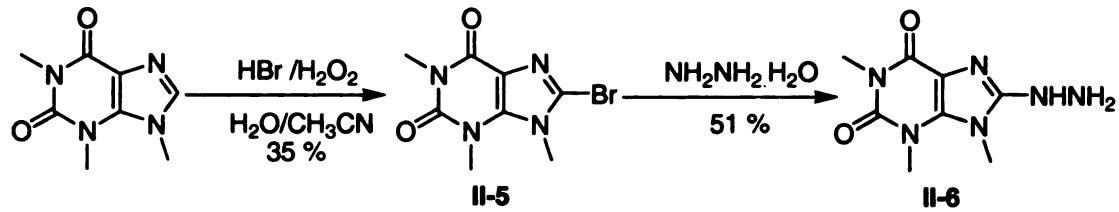

**Scheme II-2.** Synthesis of 8-phenyl theophylline (**II-11**)

Nitrosylation of the starting material gave characteristic purple-red mono-nitrosocompound **II-8** which was reduced to form the diamine **II-9**.[67] The diamine was then protected with benzoyl chloride which was finally condensed under alkaline conditions to form the imidazole moiety, yielding the desired product **II-11**. The caffeine derivatives were synthesized either using caffeine scaffold as the starting material or methylation N-7 position of the theophyllines (Scheme II-3).[68]



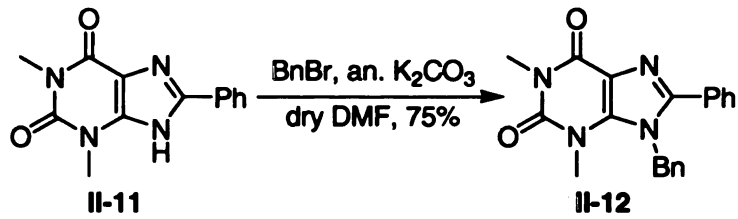

**Scheme II-3.** Synthesis of **II-4** from **II-1**.

Caffeine was subjected to oxidative bromination to give **II-5** which was further subjected to nucleophilic substitution to give **II-6** (Scheme II-4).




**Scheme II-4.** Synthesis of **II-5** and **II-6**

N-7 benzylation of **II-11** under basic conditions afforded **II-12** in good yields (Scheme II-5).[68]




**Scheme II-5.** N-Benzylation of **II-11**.

**E. Biological activity-evaluation and discussion**

Cancer chemotherapy often uses DNA damaging agents such as camptothecin, cis-platin etc. which cause induction of apoptosis in tumor cells through topoisomerase-II inhibition, DNA alkylation and often cell cycle perturbations.[69] As a cell survival response, most chemotherapeutic agents cause activation of a nuclear factor NF-κB. NF-κB comprises a family of inducible transcription factors responsible for immune and inflammatory responses.[70] It also mediates the control of cellular proliferation and apoptosis. High levels of NF-κB, result in increased expression of anti-apoptotic cellular proteins which lead to uninterrupted cellular growth. This is the onset of chemo-resistance in the tumor cells. Moreover, NF-κB also stimulates cell

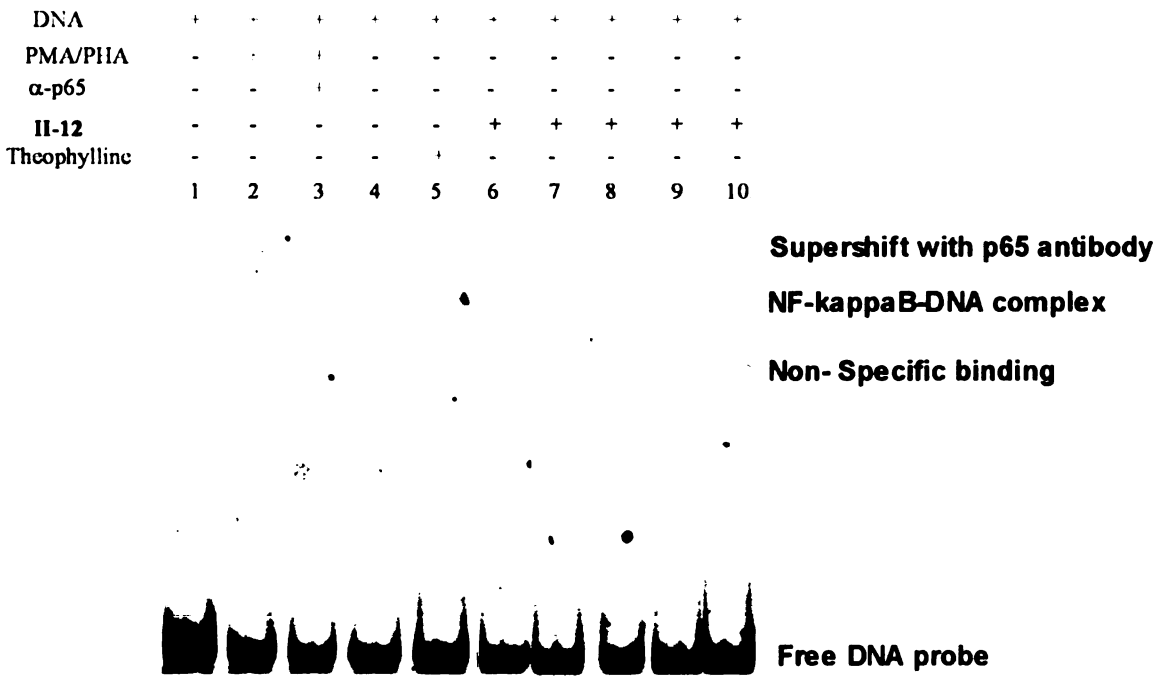
proliferation by activating target genes. Hence, NF-κB probably plays an important role in tumorigenesis through inhibition of apoptosis and stimulation of cell proliferation.

**F. NF-κB in defense and disease**

The eukaryotic transcription factor NF-κB, is involved in a variety of cellular processes and can be activated by a variety of stimuli. The regulation of NF-κB ultimately has been shown to have involvement of interactions with an inhibitory protein IκB that keeps it in bound state in the cytoplasm. The activation of NF-κB is associated with induction of phosphorylation of IκB by IκB-kinase, followed by its degradation leading to the nuclear translocation of the transcriptionally active p50/p65 subunit of the complex. In the nucleus, NF-κB binds to the target DNA elements and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control and apoptosis.[71] The direct involvement of NF-κB in regulation of inflammatory cytokines and genes responsible in growth cycle, indicates that the activation of NF-κB may play an important role in the development of chronic diseases such as rheumatoid arthritis, septic shock and cancer chemo-resistance.[72]

**G. Inhibition of Translocation of the trnascriptionally active p50/p65 heterodimer.** Theophylline and its 8-substituted derivatives were evaluated for their potential inhibitory activity against NF-κB in human Jurkat leukemia T-cells. The activation of NF-κB by PMA/PHA was confirmed in Jurkat cells using an EMSA assay.[19c, 73] Jurkat cells were exposed to phorbol myristate acetate (PMA) / phytohemagglutinin (PHA) which are known to activate NF-κB.[73]

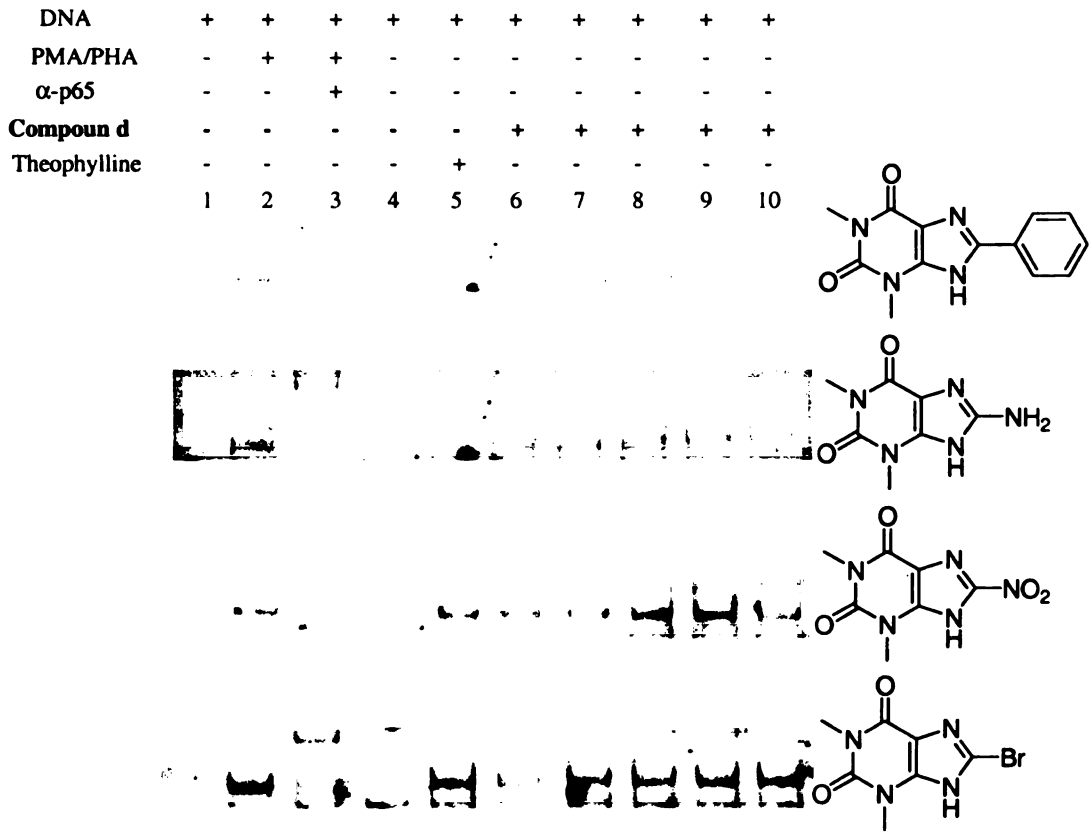
Theophylline was used as a positive control.[57, 58] The assay samples were pre-incubated with varying concentrations of the drug before activation with PMA (2.5 ng/ml)/PHA (2μg/ml). The PMA activation was performed for 4h. Cells were harvested and nuclear extracts were prepared as described by Dignam *et al.*[74] The nuclear extracts were evaluated for NF-κB-DNA binding activity utilizing the fluorescently labeled NF-κB DNA consensus sequence and run on a 4% polyacrylamide gel.




Lane1: DNA only; Lane2: DNA + activated control (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h ); Lane3: DNA + activated control (PMA(2.5 ng/ml)/PHA(2 μg/ml), 4h ) + Ab p65; Lane 4: DNA + unactivated control; Lane 5: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h ) + Theophylline, 1μM ; Lane6: DNA + activation (PMA(2.5 ng/ml)/PHA(2 μg/ml),4h ) + Compound **II-12**, 10 μM; Lane 7: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h) + Compound **II-12**, 1 μM; Lane 8: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h) + Compound **II-12**, 0.1 μM; Lane 9: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h) + Compound **II-12**, 0.01 μM; Lane10: DNA+ activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h) + Compound **II-12**, 1 nM.

**Figure II-12**. Inhibition of NF-κB- DNA binding with **II-12**.

Similar experiments were run for other theophylline derivatives. Figure II-13 illustrates p65-DNA binding for all compounds tested. PMA/PHA activation was used as a positive control (Figure II-12, lane 2, Figure II-13, lane 2). The NF-κB/DNA complex was unambiguously identified by treating the nuclear extract with a NF-κB p65 antibody following PMA/PHA activation (Figure II-12, lane 3; Figure II-13, lane 3). Treatment of the nuclear extract with the p65 antibody resulted in a significant decrease in NF-κB/DNA binding and the formation of a supershift of the complex (Figure II-12, lane 3; Figure II-13, lane 3). As a negative control, cells are left unactivated (treated only with equivolume vector DMSO for the same time as other assay plates) and the nuclear extracts was exposed to the NF-κB consensus sequence, resulting in only a slight background level of NF-κB-DNA binding (Figure III-12, lane 4; Figure II-13, lane 4). Pre-treatment of the Jurkat cells with various concentrations of the compounds **(II-1, II-2, II-3, II-11, II-12)** ranging from 10 $\mu$M to 1 nM (Figure II-12, II-13, lanes 6-10) illustrated a significant reduction in the amount of NF-κB/DNA binding as compared to that without the pre-treatment (Figure II-12, II-13, lane 2) indicating an inhibition of NF-κB activation. This effect decreased in a concentration dependent manner. Pre-treatment with theophylline, 1$\mu$M (Figure II-12, II-13, lanes 5) was used as a positive control, since it is known to inhibit IκB-phosphorylation and prevent NF-κB translocation. [57, 58]




Lane1: DNA only; Lane2: DNA + activated control (PMA (2.5 ng/ml)/PHA (2 μg/ml),4h ) ; Lane3: DNA + activated control (PMA(2.5 ng/ml)/PHA(2 μg/ml), 4h ) + Ab p65; Lane 4: DNA + unactivated control; Lane 5: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h ) + Theophylline, 1μM; Lane6: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h ) + Compound 10 μM; Lane 7: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml),4h) + Compound 1 μM; Lane 8: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml),4h) + Compound 0.1 μM; Lane 9: DNA + activation (PMA (2.5 ng/ml)/PHA (2 μg/ml), 4h) + Compound 0.01 μM; Lane10: DNA+ activation (PMA(2.5 ng/ml)/PHA (2 μg/ml), 4h) + Compound 1 nM.

**Figure II-13.** p65-DNA binding of theophylline derivatives (Compound =**II-1, II-2, II-3, II-11**).

Comparison of the various derivatives of theophylline to one another showed, that while **II-3**, **II-11**, and **II-12** inhibited the NF-κB-DNA binding significantly, **II-2** was active only at high concentrations and **II-1** was a very weak inhibitor of NF-

κB-DNA binding. These pre-liminary, qualitative EMSA results were further quantified using an ELISA based assay.

**H. Inhibition of translocation of NF-κB.**

NF-κB gets phosphorylated in the nucleus at multiple sites before it binds to the DNA. Hyperphosphorylated NF-κB binds tighter to the DNA.[75] Electromobility shift assays gave a qualitative result of whether theophyllines inhibited any step upstream of the DNA binding event (Figure I-5). A phospho-p65 based ELISA (Neogen corporation) was used to quantitate the amount of phosphorylated NF-κB in the nucleus. PDTC, a non-selective inhibitor of NF-κB was used as a positive control for inhibition of translocation of NF-κB across the nuclear membrane.[76] Figure II-14 illustrates the p65 translocation inhibition of all the 8- substituted theophylline derivatives.

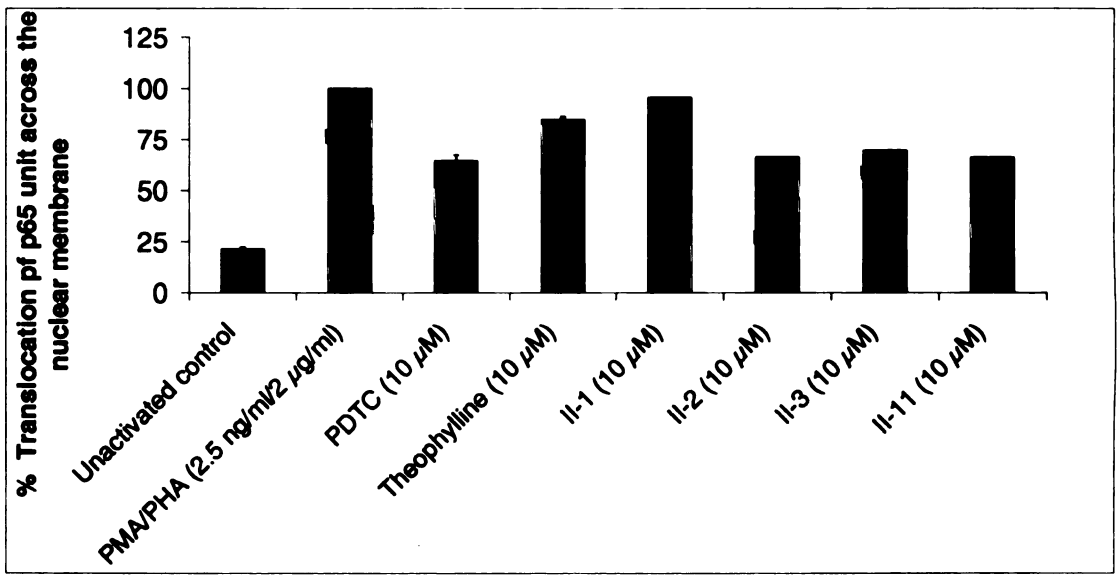
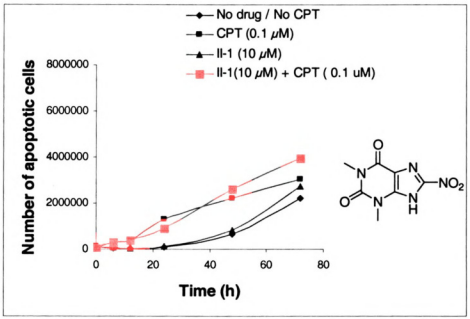



**Figure II-14.** p65 translocation measured by ELISA with unactivated control, PMA/ PHA, PDTC , theophylline and 8-substituted derivatives in presence of PMA/ PHA.

Activation with PMA/PHA for 4 h Compound **II-2, II-3** and **II-11** inhibited the translocation of NF-κB across the nuclear membrane comparable to PDTC (upto 40%), while theophylline showed only upto 15% effect at 10$\mu$M and nitrotheophylline did not show a significant effect, indicating that the 8-substitution in **II-2, II-3,** and **II-11** improved the efficacy of the xanthine nucleus in inhibiting the translocation of NF-κB.

**I. Potentiation of effect of camptothecin in CEM cells.**

Encouraged by the result that 8-substituted theophyllines are more potent than their parent scaffold, we tested if these molecules could inhibit the transcriptional activity by inhibiting the amount of anti-apoptotic factors. Figure II-15 illustrates the effect of the 8-substituted theophylline on CEM cells when incubated with CPT over a 48 hour time period.



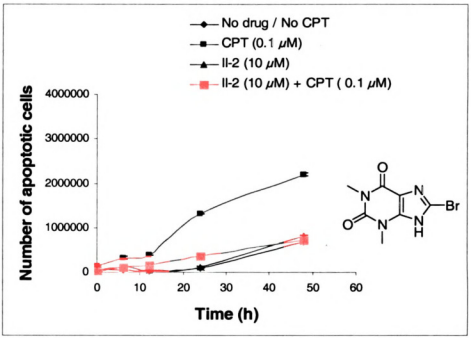




**Figure II-15**. Potentiation with **II-1** and **II-2**.

Treatment of the cells with substituted theophyllines (50 μM, 10 μM, 1 μM, 0.1 μM) had minimal apoptotic effect upto 24h. Treatment of the cells with 100 nM CPT resulted in some cell death starting after 12 hours of drug treatment (Figure II-

15). Combination treatment of the substituted theophyllines (0.1 $\mu$M) with CPT (100 nM) resulted in enhanced cell death after 48 hours (Figure II-16).

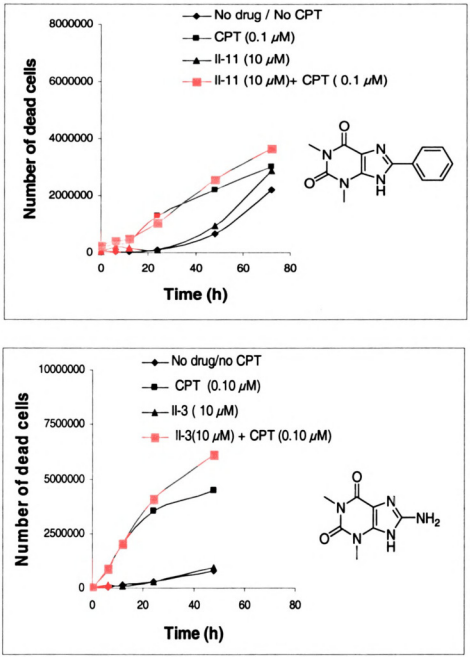



**Figure II-16**. Potentiation with **II-11** and **II-3**.

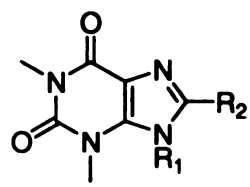
**II-1**, **II-3** and **II-11** showed modest potentiation of apoptotic response triggered by CPT. However, **II-2** protected cells from undergoing apoptotic response. This

indicates that either, over 48h, **II-2** is being metabolized into products that may not have an opposite effect and promote cell survival. Another possibility is that NF-κB inhibition can not necessarily be correlated with enhancement of apoptotic response.

Table II-3 illustrates the chemo-potentiation index as the apoptotic cell death with respect to cell death by camptothecin by itself. We have defined 'chemo-potentiation index'(CPI) as the ratio of apoptotic cell death in combination with an NF-κB inhibitor to the apoptotic cell death in presence of the chemotherapeutic drug only. Thus,

$$CPI = \text{Apoptosis}_{\text{combo treatment}} / \text{Apoptosis}_{CPT}$$

Since CPI is dependent on the relative apoptotic events with respect to the negative control (vector alone) and these in turn are dependent on the exposure (concentration * time), this number would vary based on the two variables.



| Entry | $R_1$ | $R_2$ | Chemopotentiation index after 48h @10μM |
|---|---|---|---|
| **Theophylline** | H | H | 1.17 |
| **II-1** | H | $NO_2$ | 1.3 |
| **II-2** | H | Br | 0.3 |
| **II-3** | H | $NH_2$ | 1.9 |
| **II-11** | H | Ph | 1.2 |

**Table II-3.** Chemopotentiation index after 48 h at 10 μM pretreatment of theophylline derivatives.

This data suggests that inhibition of NF-κB might lead to potentiation of chemotherapy in cancer patients. However, **II-2** showed an opposite result which may be due to a metabolism of the molecule over 48 h. While caffeine has been shown to potentiate the lethal effects of the genotoxic agents such as cis-platin, by overriding the G2 checkpoint mechanism, NF-κB inhibition hasn't been probed.[77] Our preliminary results for 8-substituted caffeines indicate similar effects as theophyllines.

**J. Methyl xanthines and septic shock**

Clinicians have used the terms 'sepsis' and septic shock without a common definition. Physiological alternations and organ dysfunction seen with bacterial infections also can result from a wide variety of clinical insults.[78a, b] This clinical state, termed the systemic inflammatory response syndrome (SIRS), is defined by two or more changes in the following factors: body temperature, heart rate, respiratory function, and peripheral leukocyte count. Sepsis is defined as the systemic host response to infection with SIRS plus a documented infection. Severe sepsis is defined as sepsis plus end-organ dysfunction or hypoperfusion.[78c] Septic shock is defined as sepsis with hypotension, despite fluid resuscitation, and evidence of inadequate tissue perfusion.[78d] While SIRS, sepsis, and septic shock commonly are associated with bacterial infection, bacteremia may not be present. Bacteremia is the presence of viable bacteria within the liquid component of blood. Bacteremia may be transient, as it commonly is after injury to a mucosal surface. Bacteremia may be primary (without an identifiable focus of infection) or, more often, secondary (with an intravascular or extravascular focus of infection).

Septicemia, sometimes referred to as bacteremia, is a syndrome that results from an acute invasion of the bloodstream by certain microorganisms or their toxic products such as LPS (lipopolysaccharide).[79] Septic shock is most commonly caused by gram-negative bacteria.[79] Staphylococci, streptococci and other gram-positive organisms, fungi and some viruses are less frequent (though notorious). Due to bacterial infection in septic shock, interaction with host cells and serum proteins initiates a series of reactions that may ultimately lead to cell injury and death. Bacteremia occurs in about 40% of patients with gram-negative bacteremia and in about 20% of patients with *Staphylococcus aureus* bacteremia.[79] Among the various mediators that are stimulated in septic shock are TNF-cachetin and IL-1, which in turn would activate NF-κB. TNF-α plays an important role in the development and pathogenesis of sepsis. Animals subjected to TNF-α show hypotension, acidosis, hyperglycemia, and vascular leak syndrome-signs in septic shock. Pentoxyphylline inhibits LPS mediated TNF-α production in rats in a dose dependent manner.[80] Hence, temporal inhibition of NF-κB which can maintain the levels of IL-2 / TNF-α and cause cytotoxicity to any of the above species of bacteria, could find applications in treatment of septic shock. Earlier studies reveal that theophylline, aminotheophylline and bromotheophylline have been used as bronchodilators against asthma and inflammation. Our studies on caffeines and theophyllines indicated that protection at N-7 in the imidazole increased the anti-microbial activity of these compounds. Azomycin, metronidazole are nitroimidazoles which have been widely used against bacteria and parasites.[81] Metronidazole is an antibiotic especially effective against anaerobic infections (infections that grow

without the presence of oxygen.) In addition, it has anti-inflammatory properties in the large intestine and is a very effective anti-diarrhea medication.




**Figure II-17**. Imidazole based anti-bacterial compounds

Encouraged by these facts we tested caffeine derivatives for anti-microbial activity and possible NF-κB inhibition with LPS.

**i) Anti-bacterial activity:** A disc-diffusion assay was used to determine the anti-bacterial activity of the xanthine based compounds. While none of the theophyllines showed any activity for any bacterial strain, caffeine based molecules showed some activity. Of all the caffeines tested, **II-4** proved to be most potent with virtually no selectivity between gram negative or positive bacteria at higher concentrations (Figure II-18). However, upon serial dilution, the compound proved inhibitory activity only against gram(+) bacteria such as M. *luteus*, S. *aureus*, B. *subtilis*. Table II-4 illustrates the anti-bacterial effect of methylxanthine- **II-4**.

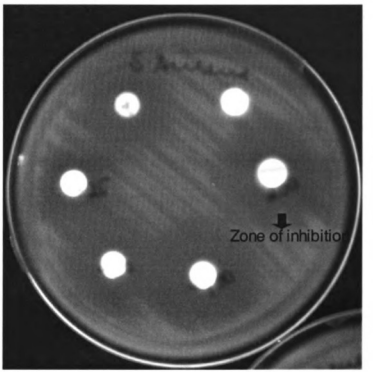



**Figure II-18.** Representative disc-diffusion assay for **II-4** against S. *aureus.* The darker regions(→) are zones of inhibition.

**II-12** showed activity only against E. *coli* at high concentrations such as 200 $\mu$g and no activity as the concentration was reduced.

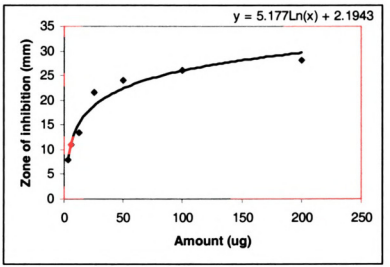
| Bacteria | Drug | Zone of inhibition (control, 10 μg) | Zone of inhibition (drug, 10 μg) |
|---|---|---|---|
| Bacillus *cereus* (+) | II-4 | 26 | 14 |
| Bacillus *subtilis* (+) | II-4 | 26 | 22.1 |
| Staphylococcus *aureus*(+) | II-4 | 17 | 9 |
| Micrococcus *luteus* (+) | II-4 | 25 | - |
| Klebsiella *pneumoneae*(-) | II-4 | - | 3.2 |
| Enterobacter *aerogenes* (-) | II-4 | - | - |
| Escherichia *coli*(-) | II-4 | 9 | - |
| Pseudomonas *aeruginosa* (-) | II-4 | - | - |

**Table II-4.** Activity of caffeines against gram (+) and gram (-) bacteria.

Further, this data was plotted to compare values with streptomycin control at 10 $\mu$g. Zone of inhibition was calculated for the **II-4** at 10 $\mu$g loading concentration.

Figure II-19 illustrates the activity against gram (+) and gram (-) bacteria as zone of inhibition (mm) as a measure of dose dependence.

**A.**




**B.**

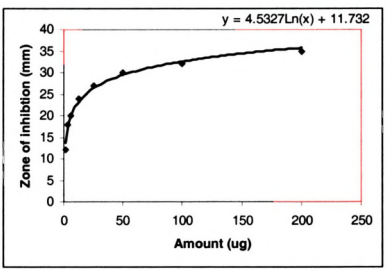
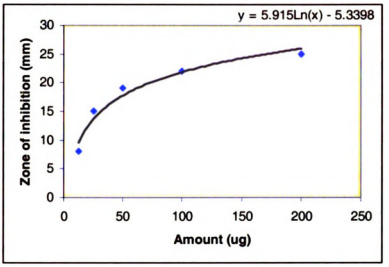



**Figure II-19.** Activity of II-4 against **A. (top)** Bacillus *cereus*, **B. (bottom)** Bacillus *subtilis*

C.




D.

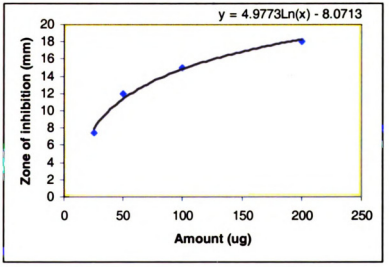
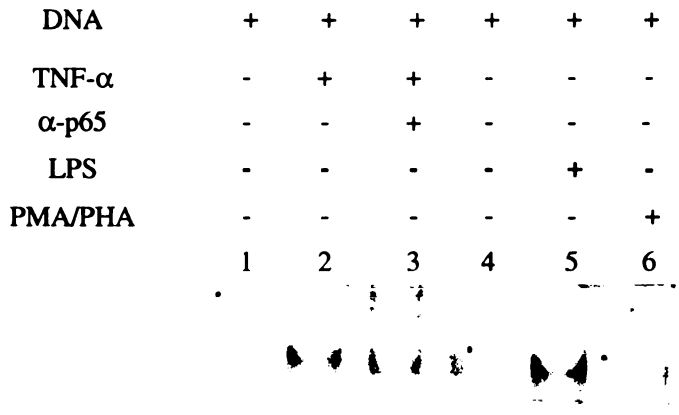



**Figure II-19(contd'):** Activity of **II-4** against **C.** Staphyllococcus *aureus,* **D.** Klebsiella *pneumoneae.*

**ii) NF-κB is activated by LPS in macrophages.**

Lipopolysaccharide is a major cell wall component of gram (-) bacteria and triggers the inflammatory responses associated with sepsis, including increased CAM (cell adhesion molecule) expression and release of IL-8 in endothelial cells.[82] Little is known, however, about the endothelial-activating properties of cell wall components of gram (+) bacteria or how these effects contribute to the pathogenesis of sepsis. The cell walls of Gram-positive bacteria are made up of a layer of peptidoglycan (PepG) embedded with teichoic acids, including lipoteichoic acid (LTA).[83] The effects of LTA and PepG (especially from S. *aureus*) on cells other than endothelial cells are quite well characterized and include induction of IL-8 release and CAM expression in monocytes.[84]

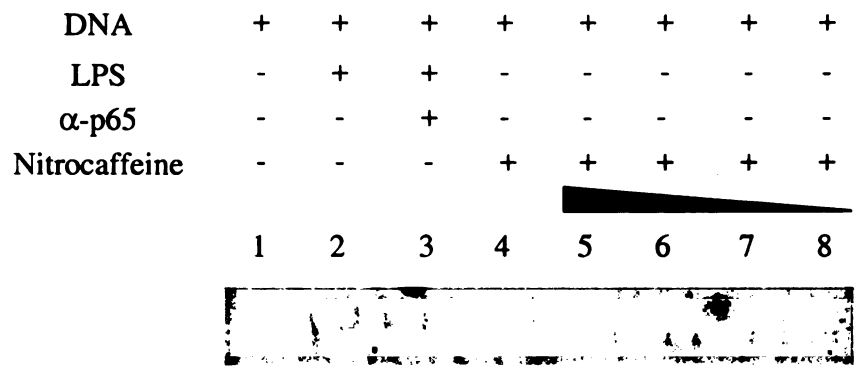



Lane1: DNA only; Lane2: DNA + activated control (TNF-α, 10 pM, 20'); Lane3: DNA + activated control (TNF-α, 10 pM, 20') + Ab p65 Lane 4: DNA + unactivated control Lane 5: DNA + activation + LPS (0.1 μg/μl, 1h) Lane6: DNA + activation PMA/PHA (2.5 ng/ml/2μg/ml, 4h).

**Figure II-20.** EMSA showing NF-κB binding in THP-1 monocytes with LPS (1μg/mL); TNF-α (2 pg/mL) was used as positive control.

**iii) Nitrocaffeine (II-4) inhibits LPS induced activation of NF-κB in macrophages.**

THP-1 monocytes were grown in RPMI-1640 medium supplemented with 7 % FBS at 37 °C at 5 % CO2 atmosphere. Cells at 1 X $10^6$/ ml were pre-incubated with the drugs for 30 minutes prior to being activated with 0.1 μM LPS. Incubation was performed for 1 h. Cells were then harvested and nuclear extracts prepared. The protein concentrations were found out and an EMSA was performed with the NF-κB consensus oligo.




Lane1: DNA only; Lane2: DNA + activated control; Lane3: DNA + activated control + Ab p65 ; Lane 4: DNA + unactivated control; Lane 5: DNA + activation + PDTC (10 μM); Lane 6: DNA + activation + **II-4** (10 μM); Lane 7: DNA + activation + **II-4** (1 μM); Lane 8: DNA + activation + **II-4** (0.1 μM); Lane 9: DNA + activation + **II-4** (0.01 μM).

**Figure II-21.** EMSA showing NF-κB binding in THP-1 monocytes upon LPS activation in presence and absence of varying concentrations of **II-4**.

From the above anti-microbial assay and inhibition of NF-κB, it can be hypothesized that caffeine based derivative **II-4** might be effective against septic shock and SIRS. Further, studies to illustrate the mechanistic details are required.

## K. Conclusion

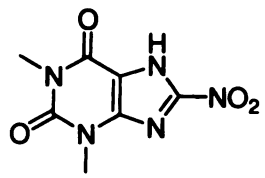
8-substituted theophyllines were found to be modest potentiators of apoptotic effects of camptothecin. Compound **II-2, II-3, II-11** inhibited the translocation of proinflammatory protein NF-κB across the nuclear membrane in PMA/PHA activated human Jurkat cells. While **II-1, II-3** and **II-11** were found to potentiate the apoptosis caused by camptothecin (as measured by chemopotentiation index in Table II-2) , **II-2** indicated just the opposite effect. These mutually opposite results provide strong evidence that the xanthine nucleus may not be solely involved in NFκB inhibition in their potentiative effect on camptothecin. It can be speculated here that inhibition of NF-κB may or may not culminate in enhancing the effect of chemotherapy.

All caffeine or N-protected scaffolds exhibited potent anti-microbial activity. **II-4** showed potent activity against *S. Aureus* (a resistant strain in clinical setting) and also showed inhibition of NF-κB translocation when activated by LPS, an important chemical secreted in bacterial infection. These results indicate that compounds like **II-4**, might find important use in treatment of septic shock. Optimal combinations of various susbtituents may lead to more potent and selective inhibitors.

## L. Experimental Section

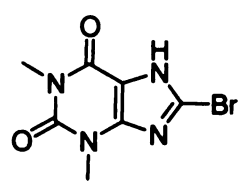
Reactions were carried out in oven-dried glassware under nitrogen atmosphere, unless otherwise noted. All commercial reagents were used without further purification. All solvents were reagent grade. THF was freshly distilled from sodium/benzophenone under nitrogen. Toluene, $CH_2Cl_2$, and TMSCl were freshly distilled from $CaH_2$ under nitrogen. All reactions were magnetically stirred and monitored by TLC with Analtech 0.25 mm pre-coated silica gel plates. Column chromatography was carried out on silica gel 60 (230–400 mesh) supplied by EM Science. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Melting points were determined on a Mel-Temp (Laboratory devices) apparatus with a microscope attachment. Infrared spectra were recorded on a Nicolet IR/42 spectrometer. $^1H$ and $^{13}C$ NMR spectra were recorded on a Varian Gemini-300 spectrometer or a Varian VXR-500 spectrometer. Chemical shifts are reported relative to the residue peaks of the solvent $CDCl_3$ ($\delta$ 7.24 for $^1H$ and $\delta$77.0 for $^{13}C$), DMSO-*d*6 ($\delta$ 2.49(5) for $^1H$ and $\delta$39.5 (7) for $^{13}C$) and $CD_3OD$ ($\delta$ 3.3 (5), 4.78 (1) for $^1H$ and $\delta$49.0 (7) for $^{13}C$). High resolution mass spectra and FAB mass spectra were obtained at the Mass Spectrometry Laboratory of the Department of Biochemistry, Michigan State University, with a JEOL AX-505H and a JEOL HX-110 double focusing mass spectrometer (JEOL USA, Peabody, MA), respectively. Gas chromatography/low resolution mass spectra were recorded on a Hewlett–Packard 5890 Series II gas chromatograph connected to a TRIO-1 EI mass spectrometer. All chemicals were obtained from Sigma-Aldrich Chemical Co. and used as received.

**Compound II-1: 1, 3-dimethyl-8-nitro-1H-purine-2, 6(3H, 7H)-dione**



A solution of theophylline (0.54 g, 3 mmol) was suspended in $Ac_2O$ (5 ml) and cooled to < 5 °C in wet ice. Conc. $HNO_3$ (0.45 ml, 10 mmol) was added dropwise with constant stirring. The starting material slowly dissolved and gave a dull yellow solid. The reaction mixture was stirred for 2 h at 0 °C and then maintained at 40 °C in a water bath for 10 minutes. The reaction mixture was brought to room temperature and was washed with EtOH/$H_2O$ (1:1, 10 ml). The bright yellow solid was filtered and dried. A small sample was recrystallized from 0.5 N HCl as yellow crystals. Yield: 49%; M.P. decomposed at 282 °C. $^1H$ NMR: (500 MHz, DMSO-*d6*) δ 3.22 (s, 3H), 3.39 (s, 3H) $^{13}C$ NMR: (74.47 MHz, DMSO-*d6*) δ 28.96,31.07, 112.38, 146.09, 148.26, 151.90, 156.32 IR: (NaCl) 2924.0, 1699.5, 1660.9, 1522.0, 1342.6, 1304.1 $cm^{-1}$. LRMS m/e ($C_7H_7N_5O_4$) (M+) 225.0.

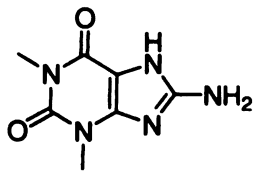
**Compound II-2: 8-bromo-1, 3-dimethyl-1H-purine-2, 6(3H, 7H)-dione**



A solution of theophylline (200 mg) in 30% $H_2O_2$ in $H_2O$ (2 eq.) was maintained at 0 °C. HBr (2 eq.) was added dropwise to the reaction mixture. The reaction was allowed to stir overnight. The white precipitate that appeared was then filtered and dried. Yield: 73 %;

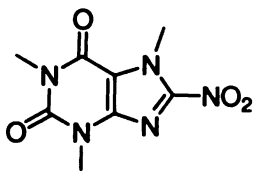
Method 2. A suspension of theophylline (1g) in water was mixed with a solution of NBS (1.09g, 6.11 mmol) at 0°C. Stirred for 3h. A white precipitate was formed which was filtered, dried, and characterized. M. P. >260°C $^{1}$H NMR: (500 MHz, DMSO-*d6*) δ 3.21 (s, 3H), 3.38 (s, 3H) $^{13}$C NMR: (74.47 MHz, DMSO-*d6*) δ 27.54, 29.62, 109.04, 123.95, 147.72, 150.70, 153.23 IR: (NaCl) 2923.3, 2854.7, 1708.1, 1648.9, 1603.1 cm$^{-1}$. LRMS m/e ($C_7H_7N_4O_2Br$) (M+) 259.8, 257.8

**Compound II-3: 8-amino-1, 3-dimethyl-1H-purine-2, 6 (3H, 7H)-dione**



A solution of **II-1** (1 g) in conc. HCl (8 mL) was maintained at 0 °C. Sn (3.5 eq.) was added in small lots to the reaction mixture. The reaction was allowed to stir for 3h with constant cooling. The white precipitate that appeared was then filtered and dried. Yield: 70%; M. P. 260 °C. $^{1}$H NMR: (500 MHz, TFA-*d1*) δ 3.92 (s, 3H), 4.07(s, 3H) $^{13}$C NMR: (74.47 MHz, DMSO-*d6*) δ 30.75, 33.61, 104.02, 147.57, 152.32, 153.25, 156.91 IR: (NaCl) 3410.0, 3105.0, 1716.9, 1664.8. LRMS m/e ($C_7H_7N_5O_4$) (M+) 195.0.

**Compound II-4: 1, 3, 7-trimethyl-8-nitro-1H-purine-2, 6 (3H, 7H)-dione**

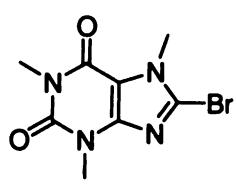


Method A: A suspension of caffeine (1.94 g, 10 mmol) was dissolved in glacial acetic acid (5 ml) at 100 °C. 65% conc. $HNO_3$ (2 ml, 44 mmol) was added

dropwise with constant stirring. The reaction mixture was heated for 1h at 100 °C. The solvent was evaporated *in vacuo*. The residue was then extracted with $CHCl_3$ and 1N $NaHCO_3$ (20 ml) twice. The organic layer was dried over $Na_2SO_4$ and evaporated. The solid obtained was recrystallised in methanol. Yield 20% (238 mg)

Method B : A solution of **II-1** (500 mg, 2.3 mmol) and anhydrous $K_2CO_3$ in dry DMF (15 ml) was allowed to stir for 10 minutes at room temperature. $CH_3I$ ( 1.63 g, 11.5 mmol) was added and the reaction mixture heated at 80 °C for 40'. The reaction mixture was further allowed to stir at room temperature for 8h. The reaction mixture was extracted with EtOAC/LiCl. The organic layer was dried over $Na_2SO_4$ and concentrated *in vacuo*. A bright yellow solid was isolated, yield 81% (430 mg); M. P.=170 °C $^1H$ NMR: (500 MHz, $CDCl_3$) δ 3.40 (s, 3H), 3.57 (s, 3H), 4.41(s, 3H) $^{13}C$ NMR: (74.47 MHz, $CDCl_3$) δ 28.41, 30.08, 36.70, 109.54, 144.54, 150.89, 155.31 IR: (NaCl) 2955.0, 1711.1, 1688.7, 1537.5, 1323.4 $cm^{-1}$. LRMS m/e ($C_7H_7N_5O_4$) (M+) 239.1. Anal. ($C_8H_9N_5O_4$) C; Calcd. H, 3.79; N, 29.28; found, H,3.65; N, 28.60.

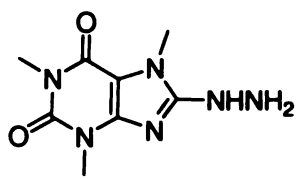
**Compound II-5:8-bromo-1, 3, 7-trimethyl-1H-purine-2, 6 (3H, 7H)-dione**




A solution of caffeine (5g, 25.8 mmol) in 30% $H_2O_2$ (2.92 ml, 28.4 mmol) in $H_2O$ (3 ml) was maintained at 0 °C. HBr (1.88 ml, 28.4 mmol) was added dropwise to

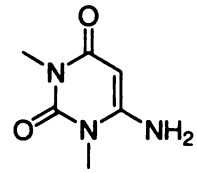
the reaction mixture. The reaction was allowed to stir overnight. The white precipitate that appeared was then filtered and dried. Yield: 35% (2.45g); M. P. 166 °C $^{1}$H NMR: (500 MHz, $CDCl_3$) δ 3.38 (s, 3H), 3.54 (s, 3H), 3.94(s, 3H) $^{13}$C NMR: (74.47 MHz, $CDCl_3$) δ 28.31, 30.17, 34.36, 109.35, 129.21, 148.15, 151.38, 154.45 IR: (NaCl) 2922.5, 2855.0, 1707.2, 1662.9, 1543.3, 1458.4cm$^{-1}$. LRMS m/e ($C_7H_7N_5O_4$) (M+) 272.0, 274.0.

**Compound II-6:8-hydrazinyl-1, 3, 7-trimethyl-1H-purine-2, 6 (3H, 7H)-dione**



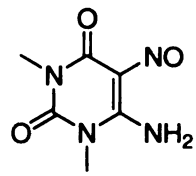

A solution of **II-5** (120 mg, 0.44 mmol) and hydrazine hydrate (374.5 mg, 7.48 mmol) in THF(20 ml) was refluxed for 1.25 h . The solvent was evaporated *in vacuo*. A greenish white solid was isolated. The solid was recrystallised in methanol, yield 51% (50 mg); M. P. decomposed at 289 °C $^{1}$H NMR: (500 MHz, DMSO-*d6*) δ 3.16(s, 3H), 3.37 (s, 3H), 3.55(s, 3H), 4.35(b, 2H), 8.05(b, 1H) $^{13}$C NMR: (74.47 MHz, DMSO-*d6*) δ 27.23, 29.38, 37.0, 148.02, 150.97, 153.15, 156.52, 168.61 IR: (NaCl) 3252.4, 2926.5, 1749.7, 1705.9 1662.9, 1616.6,1539.4 cm$^{-1}$. LRMS m/e ($C_7H_7N_5O_4$) (M+) 224.1. Anal. ($C_8H_{12}N_6O_2$) C; H; Calcd. N, 37.48; found, N,35.09.

**Compound II-7:6-amino-1, 3-dimethylpyrimidine-2, 4 (1H, 3H)-dione**



A solution of dimethyl urea(1g, 11.36 mmol) in acetic anhydride (15 ml) was heated with cyanoacetic acid (1.1g, 12.5 mmol) at 75 °C for 2h in an oilbath. A mild exothermic reaction occurred during the first stages of heating during which the heating was removed. The solvent was removed *in vacuo* and water was added. The reaction mixture was then concentrated to give a brown slurry. This was cooled in wet ice for 5 ' and neutralized with a 70% (w/v) NaOH solution. A reddish brown solid was isolated. Yield: 70% (1.23 g) M.P.decomposed at 282 °C $^1$H NMR: (300 MHz, DMSO-*d6*) δ 3.05(s, 3H), 3.22 (s, 3H), 4.67(s, 1H), 6.77(b, 2H) $^{13}$C NMR: (74.47 MHz, DMSO-*d6*) δ 27.03, 29.27, 74.87, 151.59, 154.88, 161.41, 168.61 IR: (NaCl) 3350.2, 2926.5, 1620.2 cm$^{-1}$. LRMS m/e ($C_6H_9N_3O_2$) (M+) 155.1. Anal. ($C_6H_9N_3O_2$) C; Calcd. H, 5.85; found, 6.29; N.

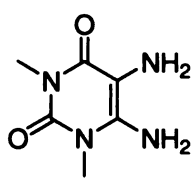
**Compound II-8:6-amino-1,3-dimethyl-5-nitrosopyrimidine-2,4(1H,3H)-dione**



A suspension of **II-7** (500 mg) was maintained at 60 °C in 50% aqueous acetic acid (15 ml) until the solid dissolved. The reaction mixture was then cooled and $NaNO_2$ ( 544 mg, 6.4 mmol) dissolved in 2 ml water was added dropwise over a period of 30' using an syringe pump (Kd Scientific Model 100, Serial No. 19016)

The solution turned deep magenta and a magenta solid appeared. The reaction mixture was stirred for 1h and then cooled to 4 °C to facilitate complete precipitation. The solid was washed with $H_2O$ and isolated, yield 93% (550 mg) M.P.=234 °C $^1$H NMR: (300 MHz, DMSO-*d6*) δ 3.23(s, 3H), 3.26 (s, 3H), 9.07(br, s, 1H), 12.94(br, s, 2H) $^{13}$C NMR: (125 MHz, DMSO-*d6*) δ 27.85, 28.69, 139.17, 146.10, 149.41, 160.26 IR: (NaCl) 3252.4, 2926.4,1704.5, 1657.1 cm$^{-1}$. LRMS m/e ($C_6H_8N_4O_3$) (M+) 184.0.

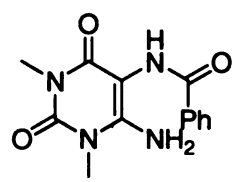
**Compound II-9:5, 6-diamino-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione**



A suspension of **II-8** (500 mg, 2.71 mmol) in 10 ml $NH_4OH$ (12.5% aqueous solution) was mixed with $Na_2S_2O_4$ (943.1 mg, 5.42 mmol) dissolved in $H_2O$(2 ml). The reaction mixture was allowed to stir for 30' and then set up on the rotary evaporator to reduce its volume to half the original volume. The orange solid dissolved and a colorless solution was obtained. This solution was then cooled to -20 °C for 30'. A bluish white precipitate was isolated; yield 80% (370 mg); M.P. decomposed at 204 °C $^1$H NMR: (300 MHz, DMSO-*d6*) δ 2.94(br, s, 2H), 3.12(s, 3H), 3.27 (s, 3H), 6.15(br, s, 2H) $^{13}$C NMR: (74.47 MHz, DMSO-*d6*) δ 27.61, 29.75, 96.03, 144.64, 149.76, 158.95 IR: (NaCl) 3397.1, 3280.2, 2922.5, 1658.9.7, 1616.5, 1498.9, 1452.6 cm$^{-1}$. LRMS m/e ($C_6H_{10}N_4O_2$) (M+) 170.0. HRMS m/e ($C_6H_{10}N_4O_2$) (M+) calcd. 170.0804; found, 170.0808; Anal.

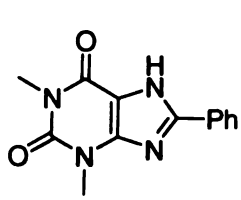
($C_6H_{10}N_4O_2$) C; Calcd.: H, 6.26; N, 32.92 found: H, Calcd. N, 37.48; found, N, 35.09.

**Compound II-10: N-(6-amino-1, 2, 3, 4-tetrahydro-1, 3-dimethyl-2, 4-dioxopyrimidin-5-yl) benzamide**



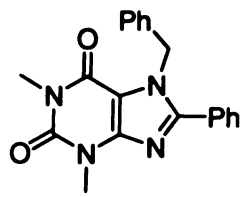
A suspension of **II-9** (800 mg, 4.7 mmol) was maintained in dry pyridine (40 ml) at 0 °C. Benzoyl chloride (599.8 $\mu$l, 5.17 mmol) was added dropwise with continuous stirring. The mixture was stirred for 12 h during which the reaction was allowed to come to room temperature. The solvent was then removed by distillation. A brown colored slurry remained which was diluted with water to yield a light brown solid. The solid was then isolated and recrystallised in 1:1 EtOH/$H_2O$. Yield: 79%(1.01 g) M. P. 225 °C $^1$H NMR: (300 MHz, DMSO-*d6*) δ 3.13(s, 3H), 3.32(s, 3H), 6.72(br, s, 2H), 7.46-7.50 (m, 3H), 7.96 (d, 2H, *J*= 6.6 Hz), 8.92 (br, s, 1H) $^{13}$C NMR: (74.47 MHz, DMSO-*d6*) δ 27.55, 30.02, 87.41, 127.94, 128.00, 131.15, 134.55, 150.63, 152.32, 159.28, 166.60 IR: (NaCl) 3252.4, 2926.5, 1697.6, 1645.5, 1576.0,1516.2 cm$^{-1}$. LRMS m/e ($C_{13}H_{14}N_4O_3$) (M+) 274.2. HRMS m/e ($C_{13}H_{14}N_4O_3$) (M+) calcd. 274.1066; found, 274.1070.

**Compound II-11:1, 3-dimethyl-8-phenyl-1H-purine-2, 6 (3H, 7H)-dione**



A solution of **II-10** (900 mg, 3.38 mmol) in 2N NaOH (15 ml)/Ethanol (5ml) was refluxed for 1h. Cooled the reaction mixture to 4°C. Diluted the reaction mixture with 2 ml $H_2O$ and acidified with glacial HOAc to pH=5. A white solid was formed which was washed with water and isolated. The solid was then recrystallised in methanol; yield 94%, (0.79 g); M. P. >260 °C $^1$H NMR: (300 MHz, DMSO-*d6*) δ 3.21(s, 3H), 3.45 (s, 3H), 7.19-8.06(m, 5H) $^{13}$C NMR: (74.47 MHz, DMSO-*d6*) δ 27.34, 28.82, 110.14, 126.32, 128.77, 128.91, 130.12, 148.48, 149.71, 151.18, 154.26 IR: (NaCl) 2926.5, 1697.6, 1643.6, 1217.2 $cm^{-1}$. LRMS m/e ($C_{13}H_{12}N_4O_2$) (M+) 256.1. HRMS m/e ($C_{13}H_{12}N_4O_2$) (M+) calcd. 256.0954; found, 256.0960; Anal. ($C_{13}H_{12}N_4O_2$) C, H, N.

**Compound II-12. 7-benzyl-1, 3-dimethyl-8-phenyl-1H-purine-2, 6(3H, 7H)-dione**




A solution of **II-11** (100 mg, 0.39 mmol) and anhydrous $K_2CO_3$ (53.9 mg, 0.39 mmol) in dry DMF (2 mL) was stirred for 10 minutes. Benzyl bromide ( 73.3 mg, 0.43 mmol) was added dropwise and the reaction mixture refluxed for 2h. The reaction mixture was cooled to room temperature and extracted in EtOAc/LiCl (3X). The organic layer was dried over $Na_2SO_4$ and evaporated *in vacuo*. The white solid was recrystallised with methanol; yield 75% (0.1 g); M. P. 179 °C white solid; $^1$H NMR: (300 MHz, $CDCl_3$) δ 3.39(s, 3H), 3.64(s, 3H), 5.62 (s, 2H),

7.02-7.54(m, 10H) $^{13}C$ NMR: (74.47 MHz, $CDCl_3$) δ 28.02, 29.80, 49.35, 107.93, 126.41, 127.90, 128.55, 128.87, 128.96, 129.21, 130.51, 136.61, 148.57, 151.72, 152.62, 155.18 IR: (NaCl) 2951.5, 1703.38, 1660.92, 1541.32, 1452.6 $cm^{-1}$. LRMS m/e ($C_{20}H_{18}N_4O_2$) (M+) 346.4.

**EMSA assay for NF-κB – DNA binding**

EMSA assay for NF-κB-DNA binding: Jurkat cells (E6-TIB clone; Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ng/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. Cells (1.6 X $10^6$ cells/mL) were subsequently treated with various concentrations of the compounds to be tested for 30 min. at 37°C and 5% $CO_2$ followed by TNF-α (0.4 pg) or PMA/PHA (Sigma-Aldrich)(1.0 μM) stimulation for an additional 30 minutes or 5 hours, respectively. The cells were harvested by centrifugation, washed in ice cold PBS and the nuclear extracts were prepared as previously described.[74] The protein concentration of the extracts was determined according to the Bradford method (1976) with BioRad reagents. Nuclear extracts (20 μg total protein) were incubated for 20 minutes at room temperature with a double stranded Cy3 labeled NF-κB consensus oligonucleotide, 5'-AGTTGAGGGGACTTTC CCAGGC-3' (0.16 pmol). The binding mixture (25 μL) contained 10 mM HEPES-NaOH pH 7.9, 4 mM tris-HCl, pH 7.9, 6.0 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.3 mg/mL bovine serum albumin and 1 μg of poly (dI.dC). The mixture was loaded on a 4% polyacrylamide gel prepared in 1X tris borate/EDTA buffer and was electrophoresed at 300 V for 20

minutes. After electrophoresis the gel was analyzed using a phosphorimager (Biorad FX plus) for detection of the NF-κB-DNA binding.

**Measurement of apoptosis using caspase 3/7 assay**

CEM cells (CCRF-CEM); Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 Media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ηg/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. DMSO was used as the vector for all drugs and was added in the control experiments. Cell cultures were treated with 10 μM, 1 μM or 0.01μM of the compounds (theophyllines) and allowed to incubate at 37°C, 5% $CO_2$ atmosphere for 30 minutes. Camptothecin was added at concentrations 0.1 μM and further incubated. An aliquote was transferred to a 96-well plate at various time points 0, 6, 12, 24, 48 hours and mixed with an equal volume of Apo-ONE™ Homogenous Caspase- 3/7 assay (Promega Corporation) reagent. The contents of the plate were gently mixed and allowed to incubate for 1 hour. The fluorescence of each well was then measured on a Molecular Imager FX Pro at 532 nm. All reported data is the average of two independent experiments unless otherwise indicated. Chemopotentiation index was found out with respect to the camptothecin control.

**Inhibition of translocation with p65-ELISA assay**

The quantity of p65/p50 heterodimer that has translocated into the nucleus was measured using a NF-κB p65 sandwich ELISA assay (Imgenex Corp.). Jurkat cells were grown to 1.0 X $10^6$ cells/mL and were subsequently treated with various concentrations of the compounds for 30 min. at 37 °C and 5% $CO_2$

followed by PMA(500 μg/ml)/PHA( 10 μg/ml) stimulation for an additional 4h, respectively. The cells were harvested after 4h and nuclear extracts were prepared as previously described by Dignam and coworkers.[74] The NF-κB p65 sandwich ELISA assay was used to monitor and quantify p65 translocation into the nucleus according to the manufacturer's protocol. Results were read on Biorad's Microplate Benchmark reader at 405 nM. PDTC is reported to be an inhibitor of NF-κB translocation and our data confirmed that it inhibited NF-κB translocation at concentrations ranging from 100 nM to 5.0 μM (data not shown).

**Disc-Diffusion Assay for Anti-Microbial Activity**

Single bacterial colonies were grown on pre-poured nutrient agar plates (23 g /L boiled (1') and autoclaved (15')) overnight. Meuller Hinton agar plates (38 g /L boiled (1') and autoclaved (15')) were prepared. Blank discs were impregnated at 100 μg, 10 μg, 1 μg, 0.1 μg and 0.01μg total compound. Commercially available streptomycin and penicillin discs were used as controls for gram positive and gram negative bacterial strains respectively. Single colonies were picked from the cultures and suspended in 5 ml sterile saline solution. (The turbidity was compared to 0.5 X McFarland standard solution containing 0.5 ml of 1.175% $BaCl_2.2H_20$ in 99.5 ml 1%$H_2SO_4$. This solution should always be stored in dark at room temperature). The control and assay discs were put on the agar plates and allowed to grow at 35 °C for 16 h. The zone of inhibition was then measured and MICs were calculated.

## M. References


1. Fredholm, B. B.; AP, I. J.; Jacobson, K. A.; Klotz, K. N.; Linden, J.,. *Pharmacol. Rev.* **2001,** *53*, (4), 527-52.

2. (a) Muller, C. E.; Thorand, M.; Qurishi, R.; Diekmann, M.; Jacobson, K. A. Padgett, W. L.; Daly, J. W. *J. Med. Chem.* **2002**, *45*, 3440-3450.(b) Fredholm, B. B.; Arslan, G.; Halldaner, L.; Kull, B.; Schulte, G.; Wasserman, W. *Naunyn-Schmiedberg's Arch. Pharmacol.* **2000**, *362*, 364-374.

3. Sajjadi, F. G.; Takabayashi, K.; Foster, A. C.; Domingo, R. C.; Firestein, G. S. *J. Immunol.* **1996,** *156*, (9), 3435-42.

4. Liang, B. T.; Jacobson, K. A. *Proc. Natl. Acad. Sci. U S A* **1998,** *95*, (12), 6995-9.

5. von Lubitz, D. K.; Simpson, K. L.; Lin, R. C. *Ann. N Y Acad. Sci.* **2001,** *939*, 85-96.

6. Fishman, P.; Bar-Yehuda, S.; Madi, L.; Cohn, I. *Anticancer Drugs* **2002,** *13*, (5), 437-43.

7. Fishman, P.; Madi, L.; Bar-Yehuda, S.; Barer, F.; Del Valle, L.; Khalili, K., *Oncogene* **2002,** *21*, (25), 4060-4.

8. Avila, M. Y.; Stone, R. A.; Civan, M. M. *Invest Ophthalmol Vis. Sci.* **2002,** *43*, (9), 3021-6.

9. Mehta, P. N.; Panitch, H. B.; Wolfson, M. R.; Shaffer, T. H. *Pediatric Res.* **1991**, *29*(5), 446-8.

10. (a) Becker, A. B.; Simons, K. J.; Gillespie, C. A.; Simons, F. E. R. *New Engl. J. Med.* **1984**, *310*(12), 743-746. (b) Simons, F. E. R.; Simons, K. J.; Becker, A. B. *Curr. Clin. Practice Ser.* **1984**, *8*, 1-8.

11. Henderson, J. C.; O'Connell, F.; Fuller, R. W. *Thorax* **1993**, *48*(8), 824-6.

12. (a) Ulus, A. T.; Aksoyek, A.; Katircioglu, S. F.; Gokce, P.; Koc, B. *Panminerva medica* **2000**, *42*(4), 253-6. (b) Broadley, Kenneth J. *Exp. Opin. Ther. Patents* **2000**, *10*(11), 1669-1692.(c) Harada, H.; Asano, O.; Hoshino, Y.; Yoshikawa, S.; Matsukura, M.; Kabasawa, Y.; Niijima, J.; Kotake, Y.; Watanabe, N.; Kawata, T.; Inoue, T.; Horizoe, T.; Yasuda, N.; Minami, H.; Nagata, K.; Murakami, M.; Nagaoka, J.; Kobayashi, S.; Tanaka, I.; Abe, S. *J. Med. Chem.* **2001**, *44*, 170-179. (d) Roberts, D. J.; Broadley, K. J. *Lancet* **1965**, *33*, 1219-20.

13. (a) Perreira, M.; Jiang, J.-K.; Klutz, A. M.; Gao, Z.-G.; Shainberg, A.; Lu, C.; Thomas, C. J.; Jacobson, K. A. *J. Med. Chem.* **2005**, *48*, 4910 -4918. (b) Yan, L.; Burbiel, J. C.; Maass, A.; Müller, C. E. *Exp. Opin. Emer. Drugs* **2003**, *8*, 537-576.

14. (a) Jacobson, K. A.; Siddiqi, S. M.; Olah, M. E.; Ji, X. D.; Melman, N.; Bellamkonda, K.; Meshulam, Y.; Stiles, G. L.; Kim, H. O. *J. Med. Chem.* **1995**, *38*, 1720-35. (b)von Lubitz, D. K.; Lin, R.C.; Popik, P.; Carter, M. F.; Jacobson, K. A. *Eur. J. Pharmacol.* **1994**, *263*, 59.

15. Burnouf, C.; Pruniaux, M. P. *Curr. Pharm. Des.* **2002**, *8*, 1255.

16. Druker, B. J.; Talpaz, M.; Resta, D. J.; Peng, B.; Buchdunger, E. Ford, J. M.; Lydon, N. B.; Kantarjian, H.; Capdeville, R ; Ohno-Jones, S.; Sawyers, C. L. *N. Engl. J. Med.* **2001**, *344*, 1031-1037.

17. Traxler, P.; Furet, P. *Pharmacol. Ther.* **1999**, *82*, 195.

18. (a) Nurse, P. *Cell* **2000**, *100*, 71-78.(b) Nurse, P. *Science*, **2000**, *289*, 1711-1716.

19. Hanahan, D.; Weinberg, R. A. *Cell* **2000**, *100*, 57-70.

20. Sherr, C. J. *Science* **1996**, *274*, 1672-1677.

21. (a) Pavletich, N. P. *J. Mol. Biol.* **1999**, *287*, 821-828.(b) Morgan, D. O. Nature, **1995**, *374*, 131-134.(c) Fisher, R. P.; Morgan, D. O. *Cell*, **1994**, *78*, 713-724.(d) Russo, A. A., Tong, L., Lee, J. O., Jeffrey, P. D.; Pavletich, N. P. Nature,**1998**, *395*, 237-243.

22. van den Heuvel, S.; Harlow, E. *Science* **1993**, *262*, 2050-2054.

23. (a)Weinberg, R. A. *Cell* **1995**, *81*, 323-330. (b) Hatakeyama, M.; Weinberg, R. A. *Prog. Cell Cycle Res.* **1995**, *1*, 9-19.

24. Cangi, M. G.; Cukor, B.; Soung, P.; Signoretti, S.; Moreira, G., Jr.; Ranashinge, M.; Cady, B.; Pagano, M.; Loda, M. *J. Clin. Invest.* **2000**, *106*, 753-761.

25. (a) Baylin, S. B.; Herman, J. G. *Trends Genet.* **2000**, *16*, 168-174. (b) Jones, P. A.; Laird, P. W. *Nat. Genet.* **1999**, *21*, 163-167.

26. (a) Foukas, L. C.; Daniele, N.; Ktori, C.; Anderson, K. E.; Jensen, J.; Shepherd, P. R. *J. Biol. Chem.* **2002**, *277*, 37124-37130.(b) Yasui, A.; Komiyama, A. *Int. J. Hematol.* **2001**, *73*, 87-92. (c) Basu, S.; Mitra, R. *Haematologia* **2000**, *30*, 225-227. (d) Sarkaria, J. N.; Busby, E. C.;

Tibetts, R. S.; Roos, P.; Taya, Y.; Karnitz, L. M.; Abraham, R. T. *Cancer Res.* **1999**, *59*, 4375–4382.

27. Blasina, A.; Proce, B. D.; Turenne, G. A.; McGowan, C. H. *Curr. Biol.* **1999**, *9*, 1135–1138.

28. Vanhaesebroeck, B.; Leevers, S.; Ahmadi, K.; Timms, J.; Katso, R.; Driscoll P. C.; Woscholski, R.; Parker, P. J.; Waterfield, M. D. *Annu. Rev. Biochem.* **2001**, *70*, 535–602.

29. Stein, R. C.; Waterfield, M. D. *Mol. Med. Today* **2000**, *6*, 347–357.

30. Hirsch, E.; Bosco, O.; Tropel, P.; Laffargue, M.; Calvez, R.; Altruda, F.; Wymann, M.; Montruchchio, G. *FASEB J.* **2001**, *15*, 2019–2022.

31. Vanhaesebroeck, B.; Welham, M. J.; Kotani, K.; Stein, R.; Warne, P. H.; Zvelebil, M. J.; Higashi, K.; Volinia, S.; Downward, J.; Waterfield, M. D. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 4330–4335.

32. Vanhaesebroeck, B.; Jones, G. E.; Allen, W. E.; Zicha, D.; Hooshmand-Rad, R.; Sawyer, C.; Wells, C.; Waterfield, M. D.; Ridley, A. J. *Nat. Cell Biol.* **1999**, *1*, 69–71.

33. Okkenhaug, K.; Bilancio, A.; Farjot, G.; Priddle, H.; Sancho, S.; Peskett, E.; Pearce, W.; Meek, S. E.; Salpekar, A.; Waterfield, M. D.; Smith, A. J. H.; Vanhaesebroeck, B. *Science* **2002**, *297*, 1031–1034.

34. O'Connor, P. M. *Cancer Surv.* **1997**, *29*, 151–182.

35. Elledge, S. J. *Science* **1996**, *274*, 1664–1672.

36. (a)Zhou, B. B.; Chaturvedi, P.; Spring, K.; Scott, S. P.; Johanson, R. A.; Mishra, R.; Mattern, M. R.; Winkler, J. D.; Khanna, K. K. *J. Biol. Chem.* **2000**, *275*, 10342-10348. (b) Nurse, P. *Cell* **1994**, *79*, 547-550.(c) Gautier, J.; Solomon, M. J.; Booher, R. N.; Bazan, J. F.; Kirschner, M. W. *Cell* **1991**, *67*, 197-211. (d) Kumagai, A.; Dunphy, W. G. *Cell* **1992**, *70*, 139-151.(e) Dalal, S. N.; Schweitzer, C. M.; Gan, J.; deCaprio, J. A. *Mol. Cell Biol.* **1999**, *19*, 4465-4479. (f) Lopez-Girona, A.; Furnari, B.; Mondesert, O.; Russell, P. **1999**, *397*, 172-175.

37. Powell, S. N.; DeFrank, J. S.; Connell, P.; Eogan, M.; Preffer, F.; Dombkowski, D.; Tang, W.; Friend, S. *Cancer Res.* **1995**, *55*, 1643–1648.

38. Jiang, X.; Lim, L. Y.; Daly, J. W.; Li, A. H.; Jadobson, K. A. *Internat. J. Oncol.* **2000**, *16*, 971-978.

39. (a) Daly, J. W.; Padgett, W. L.; Shamim, M. T. *J. Med. Chem.* **1986**, *29*, 1305-1308. (b) Shamim, M. T.; Ukena, D.; Padgett, W. L.; Daly, J. W. *J. Med. Chem.* **1989**, *32*, 1231-1237. (c) Daly, J. W.; Hide, I.; Muller, C. E.; Shamim, M. *Pharmacol.* **1991**, *42*, 309-321.

40. Choi, O. H.; Shamim, M.T.; Padgett, W. L.; Daly, J. W. *Life Sci.* **1988**, *43*, 387-398.

41. Arnaud, M .J. Prog. Drug. Res. **1987**, *31*, 273-313.

42. (a)Matsuoka, S.; Huang, M.; Elledge, S. J. *Science*, **1998**, *282*, 1893-1897. (b) Chaturvedi, P.; Eng, W. K.; Zhu, Y.; Mattern, M. R.; Mishra, R.; Hurle, M. R.; Zhang, X.; Annan, R. S.; Lu, Q.; Faucette, L. F.; Scott, G. F.; Li, X.; Carr, S. A.; Johnson, R. K. Winkler, J. D.; Zhou, B.-B. S. *Oncogene* **1999**, *18*, 4047-4054.

43. Ferrarini, M.; Heltai, S.; Zocchi, M. R.; Rugarli, C. *Int. J. Cancer* **1992**, *51*, 613-619.

44. Neckers, L.; Schulte, T. W.; Mimnaugh, E. *Invest. New Drugs*, **1999**, *17*, 361-373.

45. (a) Stebbins, C. E.; Russo, A. A.; Schneider, C.; Rosen, N.; Hartl, F. U.; Pavletich, N. P. *Cell*, **1997**, *89*, 239-250. (b) Roe, S. M.; Prodromou, C.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *J. Med. Chem.* **1999**, *42*, 260-266.

46. Neckers, L. *Trends Mol. Med.* **2002**, *8*, S55-S61.

47. (a) Munster, P. N.; Srethapandi, M.; Moasser, M. M.; Rosen, N. *Cancer Res.* **2001**, *61*, 2945-2952. (b) Srethapakdi, M.; Liu, R.; Tavorath, R.; Rosen, N. *Cancer Res.* **2000**, *60*, 3940-3946.

48. (a) Maloney, A.; Workman, P. *Exp. Opin. Biol. Ther.* **2002**, *2*, 3. (b) Banerji, U.; O'donnell, A.; Scurr, M.; Benson, C.; Brock, C.; Hanwell, J.; Stapleton, S.; Raynaud, F.; Simmons, L.; Turner, A.; Walton, M.; Workman, P.; Judson, I. *Proc. Am. Assoc. Cancer Res.* **2002**, *43*, Abst. 1352.

49. Chiosis, G.; Timaul, M. N.; Lucas, B.; Munster, P. N.; Zheng, F. F.; Sepp-Lorenzino, L.; Rosen, N. *Chem. Biol.* **2001**, *8*, 289-299.

50. Smith, V.; Sausville, E. A.; Camalier, R. F.; Fiebig, H. H.; Burger, A. M. *Eur. J. Cancer* **2002**, *38*, Suppl 7, S60-S60.

51. Chiosis, G.; Lucas, B.; Shtil, A.; Huezo, H. Rosen, N. *Bioorg. Med. Chem.* **2002**, *10*, 3555-3564.

52. Marcu, M. G.; Chadli, A.; Bouhouche, I.; Catelli, M.; Neckers, L. M. *J. Biol. Chem.* **2000**, *275*, 37181-37186.

53. Armstrong, J. I.; Bertozzi, C. R. *Curr. Opin. Drug Disc. Dev.*, **2000**, *3*, 502-515.

54. Burkart, M. D.; Wong, C. H. *Anal. Biochem.* **1999**, *274*, 131-137.

55. Fee, J. P. H. *Pharmacology for Anaesthesiologists* **2005**, 147-151.

56. (a) Coward, W. R. Sagara, H.; Church, M. K. *Clin. Exp. Allergy* **1998**, *28*, 42-46. (b)Yoshimura, T.; Usami, E.; Kurita, C.; Watanabe, S.; Nakao, T.; Kobayashi, J.; Yamazaki, F.;Nagai, H. *Biol. Pharm. Bull.* **1995**, *18*, 1405-1408.

57. (a) Collart, M.A.; Baeuerle, P.; Vassalli, P. Mol. Cell Biol. **1990**, *10*, 1498–1506. (b) Libermann, T. A.; Baltimore, D. Mol. Cell Biol. **1990**, *10*, 2327-2334. (c) Matsusaka, T.; Fujikawa, K.; Nishio, Y.; Mukaida, N.; Matsushima, K.; Kishimoto, T.; Akira, S. *Proc. Natl. Acad. Sci. USA* **1993**, *90*,10193–10197.

58. Ichiyama, T.; Hasegawa, S.; Matsubara, T.; Hayashi, T.; Furukawa, S. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2001**, *364*, 558–561.

59. (a) Yin, M.; Yamamoto, Y.; Gaynor, R. B. *Nature* **1998**, *396*, 77–80.(b) Liu, S. F.; Ye, X.; Malik, A. B. *Mol. Pharmacol.* **1999**, *55*, 658-667. (c) Roy, S.; Cain, K. J.; Chapin, R. B.; Charboneau, R. G.; Barke, R. A. *Biochem. Biophys. Res. Comm.* **1998**, *245*, 392-396.

60. Mosselhi, M. A., Pfleiderer, W. *J. Heterocyclic. Chem.* **1993**, *30*, 1221-1228.

61. Cacace, F.; Masironi, R. *Annali di Chimica* **1957**, *47*, 362-5.

62. (a) Gagauzov, I.; Peikov, P.; Davkov, D.; Sharankov, K. *Farmatsiya*, **1987**, *37*, 8-10. (b) Mukhopadhyay, S.; Ananthakrishnan, S.; Chnadalia, S. B. *Org. Proc. Res. Dev.* **1999**, *3*, 451-454.

63. (a) Farah, S. F.; McClelland, R. A. *Can. J. Chem.* **1993**, *71*, 427-32. (b) Kumar, J. S. D.; Ho, M. M.; Toyokuni, T. *Tetrahedron Lett.* **2001**, *42*, 5601-5603. (c) Yeh, M.-L.; Tang, F. -S.; Chen, S. -L.; Liu, W. -C.; Lin, L. -G. *J. Org. Chem.* **1994**, *59*, 754-757. (d) Redemann, C. T.; Redemann, C. E. *Org. Syn.* **1955** *3*, 69-72. (e) Ivanov, E. I. *Chem. Heterocyclic Compds.* **1998**, *34*, 719-722.

64. (a)Lespagnol, A.; Tudo, M.; Labiau, O.; Robelet, A.; Bizard-Gregoire, N. *Ann. Pharm. Fr.* **1968**, *26*, 207-214. (b) Ozola, V.; Ramzaeva, N.; Maurinsh, Y.; Lidaks, M. *Nucleosides Nucleotides* **1993**, *12*, 827-839.

65. Ueda, T.; Oh, R.; Nagai, S.-I.; Sakakibara, J. *J. Heterocyclic Chem.***1998**, *35*(1), 135-139.

66. (a) Kawasaki, I.; Taguchi, N.; Yoneda, Y.; Yamashita, M.; Ohta, S. *Heterocycles*, **1996**, *43*, 7, 1375.(b) Pawlowski, M.; Goryczyca, M. *Pol. J. Chem.* **1981**, *55*, 837. (c) Wolfe, J. P.; Buchwald, S. L. *J. Org. Chem.* **2000**, *65*, 1144-1157. (d) Gillet, L. C. J.; Scharer, O. D.*Org. Lett.* **2002**, *4*(24), 4205-4208.

67. (a) Daly, J. W.; Padgett, W.; Shamim, M. T.; Butts-Lamb, P.; Waters, J *J. Med. Chem.* **1985**, *28*, 487-492.

68. Kim, S. A.; Marshall, M. A.; Melman, N.; Kim, H. S.; Müller, C. E.; Linden, J.; Jacobson, K. A. *J. Med. Chem.* **2002**, *45*, 2131-2138.

69. (a) Pommier, Y., Pourquier, P., Fan, Y., and Strumberg, D. *Biochim. Biophys. Acta* **1998**, *1400*, 83-105.(b) Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723-2795.

70. (a) Boland, M. P. *Biochem. Soc. Trans.* **2001**, *29*, 674-678.(b) Guttridge, D. C.; Albanese, C.; Reuther, J. Y.; Pestell, R. G.; Baldwin, A. S., Jr. *Mol. Cell. Biol.* **1999**, *19*, 5785-5799.

71. (a) Barnes, P. J.; Karin, M. *N Engl J Med* 1997, *336*, 1066-1071.(b)Baldwin, A. S., Jr. *Annu Rev Immunol* 1996, *14*, 649-683.(c) Baeuerle, P. A.; Baltimore, D. *Science* 1988, *242*, 540-546.

72. (a) Baeuerle, P. A.; Henkel, T. *Annu. Rev. Immunol.* 1994, *12*, 141-179. (b) Tak, P. P.; Firestein, G. S. *J. Clin. Invest.* 2001, *107*, 7-11.

73. (a) Rowland, T. L.; McHugh, S. M.; Deighton, J.; Ewan, P. W.; Dearman, R. J. ;Kimber, I. *Int. Immunopharmacol.* **2001**, *1*, 49-61.

74. Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. *Nucleic Acids Res.* **1983**, *11*, 1475-1489.

75. Li, C.- C. H.; Dai, R. M.; Chen, E.; Longo, D. L. *J. Biol. Chem.* **1994**, *269*, 30089-30092.

76. Cuzzocrea, S.; Chatterjee, P. K.; Mazzon, E.; Dugo, L.; Serraino, I.; Britti, D.; Mazzullo, G.; Caputi, A. P.; Thiemermann, C. *Br. J. Pharmacol.* **2002**, *135*, 496-510.

77. Shinomiya, N.; Takemura, T.; Iwamoto, K.; Rokutanda, M. *Cytometry*, **1997**, *27*, 365-373.

78. (a) Bone, R. C. *Ann. Intern. Med.* **1991**, 115, 457-469. (b) Bone, R. C. *Crit. Care Med.* **1994**, *22*, S8-11.(c) Bone, R. C.; Sprung, C. L.; Sibbald, W. J. *Crit. Care Med.* **1992**, *20*, 724-6. (d) Bone, R. C.; Balk, R. A.; Cerra, F. B.; Dellinger, R. P.; Fein, A. M.; Knaus, W. A.; Schein, R. M.; Sibbald, W. J. *Chest* **1992**, *101*, 1644-55.

79. (a) Bone, R. C. *Arch. Intern. Med.* **1994**, *154*, 26. (b) Friedman, G., Silva, E.; Vincent. J. L. *Crit. Care Med.* **1998**, *26*, 2078. (c) Wheeler, A. P.; Bernard. G. R. *N. Engl. J. Med.* **1999**, *340*, 207.

80. Jenei, B.; Pócsik, É.; Lázár, G.; Medgyesi, G. A. *Inflamm. res.* **1997**, *46*, 287-91.

81. Samuelson, J. *Antimicrobial agents and chemotherapy*, **1999**, 1533.

82. (a) Rietschel, E. T.; Brade, H. *Sci. Am.* **1992**, *267*, 54. (b) Brown, Z.; Gerritsen, M. E.; Carley, W. W.; Strieter, R. M.; Kunkel, S. L.; Westwick, J. *Am. J. Pathol.* **1994**, *145*, 913. (c) Blease, K.; Seybold, J.; Adcock, I. M.; Hellewell, P. G.; Burke-Gaffney, A. *Am. J. Respir. Cell Mol. Biol.* **1998**, *18*, 620.

83. Bone, R. C. *J. Crit. Care Med.* **1993**, *8*, 51.

84. (a) Standiford, T. J.; Arenberg, D. A.; Danforth, J. M.; Kunkel, S. L.; VanOtteren, G. M.; Strieter, R. M. *Infect. Immun.* **1994**, *62*, 119. (b) Carratelli, C. R.; Nuzzo, I.; Bentivoglio, C.; Galdiero, M. FEMS Immunol. *Med. Microbiol.* **1996**, *16*, 309.

## CHAPTER III

# SENSITIZATION OF TUMOR CELLS TOWARDS CHEMOTHERAPY: ENHANCING THE ACTIVITY OF CAMPTOTHECIN BY NOVEL IMIDAZOLINES

*All great progress takes place when two sciences come together, and when their resemblance proclaims itself, despite the apparent disparity of their substance.*

.....*Henri Poincaré*

### A. Introduction- why is apoptosis important?

Chemotherapy for the treatment of cancer was introduced more than fifty years ago. While some forms of carcinomas have successfully been treated, treatment for common epithelial tumors of breast, colon, and lung has been less than impressive. Under most ideal circumstances, chemotherapeutic drugs are expected to target cancer cells and cause cytotoxic or cytostatic effects to reduce the tumor burden while evading damage to normal cells. However, in reality, chemotherapy suffers a range of perplexing issues including systemic toxicity, rapid metabolism and resistance.

Cell death is an indispensable process for normal embryonic development and adult homeostasis. Cells die via either of two mechanisms, necrosis or apoptosis. Insidious stimuli in and outside the cells disturb the homeostatic balance within the cells leading to a catastrophic lysis often called messy cell death. Apoptosis however, is a genetically controlled form of programmed cell death with a sequential, stepwise 'defensive mechanism' to neatly remove infected, mutated or damaged cells. [1, 2]

Conventional anticancer therapy involving chemotherapy or ionizing radiation is aimed at the induction of apoptosis in cancer cells.[3] Chemotherapeutic drugs induce a series of cellular changes to impact proliferation and cell survival and cause apoptosis. Cell death by apoptosis is accompanied by interconnected series of sequential events where cells collapse, the membrane blebs, chromatin condensation and DNA degradation are well-recognized.[2] Alterations in apoptotic pathways leading to excessive or insufficient cell death can disrupt the delicate balance between cell proliferation and cell death leading to a variety of diseases such as Alzheimer's disease and a large variety of cancers.[3, 4] In many cancers, apoptosis is abnormally down regulated, either by the mutation of pro-apoptotic proteins or by the upregulation of anti-apoptotic proteins.[5] Aberrant apoptosis provides an intrinsic survival advantage to cancer cells causing growth and expansion of the tumor, and resistance to pro-apoptotic signals including chemotherapeutic agents.[6, 7]

**B. Currently used chemotherapeutic agents.**

Current chemotherapy can be divided into either bringing out genotoxic stress via DNA topo-1 and 2 inhibitors such as camptothecin (clinically used analogs are topotecan or irinotecan), etoposide, daunomycin or DNA cross linkers such as cis-platin, oxaliplatin which ultimately culminate in an apoptotic response. Other clinically used approaches for chemotherapy include use of anti-tubulin agents such as vinca alkaloids, taxol, maystansine and rhizoxin.[8] These cause mitotic catastrophe by targeting tubulin, a 100 kDa protein and a principal subunit of microtubules which participate in spindle formation and chromosomal segregation during M-phase of cell cycle. Antimetabolites such as anti-folic acid agents, ribonucleotide reductase inhibitors etc. which indirectly

interfere with the nucleic acid biosynthesis are important chemotherapeutic agents.[8] Figure III-1 shows the common classes of drugs used in chemotherapy.

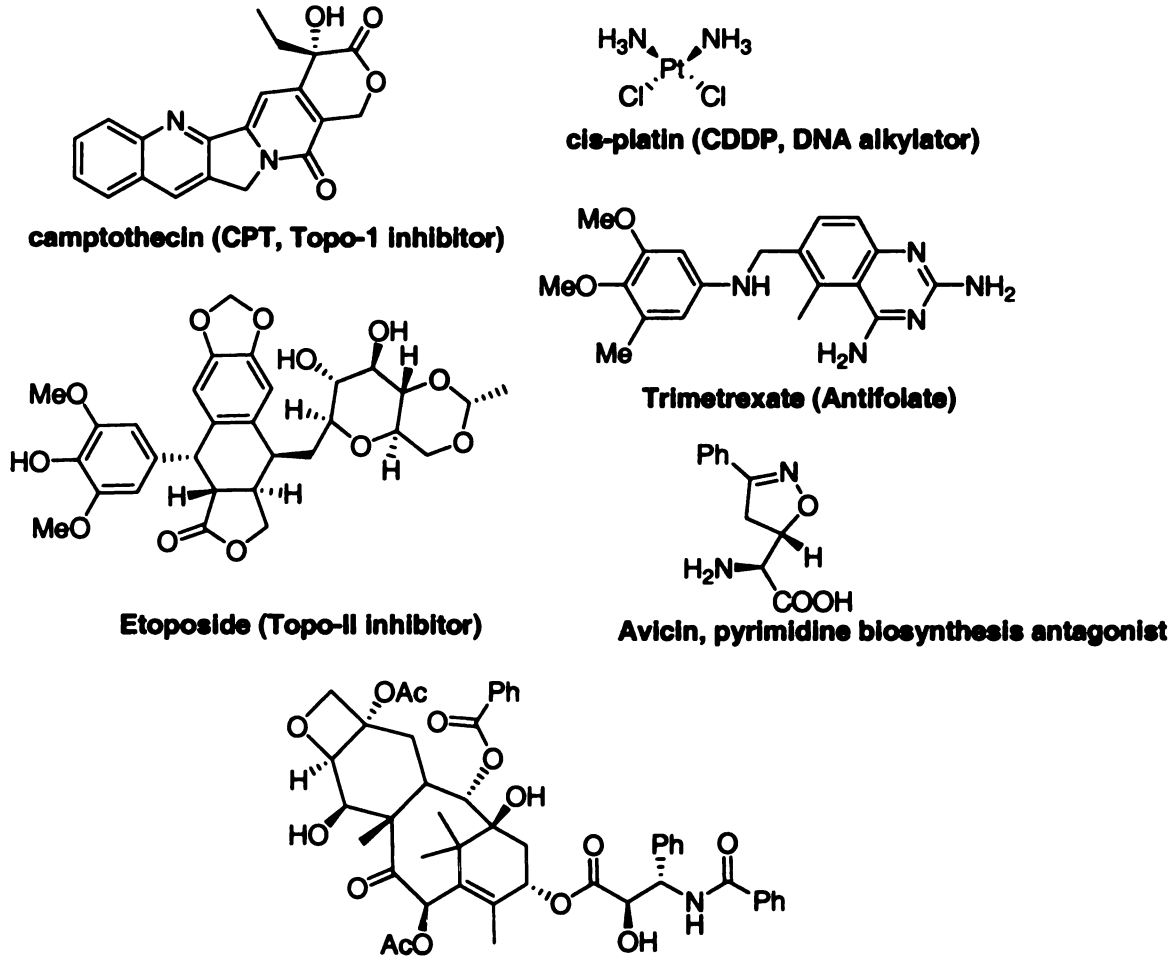



**Figure III-1.** Structures of commonly used drugs in chemotherapy.

**i) Camptothecin and its mechanism of action.**

The antitumor agent camptothecin (CPT) is a cytotoxic plant alkaloid isolated from the extracts of the fruit of *Camptotheca acuminata* that exhibits specificity for DNA topoisomerase I and induces a stable ternary topoisomerase I - DNA cleavable complex.[8, 9] Camptothecin exhibits its antitumor activity via the formation of a stable ternary topoisomerase I - DNA cleavable complex.[8, 10] Stabilization of this cleaved DNA complex is recognized as damaged DNA and initiates an apoptotic signaling pathway,

ultimately resulting in cell death.[9-11] CPT-11 and several water soluble analogues including topotecan and irinotecan have successfully passed clinical trials in the United States.[10]

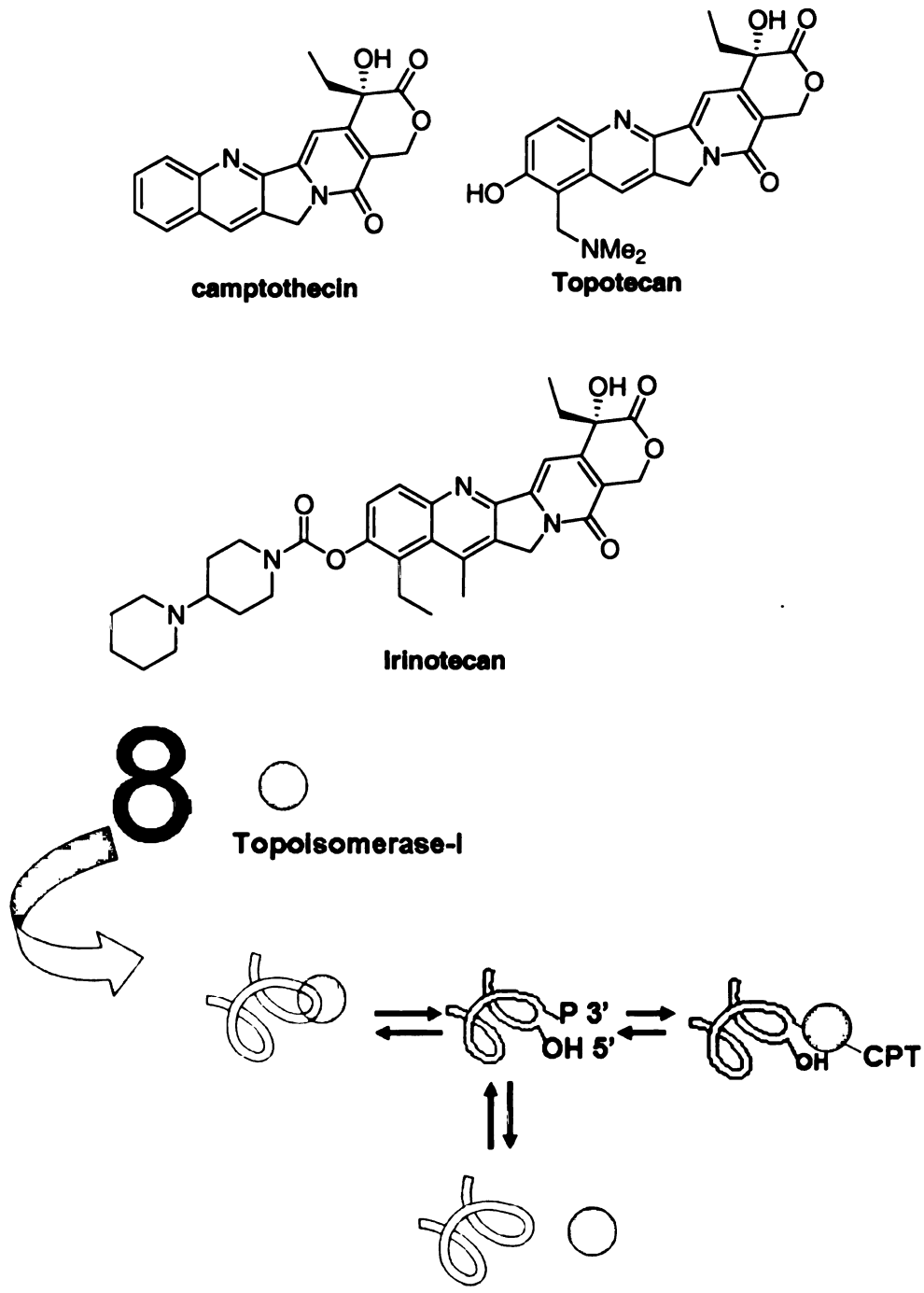



**Figure III-2.** (TOP) Structures of clinically used analogs of camptothecin. (BOTTOM) CPT stabilizes the 3'-phosphoryl end of DNA complex with protein.

Supercoiled DNA is relaxed by catalytic amounts of topoisomerase -1 over several cycles (Figure III-2) and creates a single stranded nick, unwinds the supercoil, reseals the backbone and finally dissociates from the DNA.[10d] In presence of camptothecin however, the DNA remains nicked and once collides with the replication fork does not allow DNA replication ultimately leading to cells death.[8]

**ii) Cis-platin and DNA alkylation.**

Similar to camptothecin, the clinically used DNA damaging agent, cis-platin (CDDP), exerts its anticancer activity through DNA damage via covalent binding to DNA base pairs and initiates an apoptotic signaling pathway.[12, 13] Figure III-3 shows (1,2) ,(1,3) intra-strand crosslinking and inter-strand crosslinks. Cis-platin forms cross links at N-7 of the guanine which leads to inhibition of replication, transcription, DNA repair and cell death.

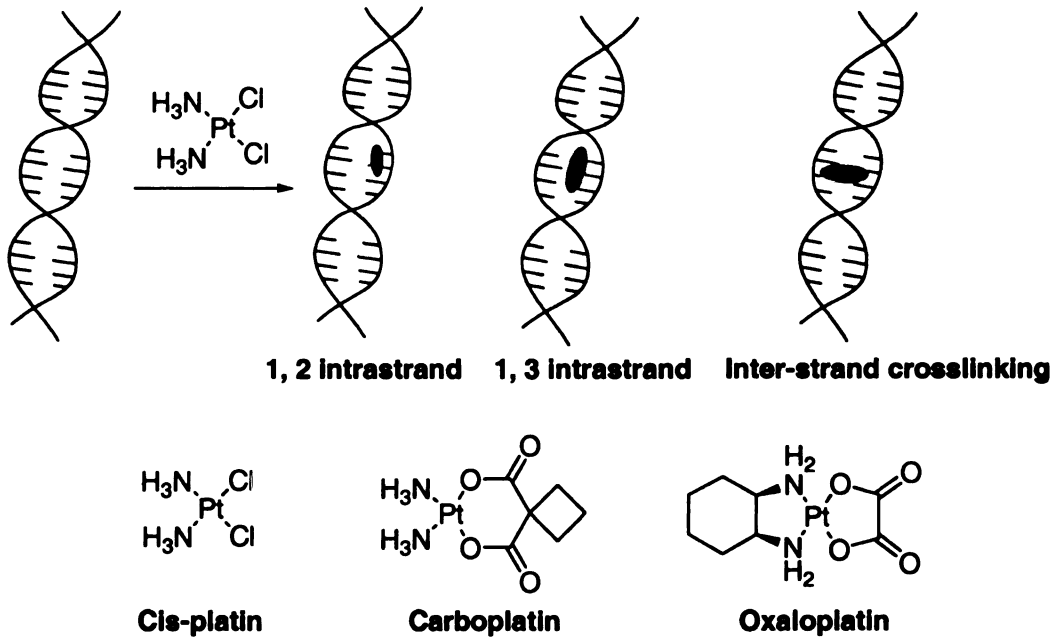



**Figure III-3.** (TOP) Intra and Inter strand cross linking of cis-platin with DNA. (BOTTOM) Water soluble analogs of cis-platin- carboplatin and oxaloplatin.

Cis-platin undergoes aquation to form $[Pt(NH_3)_2(OH_2)Cl]^+$ and $[Pt(NH_3)_2(OH_2)_2]^{2+}$ which are more reactive species than cis-platin itself. Carboplatin is more stable than cis-platin due to its six-membered ring and shows similar activity and cross resistance as cis-platin.[15] Oxaliplatin however, is active even against cis-platin resistant L1210 leukemia cell lines.[15]

Concomitant with the initiation of the apoptotic cell signal, in response to drug-induced DNA assault (both camptothecin and cis-platin), a range of signaling cascades involved in cell cycle arrest and DNA repair are also activated. These are tightly controlled by upstream regulatory pathways including the pro-apoptotic inositol triphosphate (InsP3) pathway[16] and the survival PI(3)kinase-Akt-dependent pathway as well as key transcription factors p53 and NF-κB[18, 19] and compromise the overall efficacy of chemotherapeutic anticancer treatment. Figure III-4 illustrates some signal transduction pathways when DNA damage is sensed.

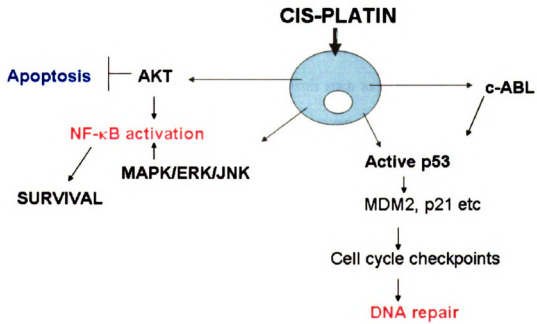



**Figure III-4.** Various signal transduction pathways are triggered upon DNA damage.

The ultimate decision whether a cell would survive or undergo cell death is dependent upon the outcome of these signal transduction pathways. Figure III-5 illustrates the future of the cell after DNA damage.

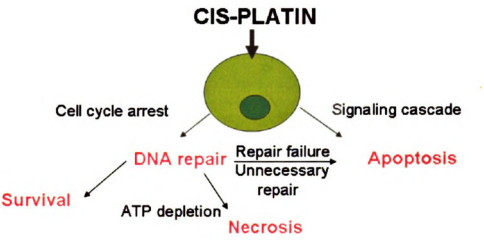



**Figure III-5.** Future of the cell after DNA damage.

**C. DNA damage, mutations and survival pathways**

Multi-drug resistance is one of the main obstacles in chemotherapy of cancer. The P-glycoprotein sets up an energy-dependent efflux mechanism which pumps the drug out of the cell. Most modulators of this category either directly bind to the P-glycoproteins or inhibit the efflux action by indirect mechanisms such as expression of *mdr1* and *mrp1* genes.[20] Thus, MDR can often be attributed to over-expression of the mdr1 gene that codes for an ATP-dependent, transmembrane P-glycoprotein (P-gp) efflux pump pathway, which rapidly exports man structurally un-related drugs from the cell, including anthracyclines, daunomycin, doxorubicin, bleomycin etc.

Its inhibition by combination of chemosensitizers or even combination of drugs for additive toxicity is an active field of research.[7] Expression of all MDR1 gene products has been associated with a poor prognosis in clinical studies. Several research groups

have demonstrated in a laboratory setting that resistance mediated by the P-glycoprotein may be modulated by a wide variety of compounds including verapamil and cyclosporine (Figure III-6), which generally have little or no effect by themselves on the tumor cells, but when used in conjunction with anti-neoplastic agents, they decrease, and in some instances even eliminate, drug resistance.

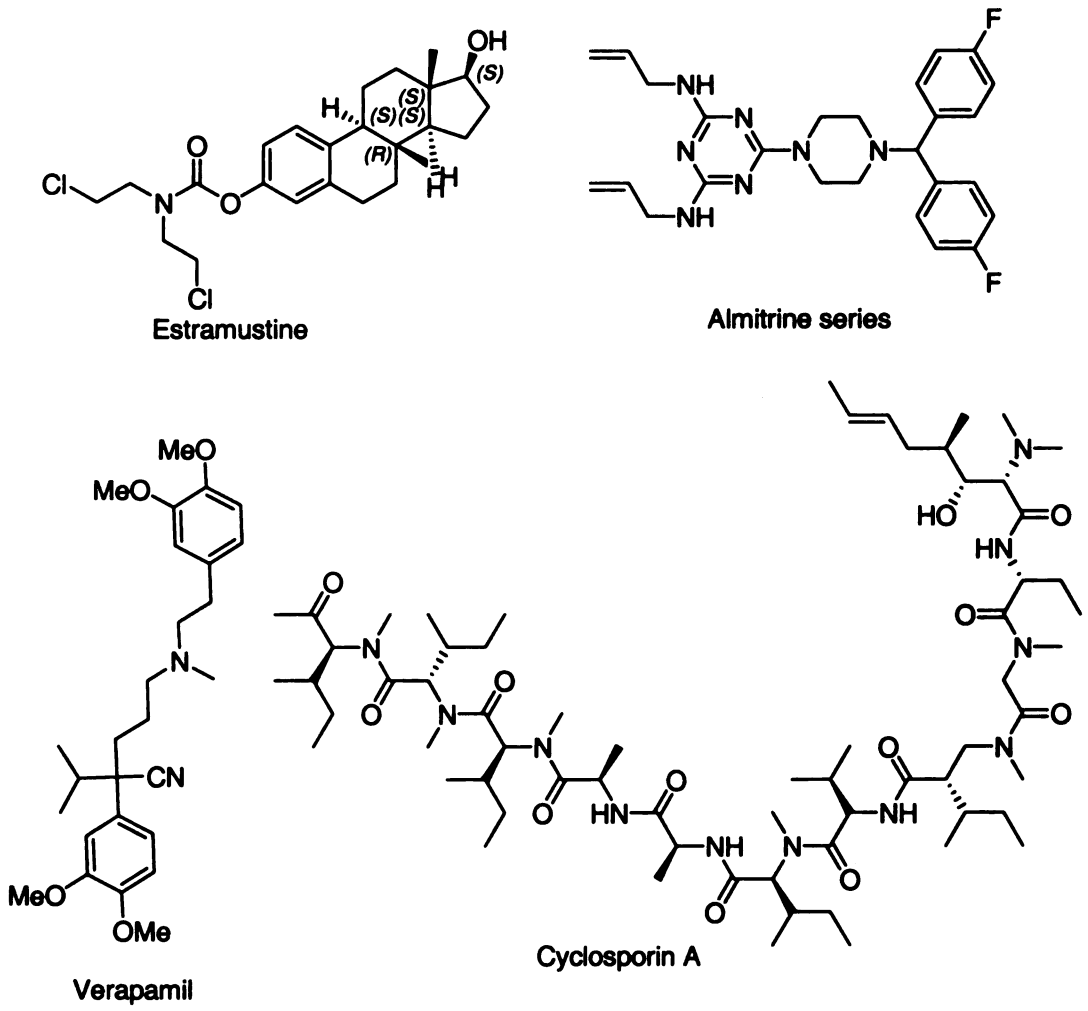



**Figure III-6.** Structures of P-glycoprotein modulators.

**i) MDR and proteasomal inhibition.**

The 26S proteosome is a highly conserved multi-catalytic protease responsible for ATP- and ubiquitin-dependent degradation of all short-lived and 70–90% of all long lived

proteins including cyclin A, B and E, p21 and p27, p53, cJun, cFos, and IκB.[22] Hence, the 26S proteosome controls cell cycle, signal transduction pathways, apoptosis and major functions of the immune system.[20b] Remarkably, the HIV protease inhibitor ritonavir was identified as an inhibitor of both the P-glycoprotein and the proteasome.[21] Owing to the inhibitory effect of molecules like ritonavir and cyclosporine A of both proteasome and P-gp activities, Pajonk *et al* investigated whether there occurs a cross specificity between P-gp and proteasome activities. The existence of this cross specificity might explain the effects of P-gp inhibitors on multiple cellular parameters that seem extrinsic to a pumping function of P-gp and provide insights for the development of more selective P-gp inhibitors.[22] They found that modulators of P-glycoproteins such as verapimil were proteasomal inhibitors and vice versa was true for MG132. Tumor cells in general exhibit altered patterns of expression of proteasome subunits than normal cells.[23] Inhibition of proteasome function induces apoptosis of tumor cells[24] and sensitizes the surviving tumor cells to the actions of chemotherapy and radiation therapy.[26] Therefore, proteasome inhibitors might overcome P-gp-related MDR, with accompanying chemo- and radiosensitizing effects. On the other hand, direct inhibition of proteasome function might be an additional major mechanism of action for anthracyclines. Such inhibition could contribute to their ability to enhance the efficacy of other chemotherapeutic drugs, independent of their ability to enhance the efficacy of other chemotherapeutic drugs, independent of their ability to reverse MDR.

Apart from multi-drug resistance, there are other oncogenic and non-oncogenic factors which contribute to drug-induced resistance. Unfortunately, due to mutations that interfere with drug-induced initiation or execution of apoptosis, the pro-apoptotic

response is abnormally down-regulated and provides an intrinsic survival advantage in many types of cancers.[6, 18]

Moreover, this intrinsic cellular resistance has also been attributed to the activation of anti-apoptotic signaling pathways concomitant to activation of apoptotic signal upon exposure to chemotherapeutic agents.[19, 27] Christman *et al* [28]observed that 8.9% of the genes of inflammation were changed upon treatment with camptothecin. This suggested that CPT may induce genes involved in inflammation. EGFR and NF-κB have been implicated as the two major anti-apoptotic signaling pathways induced in HeLa cells after CPT treatment.[28] In Hela cells, cDNA microarrays using all annotated human genes have been used to establish the association between characteristic gene expression patterns in response to camptothecin.[28] These studies provided a striking evidence that administration of camptothecin resulted in up-regulation in a large number of genes controlled by NF-κB.[28] The combination of these anti-apoptotic mechanisms has resulted in an increased dose intensity of chemotherapeutics, often without the anticipated improved therapeutic results.[29] The search for new chemotherapeutic strategies has therefore shifted to small molecules that can selectively induce apoptosis in cancer cells or retard the cellular chemoresistance.[30-32] Strategies using combinations of inducers of apoptosis and/or inhibitors of anti-apoptotic factors and traditional chemotherapeutic drugs may provide an improved alternative to conventional chemotherapy.[31, 33-36a]

**ii) NF-κB: from innocent bystander to a major culprit**

The mammalian nuclear transcription factor, NF-κB, is a multi-subunit complex involved in the regulation of gene transcription, including immune and inflammatory responses, cell growth and the regulation of apoptosis.[37, 38] Five distinct subunits of NF-κB are

found in mammalian cells, which include, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel.[38] These subunits can compose a variety of homo-or heterodimers, bound at a common sequence known as κB site, which are used to control the specificity and selectivity of specific DNA control elements.[39, 40] In most unstimulated mammalian cells NF-κB exists mainly as a homodimer (p50/p50) or heterodimer (p50/p65) in the cytoplasm in the form of an inactive complex with the inhibitory protein I-κB (Figure III-7). Many cellular stimuli including: antineoplastic agents,[19, 27, 41-48] viruses (e.g. HIV), cytokines, phorbol esters and oxidative stress, result in the IKK mediated phosphorylation of I-κB on serines 32 and 36, followed by ubiquitinylation and subsequent degradation by the 26 S proteosome.[37] Degradation of I-κB ensures the release of NF-κB.[49] Upon release, NF-κB translocates into the nucleus where the subunits bind with specific DNA control elements and initiate gene transcription (Figure III-6). Prior to DNA binding, additional protein phosphorylation events are required for optimal and specific gene transcription.[50-53] Antiapoptotic genes such as, *TRAF1*, *TRAF2*, *c-IAP1*, *c-IAP2*, *IXAP*, and *IEX-1L*, are directly regulated by NF-κB, and abrogate the apoptotic signals in response to the chemotherapeutic agents.[19, 54]

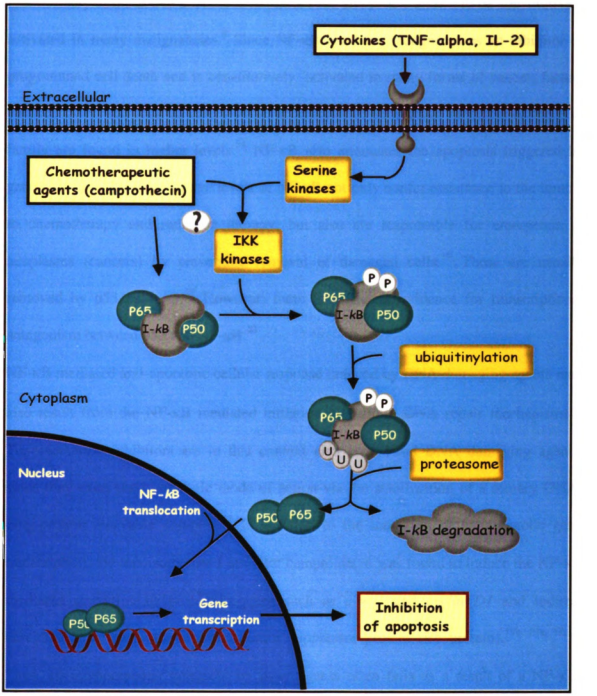



**Figure III-7.** General NF-κB activation pathway by chemotherapeutic agents.

Tumorigenesis requires six essential alterations to normal cell physiology, self-sufficiency in growth signals, insensitivity to growth inhibition, evasion of apoptosis, immortalization, sustained angiogenesis, tissue invasion and metastasis.[5] NF-κB can

induce many of the above alterations in tumor cells and is also shown to be constitutively activated in many malignancies.[5] Since NF-κB has been recognized as an inhibitor of programmed cell death and is constitutively activated in many forms of cancer, factors induced by NF-κB such as cIAPs (cellular inhibitor of apoptosis), members of the $Bcl_2$ family are found in higher levels.[51] NF-κB also attenuates the apoptosis triggered by genotoxic assaults. Constitutive levels of NF-κB not only confer resistance to the tumors to chemotherapy and radiation therapy, but also are responsible for emergence of neoplasms (cancers) by preventing removal of damaged cells.[19] These are usually removed by p53 pathway.[55] However; there is sufficient evidence for transcriptional antagonism between p53 and NF-κB.[40]

NF-κB mediated anti-apoptotic cellular response induced by DNA damaging agents may also result from the NF-κB mediated initiation of cellular DNA repair mechanisms.[49] Topoisomerase inhibitors are in this context also considered DNA damaging agents, since they exert their apoptotic mode of action via the stabilization of a ternary DNA-drug-protein cleavable complex.[11] In addition to the induction of antiapoptotic gene transcription, the topoisomerase I inhibitor camptothecin was found to induce the NF-κB mediated activation of proto-oncogenes such as *c-Myc* and *cyclin D1* and indirect deregulation of the retinoblastoma tumor suppressor protein (Rb protein).[19b, 27a, 57, 58] Thus, chemotherapeutic treatment by these agents often fails as a result of a NF-κB-mediated double stimulus, causing chemoresistance and favoring uncontrolled cell growth. Inhibition of the nuclear translocation of NF-κB, blocks this induction of gene transcription and was found to sensitize tumor cells to chemotherapeutic agents and enhance their antitumor efficacy.[34, 46, 57, 59, 60]

**D. Inflammation and cancer**

The link between inflammation and cancer dates back to 1863 when Rudolf Virchow discovered leucocytes in neoplastic tissue.[60] Inflammation environment is postulated to help in development of tumorigenesis. It has been established now that the tumor microenvironment resembles the inflammation site to a large extent with advantages towards tumor development, neovascularization and metastasis. Furthermore, this inflammation-site tumour generated microenvironment, similar to an inflammation site apart from its significant role in cancer progression and protection from the immune system, has a considerable adverse effect to the success of the various current cancer treatments. It has recently been demonstrated that the inflammatory response in cancer can greatly affect the disposition and compromise the pharmacodynamics of chemotherapeutic agents.[61]

Carcinogenesis can be divided into mainly three stages, initiation (formation of genetically stable alterations) promotion (which involves the proliferation of genetically altered cells) and progression (which involves an increase in the size of the tumor, the spreading of the tumor and the acquisition of additional genetic changes). During the first phase of tumorigenesis, tumor initiation, the carcinogens (both physical and chemical) mutate the DNA of the cell, leading to the activation of oncogenes and/or the inactivation of tumor-suppressor genes (most commonly those that encode KRAS and p53, respectively).[60] The second phase of tumorigenesis, tumor promotion, is characterized by the clonal expansion of initiated cells, owing to increased cell proliferation and/ or reduced cell death. Inflammation seems to be an important tumor promoter and several cytokines — such as interleukin-1 (IL-1), IL-6 and tumor-necrosis factor — can promote

tumor growth. Finally, invasion and metastasis, as well as an increase in tumor size, are the characteristics of the third phase of tumorigenesis, tumor progression. During this stage, additional acquired mutations can lead to further growth advantage for the cancer and it becomes malignant.[62]

**i) Role of NF-κB in cancer.**

Several of the pro-inflammatory cytokines and chemokines — such as TNF, IL-1, IL-6 (all encoded by target genes of the IκB-dependent NF-κB-activation pathway) are associated with tumour development and progression in humans and mice.[60] Furthermore, many oncogenes and carcinogens cause activation of NF-κB, whereas chemicals with known chemopreventive properties can interfere with NF-κB activation.[63] Figure III-7 illustrates the classical NF-κB activation pathway and provides a link between NF-κB, inflammation and cancer.[64] Alternatively, NF-κB-activation could occur via an upstream kinase NF-κB-inducing kinase (NIK) — which activates IKK-α homodimers, independently of either IKK-β or IKK-γ. This facilitates phosphorylation and processing of p100 to yield p65 unit.[65] Pro-inflammatory cytokines promote tumor initiation and progression. While Karin *et al* have suggested the role of NF-κB mediated transcription in tumor initiation in colon and liver cancer in two separate studies, [64, 66] Balkwill *et al* further highlighted that TNF-deficient cells were resistant to skin carcinogenesis, when the cells were subjected to 7, 12-dimethylbenzathracene as the carcinogen and phorbol ester was used as a tumor promoter.[67]

NF-κB signaling might also be crucial in tumor progression. Several NF-κB-regulated genes that encode adhesion molecules, MMPs, serine proteases, heparanase and

chemokines were shown to be essential for tumor invasion and metastasis.[68] The interaction between cancer cells and the inherent and/or adaptive immune systems is complex and outcome may not be predicted at all times. Whereas many of the previously mentioned pro-inflammatory cytokines promote tumor development, the immune system also has the means to restrict cancer development and progression through immuno-surveillance mechanisms.[69] This latter observation however, has not found clinical implications yet.

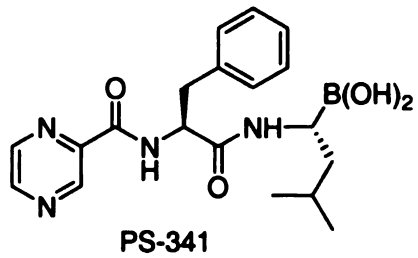
**E. Therapeutic relevance of NF-κB inhibition in cancer**

The possibility to program the immune system in such a way that it will efficiently destroy various types of cancers, with fewer side-effects has been a matter of interest to a lot of research groups. In this context, several vaccines for cancer have been developed though this strategy requires the use of adjuvants for activation of inherent immune system. [70] NSAIDs (COX-2 inhibitors) were found to be effective in reducing tumour load in patients.[71] However; these suffer of high incidence of cardiovascular side-effects, and have failed as chemopreventive drugs in large prospective.[72]

**i) Temporal control of NF-κB**

Baldwin *et al* have demonstrated the control of inducible chemoresistance through inhibition of NF-κB using a mutated form of I-κBα, a natural inhibitor of NF-κB.[56] In another study, Piette *et al* showed that the overexpression of IκB-α / mutated IκB-α regulated the cytotoxicity caused by camptothecin.[73] These pioneering studies illustrated the clinical potential of NF-κB inhibitors in combination chemotherapy. There are numerous natural and synthetic inhibitors of NF-κB reported in the literature[74] which include many antioxidants such as pyrrolinedithiocarbamate or PDTC,[75a] kinase

inhibitors such as hymenialdisine and analogues,[76] SC-514,[77] inhibitors of I-κB degradation such as the proteosome inhibitors lactacystin[78] and PS-341[79] and the sesquiterpene lactones IKK inhibitors illustrated by parthenolide.[80] Even though many of these agents indicate inhibition of the anti-apoptotic transcription factor, enhancement of chemotherapeutics has been limited. The most successful example of combination therapy using chemotherapeutic agents with NF-κB inhibitors has been illustrated by the proteasome inhibitor bortezomib® (previously called velcade and PS-341), which was approved by FDA in 2003 for treatment of multiple myeloma as a single agents and is currently in Phase II clinical trials in the US.[59, 81, 82]



PS-341

**Figure III-8.** Structure of Bortezomib® (PS-341).

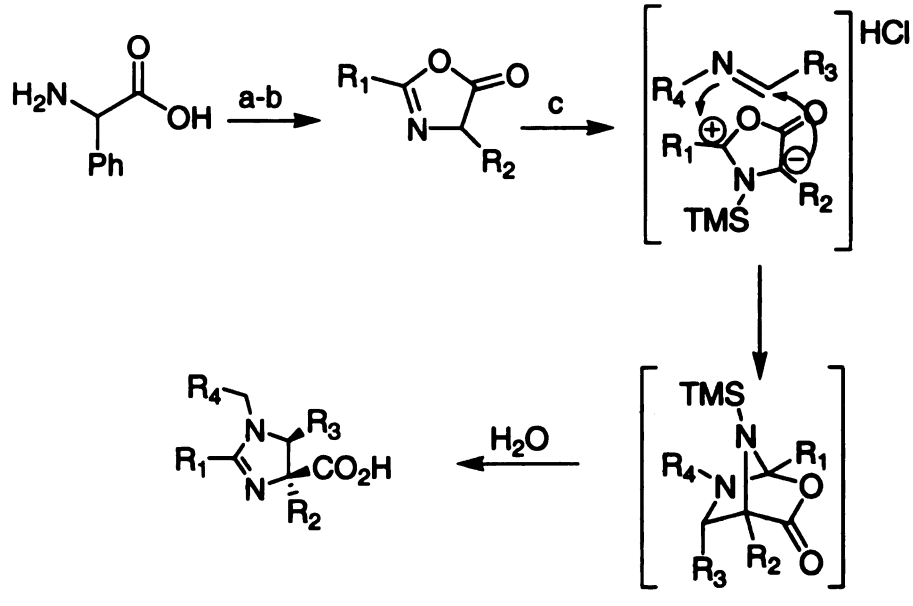
Bortezomib inhibits the nuclear translocation of NF-κB *via* the inhibition of the 26 S proteasome-mediated degradation of I-κB (Figure III-7). Since bortezomib exhibits cell significant cytotoxicity it may be used as a single agent or in combination regiments with classical anticancer agents providing a more than additive apoptotic response.[20b, 83]

**F. NF-κB modulation and enhancement of efficacy of DNA damaging agents.**

**i) Synthesis of imidazolines**

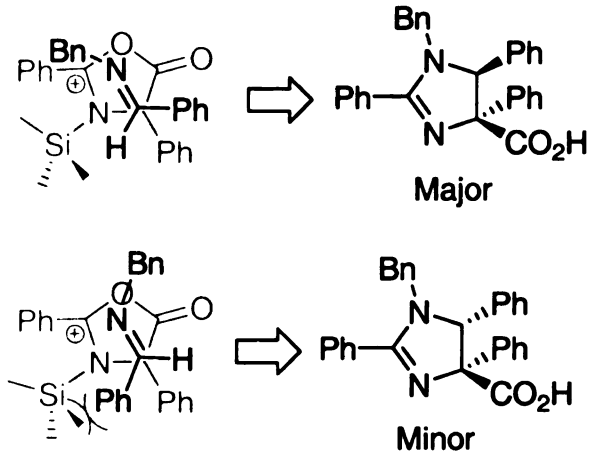
The *trans*-imidazoline scaffold was prepared fir the first time by Satyamaheshwar Peddibhotla in our lab, *via* a highly diastereoselective multicomponent, silicon mediated 1,3-dipolar cycloaddition reaction (Scheme III-1).[84] This novel one pot synthesis was

used to generate a large library of imidazoline scaffolds. The low molecular weight scaffold contains a four-point diversity applicable to alkyl, aryl, acyl and heterocyclic substitutions. After screening a small number of Lewis acids we found that TMSCl promotes the reaction of oxazolones and imines to afford the imidazolines scaffolds in good yields as single diastereomers (Scheme III-1).




**Scheme III-1**. Synthesis of imidazoline, a) Acyl chloride, NaOH (aq), $Et_2O$, overnight; b) EDCI, $CH_2Cl_2$, 2h, room temperature, 65-75 % overall; c) TMSCl, $R_4NH_2$, $R_3CHO$, $CH_2Cl_2$, reflux 4h, room temperature overnight, 45-80%.

Imidazolines were isolated as a single diastereomer and the diastereoselectivity appears to primarily arise from the steric interaction of the bulky silyl group of the azlactone and the phenyl moiety of the imine (Scheme III-2).




**Scheme III-2.** Proposed rational for diastereoselectivity of trans-imidazolines compound III (1-8).* Note: only representative imidazolines are being shown in this thesis.

**ii) Biological Activity –sensitization of chemotherapeutic agents**

**a) Imidazolines and inflammation**

Antagonizing pro-inflammatory cytokines such as TNF-α and interleukins have been recognized as valid goals in the development of new drug candidates for the treatment of chronic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease.[85a] Both imidazolines and imidazoles have been shown to have anti-inflammatory activity.[86] Among the various enzymes as targets for anti-inflammation therapy, a serine/threonine kinase termed p38 MAP (mitogen-activated protein) kinase has become one of the main targets,

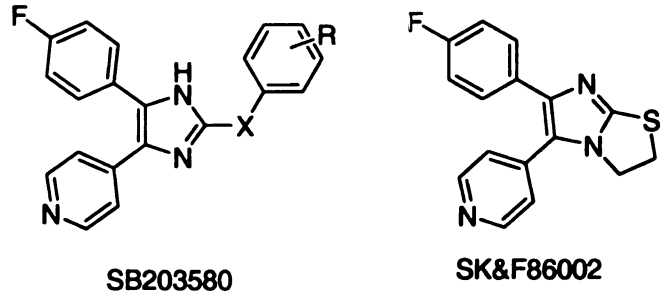



**Figure III-9**. Structures of imidazole based anti-inflammatory compounds

owing to its importance in the biosynthesis and release of both TNF-α and IL-1β.[85b] Exemplified by SB 203580 (Figure III-9), several inhibitors of p38 MAP kinase with an imidazoline or imidazole core ring have been synthesized.[85c] SB 203580 binds in the ATP pocket of p38 MAP kinase.[85d]

Based on the link between inflammation and cancer and imidazoles and imidazolines being anti-inflammatory compounds, we probed imidazolines for anti-tumor activity.

**b) Cytotoxicity of imidazolines**

The library obtained via TMSCl-mediated cycloaddition reaction, was evaluated for their biological activity. Upon screening of these agents using the standard trypan blue exclusion method, we found that these imidazoline scaffolds exhibited no apparent cytotoxicity (Figure III-10) when used as single agents,

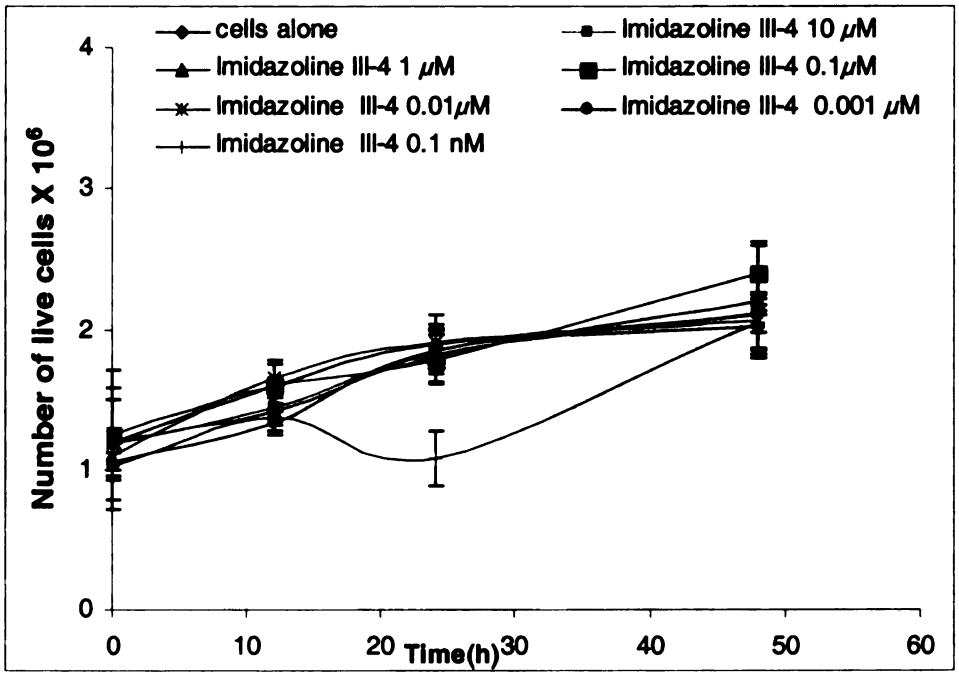



**Figure III-10**. Imidazolines are not cytotoxic. Cells were incubated with various concentrations of III-4 (10μM, 1μM, 0.1 μM, 0.01 μM, 1 nM, 0.1 nM) for 48 h. Time points represent averages over two separate experiments.

however further investigations revealed that this agents drastically enhanced the level of apoptosis induced by chemotherapeutics.

**c) Sensitization of cancer cells toward camptothecin**

The imidazolines (III-1- III-8) were evaluated for their ability to enhance the activity of camptothecin (CPT) in cancer cells by evaluating the level of apoptosis induced by camptothecin in the presence and absence of imidazolines. Induction of apoptosis is the hallmark of most chemotherapeutic agents, including camptothecin.[9, 87] Investigation of the imidazolines as apoptotic modulators, revealed that these agents drastically enhanced the level of apoptosis induced by camptothecin (red squares, Figure III-11). The level of induction of apoptotic cell death was measured using a caspase-based biphasic screen using the commercially available caspase based Apo-One assay™ from Promega Corporation. Caspase activation plays a central role in the execution of apoptosis *via* the proteolytic cleavage of multiple protein substrates by caspase-3, -6 and –7. [88] As the first phase, the optimization of chemotherapeutic drug was carried out such that it perturbs the cell machinery but does not cause extensive apoptosis. This was done using both caspase based assay as well as MTS assay from Promega Corporation, WI, which takes advantage of the reduction potential of the live cells (appendix 1). Treatment of the CEM leukemia T-cells with compound **III-4** (or any other trans-imidazolines) did not induce significant amounts of apoptosis (tested up to 10 $\mu$M for 48 h by cell count, Figure III-10), indicating that imidazolines exhibits no significant cell cytotoxicity. The imidazoline **III-4** was subsequently screened for its ability to enhance apoptosis induced by the chemotherapeutic agent, camptothecin (CPT).[10a] As described previously (also illustrated in Figure III-7) chemotherapeutic agents such as camptothecin activate the anti-apoptotic

factor, NF-κB.[19c] Camptothecin causes cell death via apoptotic mechanisms which undergo final execution through the activation of caspases.[1] Caspases are constitutively present in all cells that reside in the cytosol as a proenzyme. These convert into fully functional caspases upon proteolytic cleavage.[1a, 88] Caspases such as caspase-3 is often called 'executioner' or 'effector' of apoptosis.[1a] Cleavage of NF-κB regulates programmed cell death *via* cross talk with the caspases.[89a] Caspase-mediated cleavage of RelA and IKK-β can prevent NF-κB activation.[89b,c] Caspases can also cleave IκB to generate a degradation-resistant NF-κB inhibitor.[89d] Hence, as shown in Figure III-11, active caspase-3 and -7 were measured using a profluorescent caspase-3/7 consensus substrate, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) rhodamine 110 (Z-DEVD-R110). The lysis buffer efficiently lyses cultured mammalian cells and supports optimal caspase-3/7 enzymatic activity. The substrate and buffer were combined to make the reagent that was added to the samples. Upon cleavage on the C-terminal side of the aspartate residue in the DEVD peptide substrate sequence by caspase-3/7 enzymes, the rhodamine 110 becomes fluorescent when excited at a wavelength of 498nm. The amount of fluorescent product generated is representative of the amount of active caspase-3/7 present and the overall apoptosis undergone. In order to find out the efficacy of various imidazolines, we defined a threshhold index to qualitatively find any significant lead molecules. Chemopotentiation index (CI) was defined as a basic result of the assay to determine if an imidazoline could potentiate the efficacy of camptothecin in leukemia cells.

$$\text{Chemopotentiation Index} = \text{Apoptosis}_{\text{imidazoline}\rightarrow/\text{CPT}} / \text{Apoptosis}_{\text{CPT}}$$

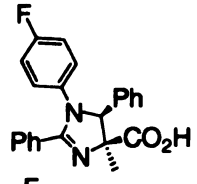
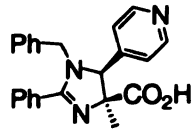
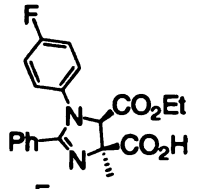
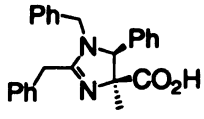
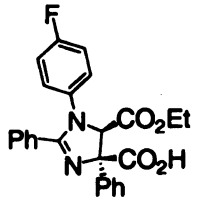
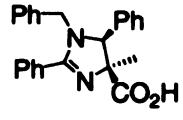
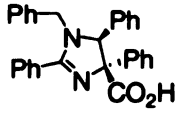
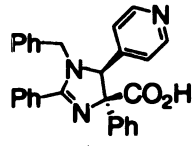
| Entry | trans-imidazoline | Chemopotentiation Index (1μM, 48h) | Entry | trans-imidazoline | Chemopotentiation Index (1μM, 48h) |
|---|---|---|---|---|---|
| III-1 |  | 1.4 (+ / - 0.83) | III-5 |  | 2.44 (+ / - 0.01) |
| III-2 |  | 2.3 (+ / - 0.93) | III-6 |  | 0.97 (+ / - 0.01) |
| III-3 |  | 2.3 (+ / - 0.11) | III-7 |  | 1.05 (+ / - 0.002) |
| III-4 |  | 3.98 (+ / - 0.85) | III-8 |  | 1.4 (+ / - 0.03) |

**Table III-1.** Chemopotentiation index of imidazolines with camptothecin.

Enhancement of CPT-induced apoptosis in CEM cells was first investigated at concentrations of ≤10 nM CPT, at which CPT has been reported to cause DNA aberrations but no significant levels of apoptosis in leukemia cells.[90] Figure III-11 illustrates the effect of the imidazoline on CEM cells when incubated with CPT over a 48 hour time period. Clearly, upto 48h treatment with imidazolines as a single agent did not activate caspase-3 indicating that treatment of the cells with compound III-3 had no effect on the level of apoptosis. Treatment of the cells with 10 nM CPT resulted in some cell death starting after 12 hours of drug treatment (Figure III-11).

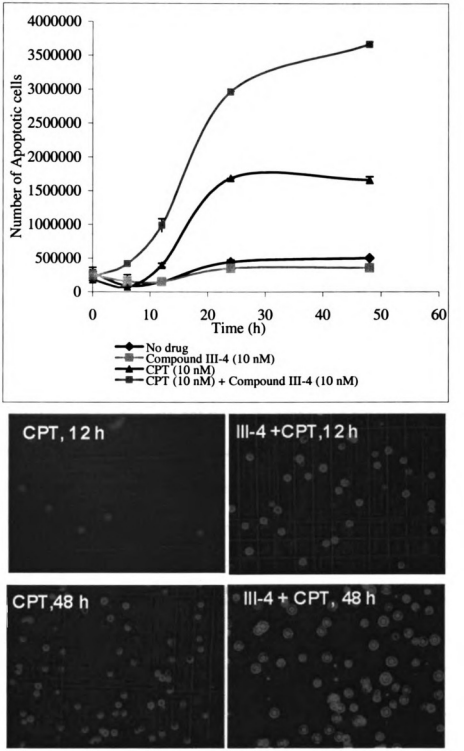



**Figure III-11a.** Sensitization of CEM cells to camptothecin by imidazoline **III-4**, measured over 48 hours. Data reported as an average of two independent experiments (error margins are included) Figure III-11 illustrates cell death through apoptosis as a function of time for cells alone (dark blue triangle), imidazoline only (pink squares), CPT (10 nM) (light blue triangle) and CPT (10 nM) in presence of imidazoline (10 nM) (red

squares). **III-11b.** Combination pre-treatment with **III-4**, 48h, all treatments stained with YoPro-1 and visualized using fluorescence microscopy.

Combination treatment of the non-cytotoxic imidazoline **III-4** (10 nM) with CPT (10 nM) resulted in enhancement of apoptotic cell death after 48 hours (red squares, Figure III-11).

A similar observation was made when the cells were pre-incubated with **III-4** (100 nM) followed by CPT treatment and stained with Yo-Pro dye to be visualized under a fluorescence microscope (Figure III-11b). The membrane integrity was compromised upto 48 h (a characteristic feature of apoptosis) and caused increase in fluorescence. Additional experiments were performed in order to quantify this enhancement of the apoptotic signal due to treatment with **III-4** in leukemia cells. Briefly, the study was divided into two sets of experiments, one involving continuous exposure of the agents to the cells and another subjecting the cells to an initial exposure of the drug, followed by removal of drug and supplementing with fresh media. Cells were pre-incubated with 1, 10 and 100 nM concentrations of the imidazoline **III-4** followed by exposure to a titration range of CPT concentrations. Various time points were obtained and the data was analyzed. Table III-2 and Figure III-12 illustrate the effect on $CC_{95}$ for camptothecin on cells pre-treated with imidazoline.

| Experiment | $CC_{95}$(nM) CPT | $CC_{95}$(nM) CPT+0.1$\mu$M III-4 | $CC_{95}$(nM) CPT+ 10nM III-4 | $CC_{95}$(nM) CPT+1nM III-4 | $CC_{95}$(nM) III-4 |
|---|---|---|---|---|---|
| Continuous exposure, 48h | 44.3 | 0.59 | 0.98 | 1.52 | >10,000 |
| Cells washed after 1h | 47.8 | 2.29 | NT | NT | >10,000 |

**Table III-2.** Comparison of cytotoxic concentration causing 95% cell cytotoxicity in

continuous exposure and washed cells and is an average of two independent experiments. NT: Not tested

The $CC_{95}$ of CPT was increased by 75.1 fold, when the cells were pre-treated with 0.1 $\mu$M imidazoline for 1 hour. This fold enhancement was reduced to 29.1 fold enhancement of efficacy when the amount of imidazoline was decreased to 1 nM (Table III-1, see appendix 2 for graphs). As anticipated, the continuous exposure resulted in overall higher cell cytotoxicity due to a longer exposure time. When the cells were washed with 1XPBS after 1 h of camptothecin treatment, the $CC_{95}$ dropped to 20.9 folds enhancement of CPT induced cell death ($CC_{95}$).

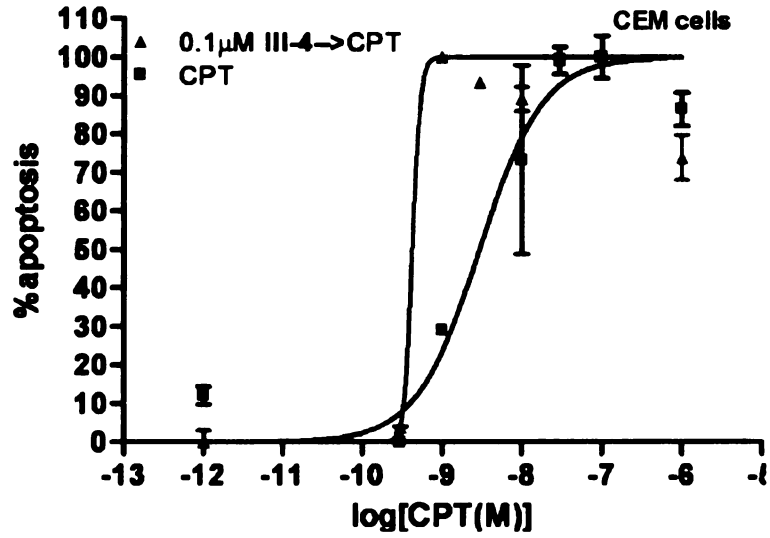



**Figure III-12.** $CC_{95}$ curves for CPT alone and combination treatment with III-4. The red triangles show combination treatment of imidazoline and camptothecin. Blue squares are camptothecin alone. Values are averages of two independent experiments. P=0.0024

Similar potentiation was seen with pre-treatment of the proteasomal inhibitor, ALLN followed by camptothecin treatment. It is important to note however, that higher concentrations of ALLN caused significant additive cytotoxicity (see appendix 3).

**d) Cell death determined by Trypan Blue Exclusion**

Parallel to the Apo-One assay, cells were counted by the Trypan Blue Exclusion method to find out the number of viable cells. Cells were pre-incubated with 0.1 $\mu$M , 0.01 $\mu$M and 1 nM imidazoline **III-4**, then, various concentrations of camptothecin were added and allowed to incubate for 0, 24, 48 h. Well-mixed aliquots of these cell cultures were then mixed with equal volume of 0.04% trypan blue dye and only the transparent cells (viable) were counted with a hemacytometer under the microscope. Results indicated that the control with higher concentrations of CPT (50 $\mu$M) caused significant reduction in cell viability (pink squares, Figure III-13). Moderate concentrations of CPT (0.01 $\mu$M) induced cell death upto 48h, though ~40% of the cell population passed the trypan blue test and was viable (green triangles, Figure III-13). Clearly, a pre-treatment with 0.1 $\mu$M **III-4** reduced the cell viability by nearly 20% starting at 24h which was maintained over 48 h (red squares, Figure III-13).

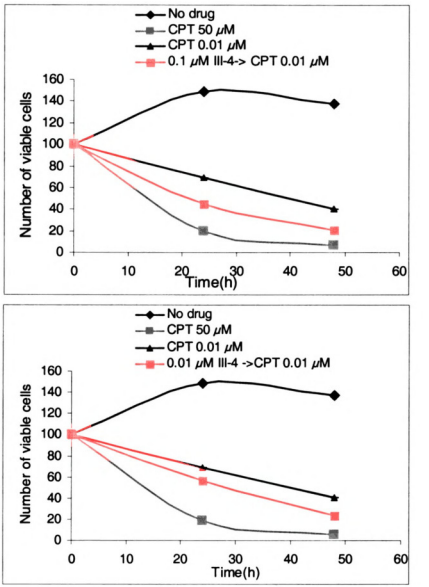



**Figure III-13.** Measurement of number of viable cells using the Trypan Blue Method

**e) Pathway of apoptotic modulation.**

In order to determine the potential molecular pathway through which the imidazoline exert its apoptotic modulation, we evaluated its ability to inhibit CPT induction of NF-κB activation. Inhibition of the NF-κB signaling cascade may occur at several different steps in its activation pathway (Figure III-7). In order to determine where the imidazoline

intersected the NF-κB activation pathway we examined the effect of imidazoline on CPT induced NF-κB activation in each of the steps shown in Figure III-7.

**i. NF-κB activation by camptothecin.**

Activation of NF-κB by chemotherapeutic agents such as the camptothecin has well been documented by several groups in different cell lines, including CEM leukemia T-cells.[19] The stabilization of a topoisomerase I – DNA cleavable complex by camptothecin activates cell survival signaling pathways mediated by the nuclear transcription factor NF-κB.[19, 27d] The activation of NF-κB by camptothecin was confirmed in CEM cells using an EMSA assay.[19c, 36a] The cell nuclei were isolated and evaluated for NF-κB-DNA binding activity utilizing the fluorescently labeled NF-κB DNA consensus sequence. TNFα activation was used as a positive control (Figure III-14a, lane 2). The NF-κB/DNA complex was unambiguously identified by treating the nuclear extract with a NF-κB p65 antibody following TNFα activation and CPT activation (Figure III-14a, lane 3 and 6, respectively). Treatment of the nuclear extract with the p65 antibody resulted in a significant decrease in NF-κB/DNA binding and the formation of a supershift of the complex (Figure III-14a, lane 3 and 6). As a negative control, cells are left unactivated and the nuclear extracts was exposed to the NF-κB consensus sequence, resulting in only a slight background level of NF-κB-DNA binding (Figure III-14a, lane 4). Treatment of the CEM cells with camptothecin concentrations ranging from 10 μM to 0.1 nM (Figure III-14a, lanes 5, 7-10) illustrated a significant amount of NF-κB/DNA binding due to NF-κB activation. This activation decreased as the concentration of CPT decreased with significant activation upto 0.01 μM CPT and virtually weak /no activation at 1 nM and 0.1 nM respectively. Non-specific binding was minimal due to the addition of 1 μg poly

dI.dC. Miyamoto and coworkers have shown that activation of NF-κB also led to an increased NF-κB mediated transcription.[19c] We confirmed similar results in HeLa cells which have been discussed later in this chapter. These results indicate that activation of NF-κB in CEM cells translates into increased NF-κB mediated transcription.

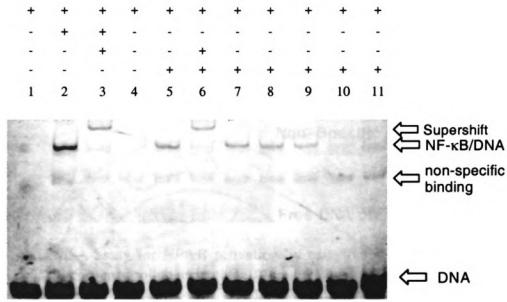



**Figure III-14a.** EMSA assay for NF-κB activation by camptothecin (CPT) in CEM cells. Lane 1: NF-κB consensus oligonucleotide (0.16 pmol/λ); Lane 2: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α); Lane 3: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α + p65 antibody; Lane 4: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (unactivated); Lane 5: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT); Lane 6: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT)+ p65 antibody; Lane 7: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1 μM CPT); Lane 8: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.1 μM CPT); Lane 9: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.01 μM CPT); Lane10: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1 nM CPT) ; Lane 11: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.1 nM CPT). All incubations with CPT were performed for 2h. The positive control with TNF-α was incubated for 15'.

A similar experiment was carried out in Jurkat cells, another leukemia cell line; however, no activation of NF-κB was seen in this T-cell line. The cell nuclei were isolated and evaluated for NF-κB-DNA binding activity utilizing the fluorescently labeled NF-κB

DNA consensus sequence. TNF-α activation was used as a positive control (Figure III-14b, lane 2). The NF-κB/DNA complex was unambiguously identified by treating the nuclear extract with a NF-κB p65 antibody following TNF-α activation (Figure III-14b, lane 3, respectively).

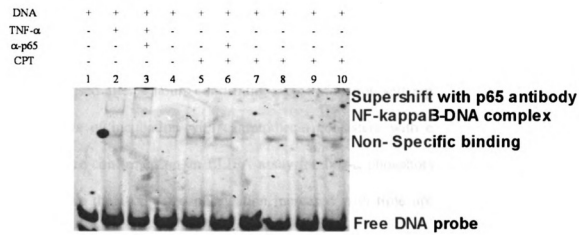



**Figure III-14b.** EMSA assay for NF-κB activation by camptothecin (CPT) in Jurkat cells. Lane 1: NF-κB consensus oligonucleotide (0.16 pmol/λ); Lane 2: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α); Lane 3: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α + p65 antibody; Lane 4: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (unactivated); Lane 5: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT); Lane 6: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT)+ p65 antibody; Lane 7: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1 μM CPT); Lane 8: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.1 μM CPT); Lane 9: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (0.01 μM CPT); Lane10: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1 nM CPT) ; All incubations with CPT were performed for 2h. The positive control with TNF-α was incubated for 15'.

Treatment of the nuclear extract with the p65 antibody resulted in a significant decrease in NF-κB/DNA binding (Figure III-14b, lane 3). As a negative control, cells are left unactivated and the nuclear extracts was exposed to the NF-κB consensus sequence, resulting in only a slight background level of NF-κB-DNA binding (Figure III-14, lane 4). Treatment of the Jurkat cells with camptothecin concentrations ranging from 10 μM

to 1 nM (Figure III-14b, lanes 5, 7-10) illustrated no NF-κB/DNA binding indicating that CPT could not activate NF-κB in Jurkat cells.

**ii. NF-κB activation by CPT in CEM cells is transient and a function of time.**

Activation of NF-κB is transient and depends on exposure time. This was confirmed by treating CEM cells at log phase with 10 μM camptothecin or DMSO for 30 minutes and 1, 2, 4, 6, 14, and 24 hours (Figure III-15a). Maximal NF-κB activation was at 2 hours and decreased after 4 hours confirming a post-induction self repression loop during the early hours of incubation with camptothecin consistent with earlier reports.[19c] These results were confirmed on an ELISA assay for IκB-α phosphorylation also (Figure III-15b), where the I-κBα phosphorylation increased with time upto 60 minutes and then began to decrease rapidly owing to proteasomal degradation (Figure III-7).

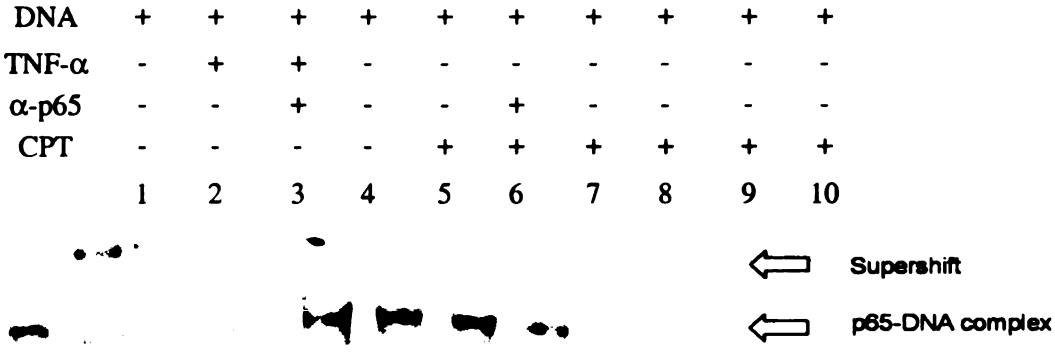



**Figure III-15a.** EMSA assay for NF-κB activation by camptothecin (CPT) in CEM cells, Time course. Lane 1: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α); Lane 2: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α + p65 antibody; Lane 3: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (unactivated); Lane 4: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT, 30'); Lane 5: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT, 1h); Lane 6: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT, 2h); Lane 7: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT, 4h); Lane 8: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT, 6h); Lane 9: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10μM CPT, 14h) ; Lane 10: gel shift for NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (10 μM CPT, 24h). All incubations with CPT at 10μM. The positive control with TNF-α was incubated for 20'.

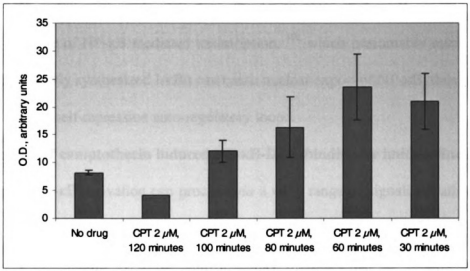



**Figure III-15b.** Time dependent activation of IκB-α.

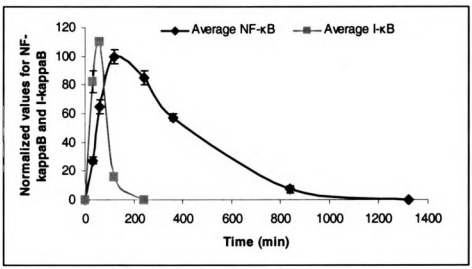



**Figure III-15c.** Comparison of IκB degradation and NF-κB-DNA binding over time.

The kinetics of NF-κB activation and IκB-degradation depend on the activation signal and its concentration. Figure (III-15c) illustrates the degradation of I-κBα (pink triangles) monitored via ELISA based studies (Figure III-15b) over 2h and increase in NF-κB (blue rhombus) with most optimum value for at 2h (Figure III-15a). From Figure III-15, it can be deduced that as I-κB degrades as a function of time, NF-κB accumulates in the

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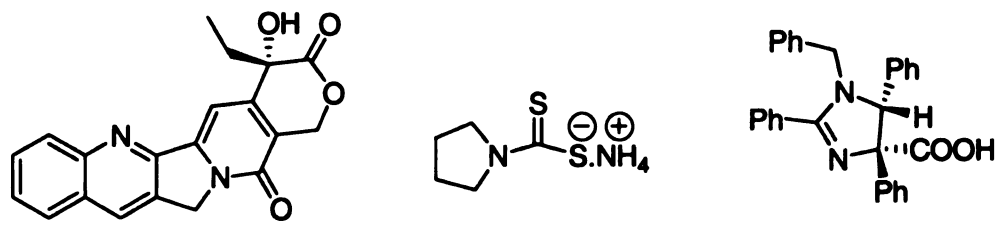
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nucleus with a maximal accumulation at 2h. An increase in NF-κB binding leads to efficient increase of NF-κB mediated transcription, [19c] which presumably transcribes for I-κB-α. This newly synthesized I-κBα can cause nuclear export of NF-κB, thus setting up a post-induction self repression auto-regulatory loop.

**iii. Inhibition of camptothecin induced NF-κB-DNA binding by imidazoline III-4.**

Induction of NF-κB activation can proceed *via* a wide range of signaling pathways.[91, 92] Thus, inhibition of NF-κB activation can proceed via the mediation of many different pathways.[74] Modulators of these pathways may therefore act as general activation inhibitors, whereas others may inhibit specific induction pathways.[74] In order to investigate whether the imidazoline **III-4** inhibits the specific pathway of camptothecin induced NF-κB activation, we examined the inhibition of camptothecin induced NF-κB – DNA binding in the presence of the compound **III-4**. CEM cells were treated with various concentration of imidazoline **III-4**, 30 minutes prior to the treatment by camptothecin (1 $\mu$M). Pyrrolidine dithiocarboxylic acid (PDTC, Figure III-16) is a non-selective NF-κB inhibitor[75a] and was used as a positive control.




camptothecin (CPT) | pyrrolidinedithiocarbamate (PDTC) | III-4(SP_4_84)

**Figure III-16.** Structures of CPT, PDTC and imidazoline **III-4**.

After a 2 hour incubation period, the nuclei were isolated and treated with the labeled κB consensus sequence. As illustrated in Figure III-17, the addition of imidazoline **III-4** inhibited camptothecin induced NF-κB nuclear binding in a dose dependent manner.



DNA only (lane 1, Figure III-17) served as a negative control lane for the gel shift. TNF-α activated NF-κB (lane 2, Figure III-17) was used a positive control for gel shift which was unambiguously determined with a p65 antibody used to provide a supershift (lane 3, Figure III-17) and the unactivated control (lane 4, Figure III-17).

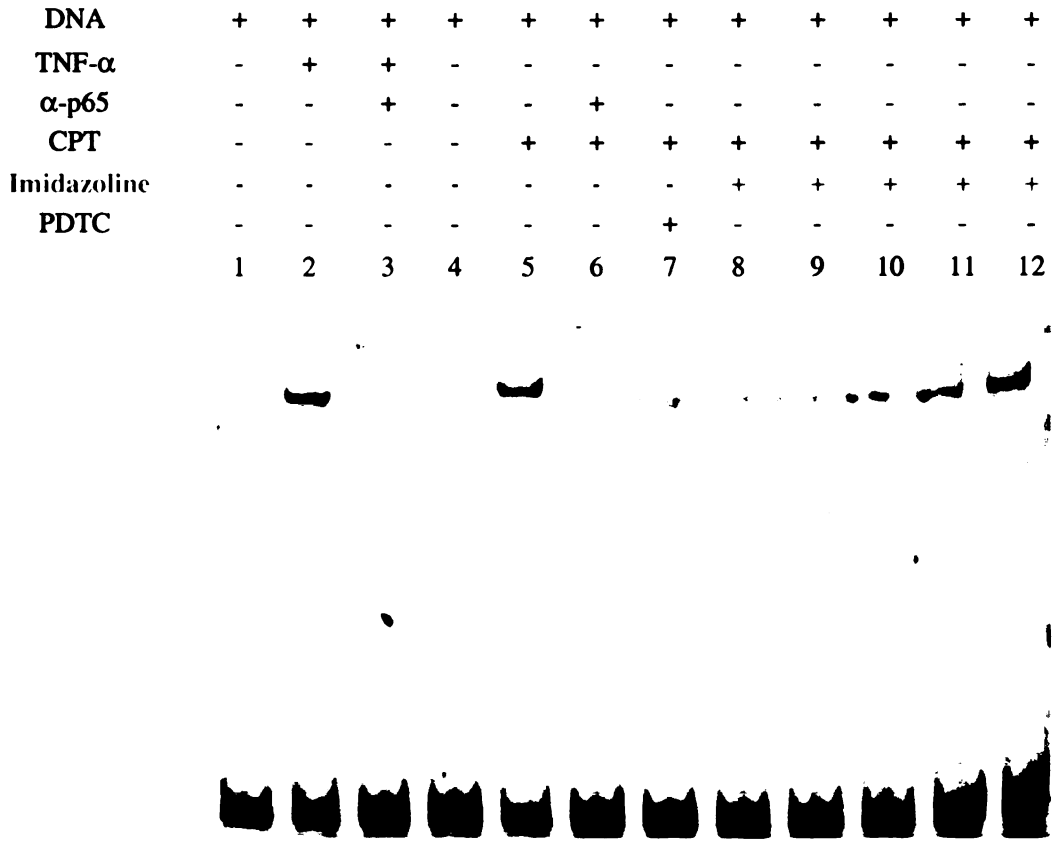


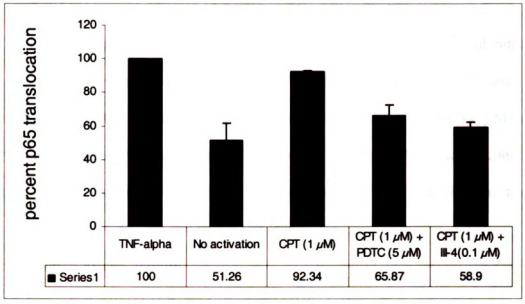

**Figure III-17.** Inhibition of CPT activated NF-κB binding by imidazoline **III-4** using EMSA. Lane1: κB consensus oligonucleotide (0.16 pmol/λ); Lane2: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α; Lane3: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (TNF-α)+ p65 antibody Lane4: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract(no activation); Lane 5: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT); Lane6: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT)+ antibody p65; Lane 7: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT + 1 μM PDTC); Lane 8: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT + 1.0 μM imidazoline **III-4**); Lane 9: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT + 0.1 μM imidazoline **III-4**): Lane 10: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT + 0.01 μM imidazoline **III-4**); Lane 11: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT + 1.0 nM imidazoline **III-4**); Lane 12: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (1.0 μM CPT + 0.1 nM imidazoline **III-4**) All incubations with CPT were performed for 2h. The positive control with TNF-α was incubated for 0.5h.

Treatment of the cells with camptothecin resulted in the activation of NF-κB as indicated by the strong band of the NF-κB-DNA complex (lane 5, Figure III-17). Inhibition of DNA binding in the presence of the non-selective NF-κB inhibitor PDTC resulted in a decrease of binding as anticipated (lane 6, Figure III-17). A similar decrease of camptothecin induced DNA binding upon treatment of imidazoline **III-4** (concentrations ranging from 1 $\mu$M to 0.1 nM) is clearly illustrated in lanes 8-12. Comparison of lane 5 (activated by 1 $\mu$M CPT) with lane 7 (activated by 1 $\mu$M CPT + 1 $\mu$M compound **III-4**) indicates a significant decrease in NF-κB-DNA complex formation. This effect reduced as the concentration of imidazoline **III-4** was decreased; indicating that the imidazoline **III-4** inhibited the CPT mediated activation of NF-κB in a dose dependent manner.

**iv) Inhibition of camptothecin induced nuclear translocation of NF-κB by imidazoline III-4**

The previously described EMSA studies indicate that imidazoline **III-4** inhibits the binding of NF-κB to its consensus sequence. This may be due to a direct inhibition of DNA binding or due to the absence of NF-κB in the nucleus. We therefore investigated the ability of compound **III-4** to inhibit the nuclear translocation of NF-κB using a p65 sandwich ELISA assay (Figure III-18). The ELISA captures the phosphorylated p65 (an active form of NF-κB for DNA binding) which is detected specifically using a secondary antibody. This antibody conjugate is further treated with alkaline phosphatase antibody which liberates p-nitrophenol from chromagen, p-nitrophenylphosphate. Hence, the amount of color developed due to p-nitrophenol generated can be correlated to the amount of p65 unit present in the sample.

Activation of NF-κB was carried out for a period of 0.5 hours for TNF-α and 2 hours for camptothecin after which the nuclear extracts were isolated. TNF-α activation of NF-κB was used as positive control and the level translocated p65 was normalized to 100%. The negative control included the level of nuclear p65 without activation (0% translocation).[76] Treatment of the cells with TNF-α or CPT (1.0 μM) resulted in a significant increase in nuclear NF-κB as illustrated in Figure III-7 (1st and 3rd column, Figure III-18a). Pretreatment of the cells with the non-selective NF-κB inhibitor PDTC (5 μM) indicated a significant inhibition of nuclear translocation after CPT activation (4th column). Pretreatment of the cells with the imidazoline **III-4**, 30 minutes prior to activation resulted in a significant inhibition of translocation (5th column). Various concentrations ranging of the imidazoline **III-4** (as low as 1 nM) resulted in inhibition of translocation. Similar results were obtained with HeLa cells activated with TNF-α (Figure III-18b) as well as Jurkat cells activated with PMA/PHA (data not shown). Clearly, figure (III-18a and III-18b) illustrate that both TNF-α and CPT were able to induce NF-κB translocation across the nuclear membrane when compared to the negative control in both CEM and HeLa cells (column 2, Figure III-18 a and b).



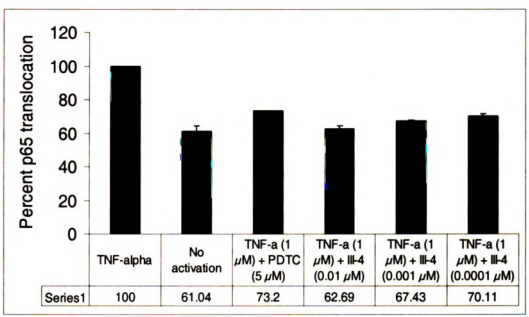




**Figure III-18a**. TOP: Level of nuclear translocation measured by p65 ELISA assay of compound **III-4** in CEM cells with activation by camptothecin. All values have been normalized to the positive control to show percentage translocation. Data reported as an average of two independent experiments. **III-18b**. BOTTOM: Level of nuclear translocation measured by p65 ELISA assay of compound **III-4** in HeLa cells with activation by TNF-α. All values have been normalized to the positive control to show percentage translocation. Data reported as an average of two independent experiments.

However, the kinetics of TNF-α and CPT are different in T-cells and are a function of both time and concentration. PDTC (a non-specific kinase inhibitor) [75a] inhibited the nuclear translocation of NF-κB caused by CPT (1 μM) and TNF-α in CEM and HeLa cells respectively. Comparison of activation of CEM cells with CPT (1 μM) upon pretreatment with imidazoline (column 5, Figure III-18a) to CPT (1 μM) (column 3, Figure III-18a) indicates indicate that the imidazoline **III-4** inhibits the nuclear translocation of NF-κB at the low nanomolar level.

**v) Inhibition of kinase activity**

Protein kinases play an essential role in the regulation of a variety of cellular processes such as transcription involving cellular proliferation, growth and metabolism, and apoptosis.[75b] Abberant roles in the activity of kinases can lead to diseases such as cancer, alzheimer's disease and diabetes.[75c] Kinases are active participants in activation of NF-κB (Figure III-7). I-κB phosphorylation requires the activation of endogenous kinases IKKα and IKKβ.[19f, 92, 93] Inhibition of I-κB phosphorylation will result in the sequestering of NF-κB in the cytoplasm. We therefore evaluated the imidazoline **III-4** for its effect on the phosphorylation of the inhibitory protein I-κB by its endogenous kinases IKKα and IKKβ. No inhibition of kinase activity was observed (tested at 10 μM). Alternatively, inhibition of NF-κB translocation may be the result of the upstream inhibition of kinase activity in a variety of kinase pathways. We therefore evaluated the imidazoline **III-4** for its inhibition of the several kinases either related to the NF-κB pathway or other apoptotic signaling pathways. Compound **III-4** was sent to Upstate NY for the kinases assay. The kinases tested included: AMPK, Axl, Blk, CDK2/cyclinE, CDK5/p35, c-RAF, Fes, Flt3, Fyn, IGF-1R, IR, JNK1α1, JNK2α2, JNK3, Lck, Lyn (human), MAPK1,

MAPK2 (human), MEK1, MKK4, MKK6, MKK7b, MSK1, p70S6K, PDK1, PAK2, PKA (human), PKBβ, PKCα, PKCψ, PKCδ, PKCε, PKCmu, SGK, Syk, TrkB, Yes(h), ZAP-70. Table III-3 shows the kinase inhibition for the above mentioned kinases at 1 μM **III-4**.

| Kinase | % inhibition | SD | Kinase | % inhibition | SD |
|---|---|---|---|---|---|
| Control | 100 | -- | MAPK2(human) | 101 | ± 4 |
| AMPK | 93 | ± 2 | MEK1 | 99 | ± 3 |
| Axl(h) | 96 | ± 3 | MKK4 | 96 | ± 9 |
| Blk(m) | 118 | ± 14 | MKK6 | 96 | ± 5 |
| CDK2/cyclinE(h) | 98 | ± 1 | MKK7b | 71 | ± 6 |
| CDK5/p53(h) | 95 | ± 4 | MSK1 | 103 | ± 1 |
| c-RAF | 100 | ± 1 | p70S6K | 96 | ± 4 |
| Fes | 96 | ± 15 | PDK1 | 99 | ± 6 |
| Flt3 | 110 | ± 2 | PAK2 | 101 | ±12 |
| Fyn | 100 | ± 2 | PKA | 83 | ± 9 |
| IGF-1R | 110 | ± 11 | PKBβ | 100 | ± 0 |
| IKK(α) | 101 | ± 1 | PKCα | 108 | ± 1 |
| IKK(β) | 85 | ± 6 | PKCψ | 71 | ± 7 |
| IR | 102 | ± 7 | PKCδ | 100 | ± 2 |
| JNK1α1 | 95 | ± 1 | PKCε | 99 | ± 1 |
| JNK2α2 | 99 | ± 0 | PKCμ | 99 | ± 2 |
| JNK3 | 112 | ± 10 | SGK | 98 | ± 6 |
| Lck | 80 | ± 1 | SyK | 85 | ± 1 |
| Lyn(human) | 72 | ± 7 | TrkB | 103 | ±12 |
| MAPK1 | 95 | ± 6 | Yes(h) | 105 | ± 7 |
| | | | ZAP-70 | 111 | ± 4 |

**Table III-3**. Kinase inhibition data for **III-4** against kinases from NF-κB signaling and apoptosis modulation.

No inhibition of any of these kinases was detected (tested up to 1 μM concentrations of the imidazoline). Based on these in vitro studies it is unlikely that imidazoline **III-4** inhibits nuclear translocation via the inhibition of kinase activity.

**vi. Effect of imidazoline III-4 on I-κBα phosphorylation**

We have demonstrated that imidazoline **III-4** inhibits the translocation of NF-κB across the nuclear membrane in response to stimulation by camptothecin. The in vitro studies for kinase inhibition did not show any activity of kinases The translocation of NF-κB requires the phosphorylation of its inhibitory protein, I-κB, followed by the subsequent degradation by the 26S proteosome resulting in the liberation of NF-κB.[94] Thus, the effect of the imidazoline on I-κB phosphorylation and degradation was investigated using a pS32 ELISA on cytoplasmic extracts. The experiment was designed in a manner that both the phosphorylation event of IκB-α as well as its degradation could be targeted at the same time. This was achieved by utilizing two different controls, ALLN (N-acetyl-Leu-Leu-norleucinal) a proteasomal inhibitor[19c, 95, 96] and parthenolide, a specific inhibitor for IKK-kinase.[80] A time course experiment using 2 μM CPT was performed to optimize the levels of I-κB-α degradation as a function of time. The results indicated that 2 hours was the most optimum time period for I-κB phosphorylation (Figure III- 15b) at 2 μM CPT. Further, cells were incubated with varying amounts of ALLN (100 μM, 50 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM) or parthenolide (100 μM, 50 μM, 20 μM, 5 μM, 1μM, 0.1 μM, 0.01 μM) and treated with CPT (2 μM) for 60' to get IκB-phosphorylation profiles of the two controls. As anticipated, the proteasome inhibitor, ALLN showed accumulation of phosphorylated I-κB ($IC_{50}$=0.2 μM) whereas the IKK kinase inhibitor indicated a decrease in phosphorylation ($IC_{50}$=13.5 μM) (Figure III-19a and b). Parallel to the I-κB-α phosphorylation assays, we determined the cytotoxicity of both ALLN and parthenolide in CEM cells to ascertain the lowering in value is not seen due to cytotoxic effects of the drugs especially parthenolide (see appendix 4).

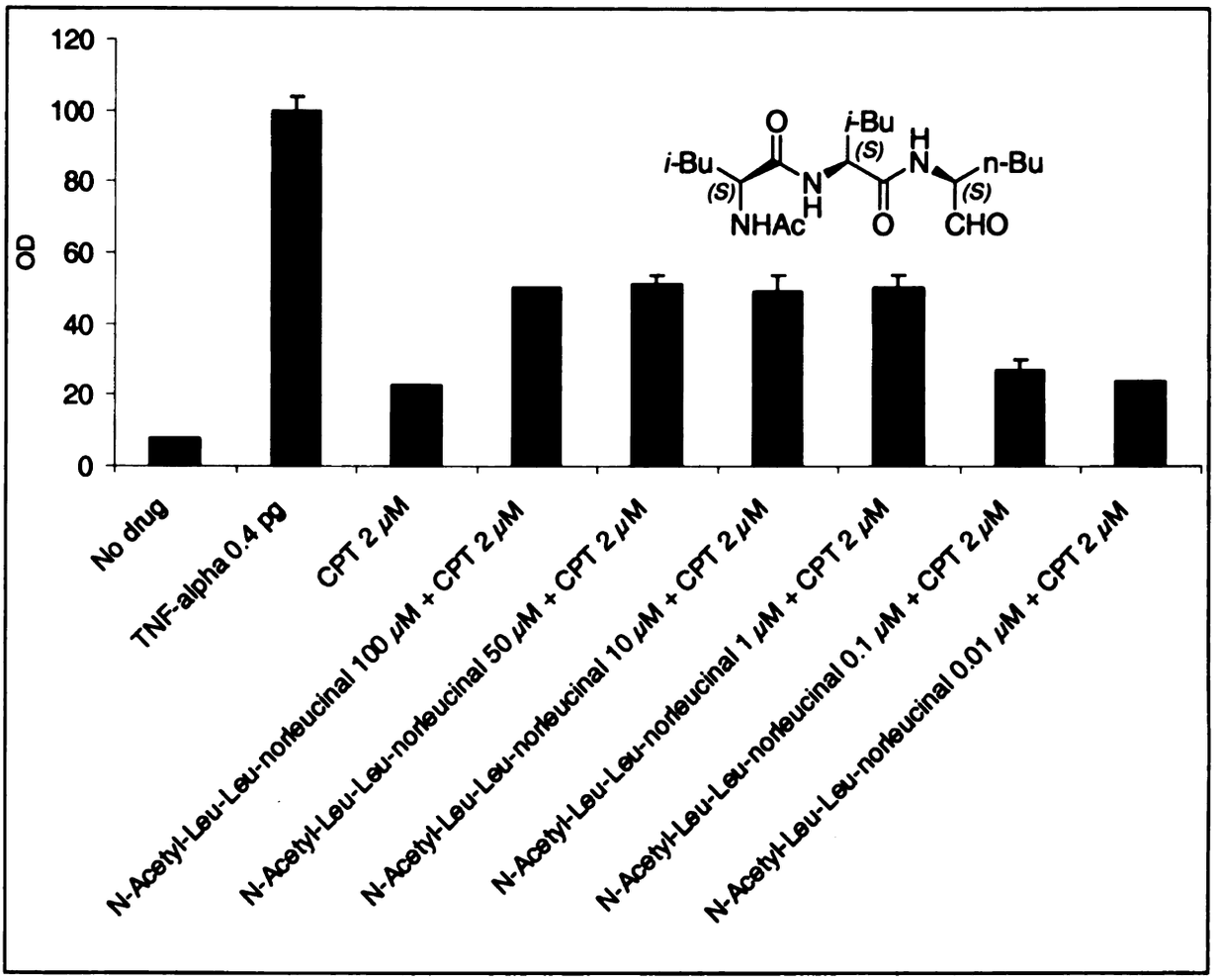
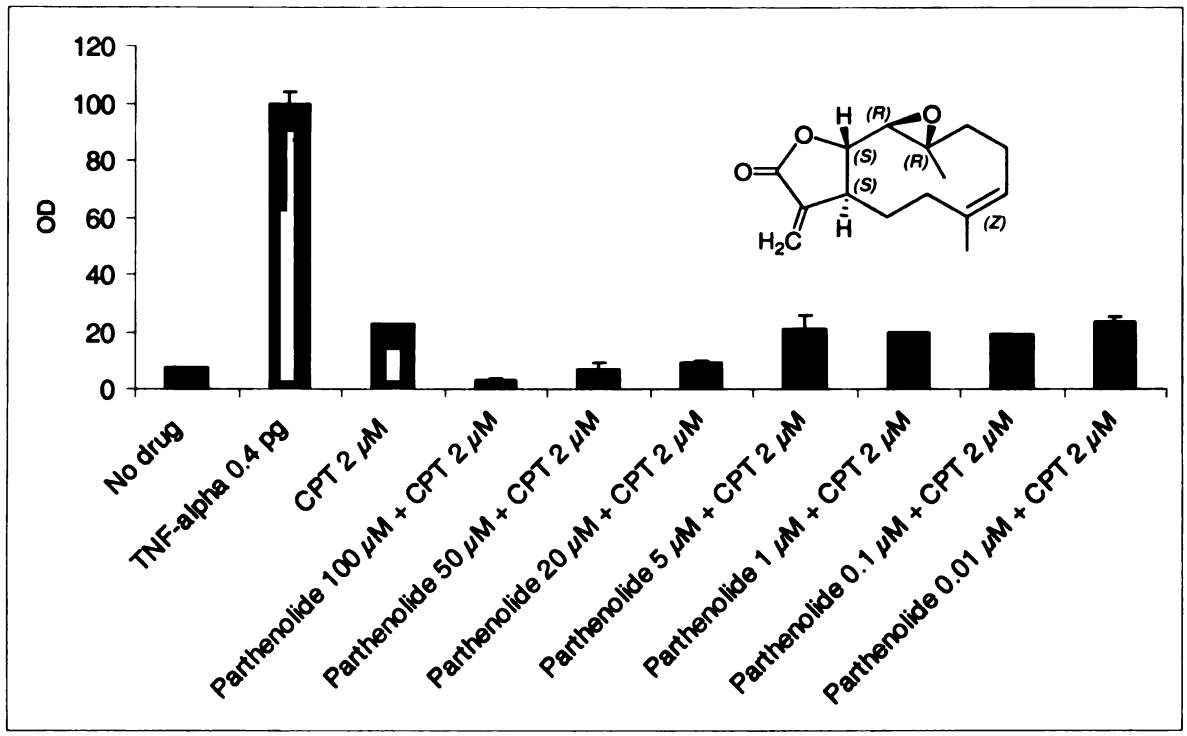



**Figure III-19a**. Phosphorylated I-κB (at serine 32 and serine 36) in presence and absence of various concentrations of ALLN upon activation by CPT. All values were normalized to maximum accumulation seen in ALLN control. All experiments were run in duplicate and data reported as the average of the two independent experiments. Activation of NF-κB was initiated using camptothecin (2 μM) and TNF-α (1 pg).




**III-19b.** Phosphorylated I-κB (at serine 32 and serine 36) in presence and absence of various concentrations of Parthenolide upon activation by CPT. All values were normalized to maximum accumulation seen in ALLN control. All experiments were run in duplicate and data reported as the average of the two independent experiments. Activation of NF-κB was initiated using camptothecin (2 μM) and TNF-α (1 pg).

Finally, we investigated the effect of imidazoline **III-4** on I-κBα phosphorylation. Cytoplasmic extracts from CPT treated cells with or without a pre-treatment with imidazoline were analyzed for phosphorylation at ser-32 and 36 (Figure III-20). ALLN, a proteasome inhibitor[95, 96] and parthenolide, an IKK inhibitor[80] were used as controls to mark the two steps involved in I-κB degradation leading to NF-κB translocation and gene transcription. Parallel to the pS32 ELISA, the total I-κB levels in were measured to accurately determine the percent phosphorylation. Upon pretreatment of the cells with imidazoline **III-4**, accumulation of phosphorylated I-κBα was observed (Figure III-20).

The values were normalized to the total amount of I-κB resulting in an $IC_{50}$ value I-κB phosphorylation of 0.19 μM.

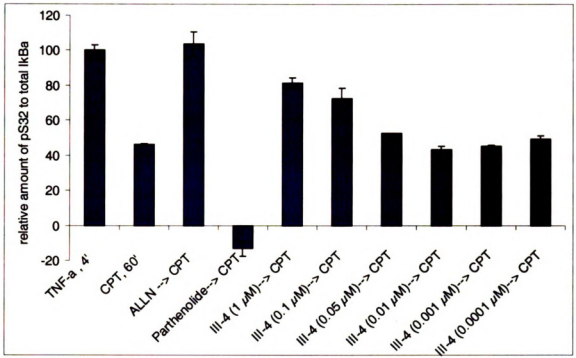



**Figure III-20.** Relative amount of phosphorylated I-κB (at serine 32 and serine 36) in presence and absence of imidazoline **III-4** upon activation by CPT. All values were normalized to maximum accumulation seen in ALLN control. All experiments were run in duplicate and data reported as the average of the two independent experiments. Activation of NF-κB was initiated using camptothecin (2 μM). ALLN (50 μM) and Parthenolide (50 μM) were used as controls for proteasomal and IKK inhibition, respectively. The control for parthenolide shows a negative bar due to its cell cytotoxicity over the course of the experiment.

Similar experiments were performed using TNF-α to activate the leukemia cells. Both TNF-α and camptothecin are known to activate NF-κB by I-κB degradation following phosphorylation at ser32 and ser36 in CEM cells though with varying kinetics.[73] Consistent with these studies we found that the imidazolines inhibited the NF-κB signaling pathway in TNF-α activated cells more effectively than with camptothecin illustrated by an $IC_{50}$ value of 2.1 nM (see appendix 5). These studies indicate that

imidazoline **III-4** inhibits nuclear translocation of NF-κB via the inhibition of I-κB degradation of phosphorylated IκBα.

**vii. Inhibition of CPT induced NF-κB transcription by imidazoline III-4**

To demonstrate the ability of imidazoline **III-4** to affect NF-κB mediated gene transcription we evaluated the inhibition of NF-κB mediated luciferase production using a luciferase reporter assay. HeLa cells were transfected with 6 X κB driven reporter gene pNF-κB-Luc and were activated with TNF-α or camptothecin (3 μM) in presence and absence of imidazoline **III-4**, parthenolide, ALLN or DMSO control and luciferase production was assayed using Dual – Glo reporter assay (Promega Corporation). A constitutively activated plasmid pFC-MEKK (Stratagene) was used as a positive control for transfection. pCIS-CK (Stratagene) was used as the negative control for the κB-driven reporter gene. pRL-TK was used as a co-reporter (Promega Corporation).

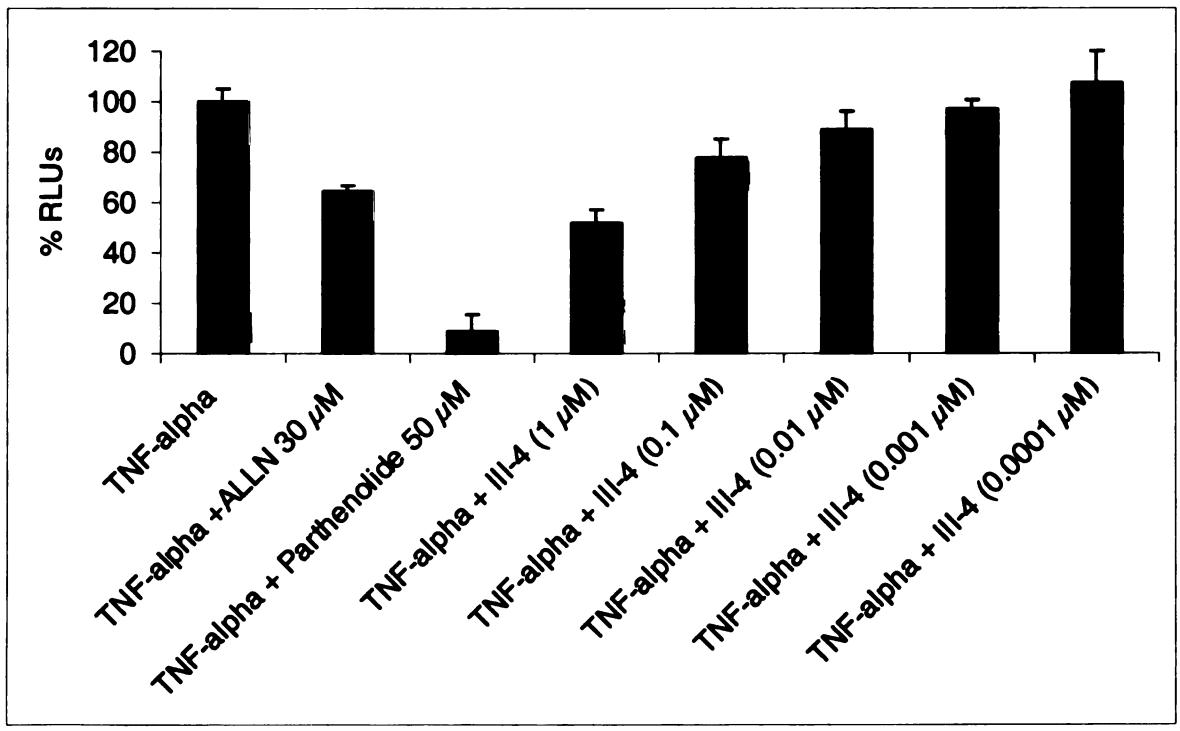



**Figure III-21**. Measuring transcriptional activity of NF-κB via Luciferase reporter assay in presence and absence of imidazoline **III-4**.

The imidazoline inhibited the NF-κB induced transcription by 52% and an $IC_{50}$ value of 0.95 $\mu$M when cells were activated by TNF-α. When the same assay was employed to measure the transcriptional activity of NF-κB with respect to activation with CPT, a similar effect in decrease in luciferase activity was seen (Figure III-22).

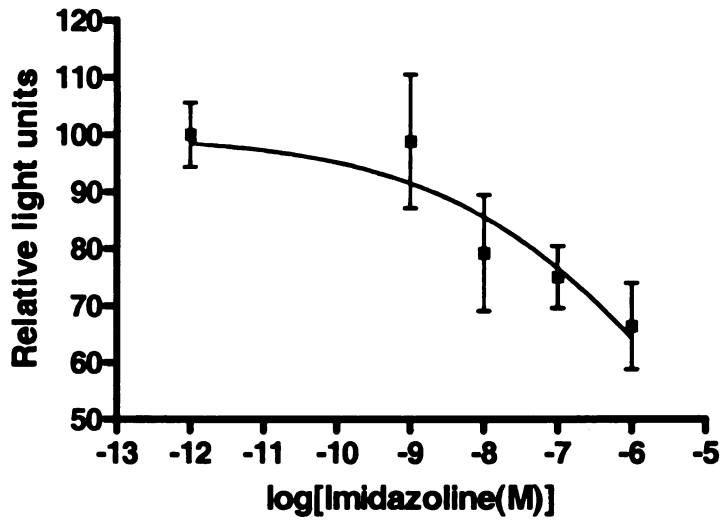



**Figure III-22.** Imidazoline **III-4** inhibits NF-κB induced transcription in a concentration dependent manner. Relative light units have been normalized to co-reporter. Values have been normalized to the positive control (constitutively activated plasmid pFC-MEKK). All values are averages of two independent experiments.

The imidazoline inhibited the CPT induced NF-κB transcription by up to 35% providing an $IC_{50}$ value of 9.8 $\mu$M (Figure III-22).

**viii. Luciferase enzyme assay: Imidazoline does not affect the luciferase enzyme directly**

To confirm the inhibition of NF-κB mediated luciferase production by imidazoline **III-4** and the luciferase enzyme itself, we incubated purified luciferase enzyme with various concentration of imidazoline and the vehicle (DMSO) at 0°C for 1h. These were then

analyzed for luciferase activity. Imidazolines did not appear to affect the activity of the enzyme (Figure III-23).

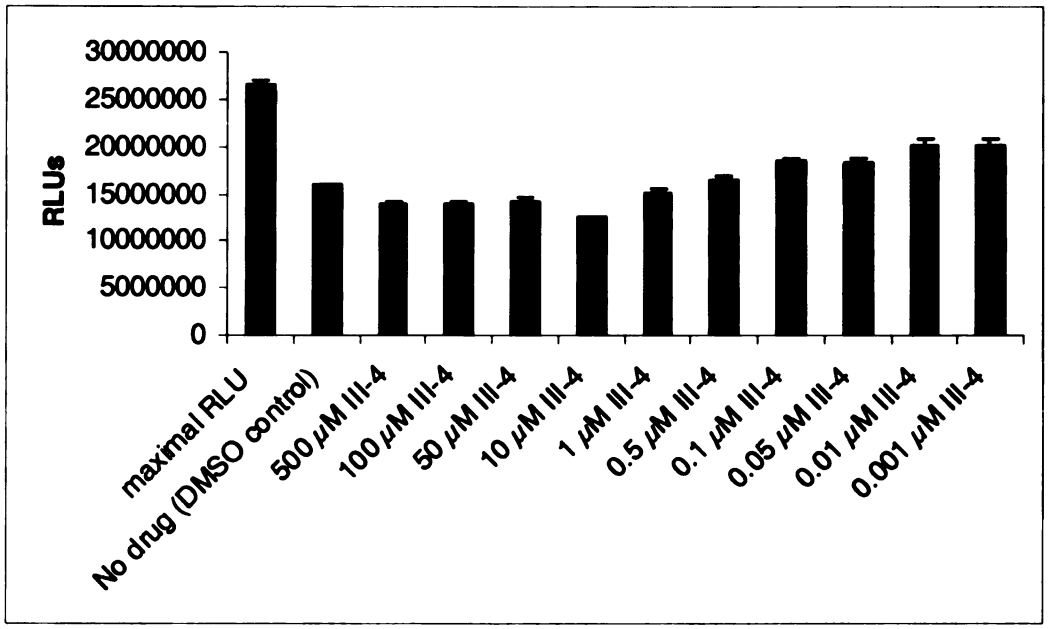



**Figure III-23.** Imidazoline **III-4** does not directly bind to the luciferase enzyme.

From the above assays we found that the imidazoline scaffold as a potent inhibitor of the NF-κB signaling pathway via the inhibition of the degradation of its inhibitory protein IκB.[36a] In order to determine the scope of this adjuvant therapy, we further investigated the effect of imidazoline **III-4** in a variety of cell lines and its effect on several clinically used chemotherapeutic drugs. We were able to show the ability of imidazoline **III-4** to selectively sensitize cancer cells towards CPT and CDDP in CEM (acute leukemia), HT1080 (fibrosarcoma), and HeLa (cervical carcinoma) cells but not in the non- cancerous SL89 (fibroblast) cell line. Figure III-24 (bottom) illustrates the effect of the imidazoline **III-4** on CEM cells when incubated with CPT over a 48 hour time period. Enhancement of CPT-induced apoptosis in CEM cells was investigated at concentrations ≤10 nM CPT, at which it causes DNA aberrations

but no significant apoptosis in leukemia cells [24] We had shown previously that treatment of the cells with the imidazolines had no effect on the level of apoptosis (Figure III-10, III-11, III-24) indicating that imidazolines do not exhibit significant cell cytotoxicity. Treatment with 10 nM CPT resulted in some cell death starting after 12 hours. Combination treatment of the non-cytotoxic imidazoline **III-4** (10 nM) with CPT (10 nM) resulted in complete apoptotic cell death after 48 hours (Figure III-24, bottom).

**ix. Post treatment with imidazoline III-4 does not potentiate the CPT induced apoptosis.**

Previously, we had shown that pre-treatment of CEM cells with imidazoline **III-4** inhibited the DNA binding activity of NF-κB (Figure III-17), degradation of I-κBα (Figure III-20) and transcriptional activity of NF-κB (Figure III-22) induced by CPT.[36a] To further evaluate the relationship between the NF-κB signaling events and apoptosis induction by CPT, we determined the effect of pre- and post-treatment with imidazoline on CPT induced apoptosis in CEM cells. Figure III-24 demonstrates that pretreatment of the cells with imidazoline **III-4** significantly enhances apoptosis, whereas addition of the imidazoline 1h after CPT treatment does not effect the induction of apoptosis (for both washed and continuous exposure, also see appendix 6). This differential effect of pre- or post-treatment with **III-4** indicates that the pro-survival activation of NF-κB by CPT precedes the apoptotic signaling in CEM cells. The inhibition due to imidazoline **III-4** in the DNA binding activity of NF-κB (Figure III-17), degradation of I-κBα (Figure III-20) and transcriptional activity of NF-κB (Figure III-22) induced by CPT and enhancement of apoptosis upon pre-treatment of

CEM cells with **III-4** suggests that activation of NF-κB is required for apoptosis in CEM cells. This effect correlates to the fact that CPT induced NF-κB activation in CEM cells is seen upto 6h (most optimum at 2h, Figure III-15a) while the apoptotic response in CEM cells does not trigger off until after 6h. The combination pre-treatment however, showed enhancement of apoptotic signal starting 6h.

Combined together, these results indicate that the apoptosis induction in CEM leukemia cells by CPT is dependent on prior activation of NF- κB signaling cascade.

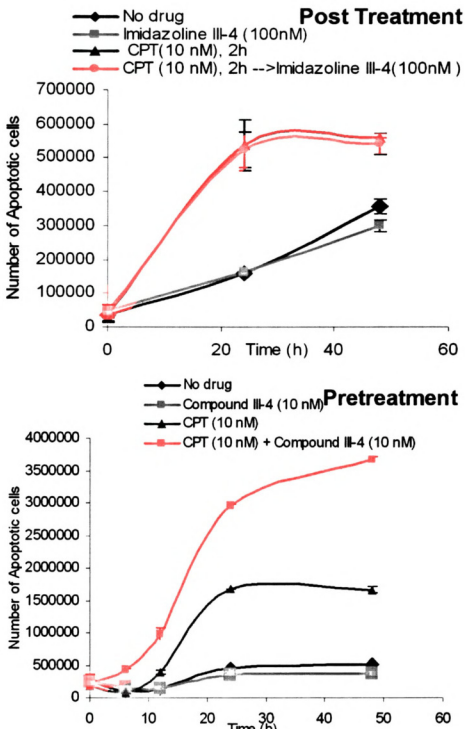



**Figure III-24a (top).** Caspase 3/7 activity in CEM cells post-treated with no drug control, imidazoline **III-4** (10 nM), CPT (10 nM), and the combination of imidazoline **III-4** (10 nM) and CPT (10 nM) over 48 hours; **III-24b (bottom).** Caspase 3/7 activity in CEM cells pre-treated with no drug control, imidazoline **III-4** (10 nM), CPT (10 nM), and the combination of imidazoline **III-4** (10 nM) and CPT (10 nM) over 48 hours.

## x. Potentiation by Imidazoline scaffold requires DNA damage

We investigated if imidazoline **III-4** could potentiate drug-mediated cell death by various anti-cancer agents including taxol (anti-tubulin agent, which inhibits the depolymerization of microtubules into tubulin resulting in blockage of the cell's ability to break down the mitotic spindle during mitosis), vincristine (anti-tubulin agent, which prevents the cell from making the spindles it needs to be able to move its chromosomes around as it divides), camptothecin (topoisomerase-1 inhibitor, which prevents the uncoiling of super-coiled DNA during DNA replication, transcription etc.) and cisplatin (DNA cross-linking agent, which inhibits DNA replication).[8-15] In CEM cells, agents that induced DNA-damage, (cis-platin and camptothecin) consistently showed a strong enhancement of apoptosis, on pre-treatment with imidazoline (Figure III-24 bottom). However, **III-4** failed to significantly enhance tubulin inhibitors such as taxol and vincristine in CEM cells (Figure III-25). As indicated previously, imidazoline **III-4** does not initiate an apoptotic response s a single agent (column 2, Figure III-25). Columns 3-8 (Figure III-25 top) indicate that various concentrations of taxol (1$\mu$M, 0.1 $\mu$M, 0.01 $\mu$M, 1 nM, 0.1 nM, 0.01 nM) increase the apoptotic cells in culture. Pre-treatment with **III-4** (10 nM) followed by incubation with various concentration of taxol (1 nM, 0.1 nM, 0.01 nM) did not show an appreciable increase in apoptosis. This result was not limited to the anti-tubulin agent taxol, but was further seen in the vinca alkaloids-vincristine (Figure III-25, bottom) and vinblastine. Only sub-nanomolar concentrations of taxol and vinca alkaloids were studied since earlier optimization with cell viability assays showed severe cell toxicity at higher concentrations of the drug.

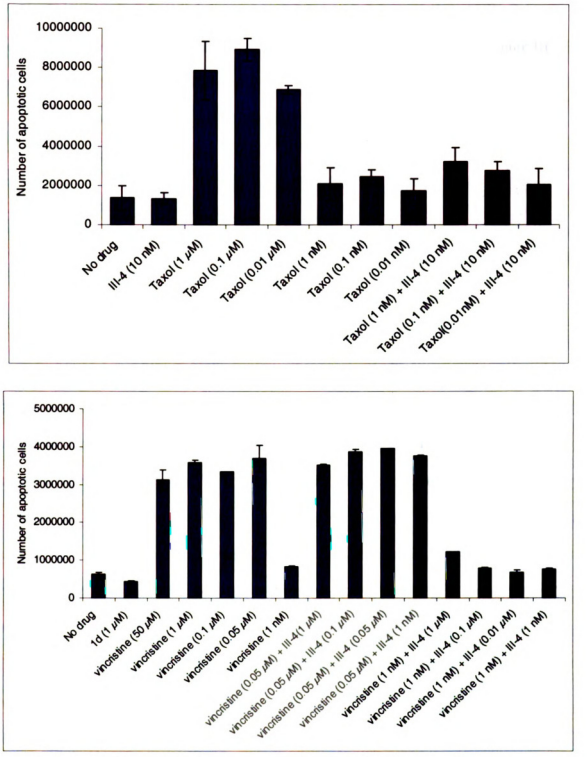



**Figure III-25**. Imidazoline **III-4** does not sensitize CEM cells to tubulin inhibitors.

Further investigation revealed that unlike CPT, taxol did not activate NF-κB in CEM cells. CPT activated NF-κB in a dose dependent manner (Figure III-14 and Figure III-26).

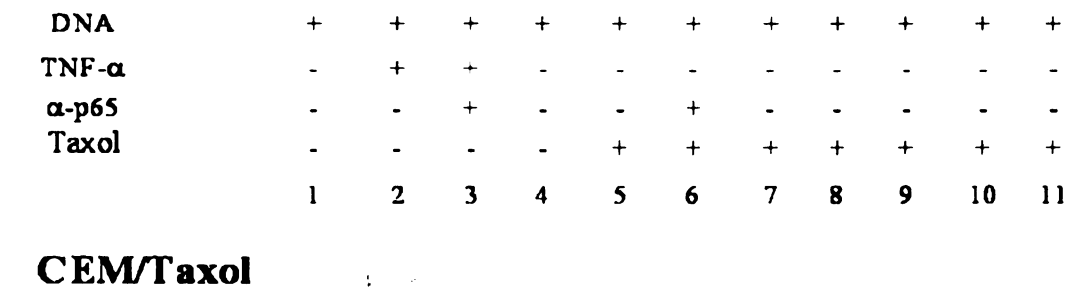



**Figure III-26.** EMSA for activation of NF-κB by various concentrations of Taxol. TNF-α was used as a positive control.

The cell nuclei were isolated and evaluated for NF-κB-DNA binding activity utilizing the fluorescently labeled NF-κB DNA consensus sequence. TNFα activation was used as a positive control (Figure III-26, lane 2). The NF-κB/DNA complex was unambiguously identified by treating the nuclear extract with a NF-κB p65 antibody following TNFα activation (Figure III-26, lane 3). Treatment of the nuclear extract with the p65 antibody resulted in a significant decrease in NF-κB/DNA binding and the formation of a supershift of the complex. As a negative control, cells are left unactivated and the nuclear extracts were exposed to the NF-κB consensus sequence, resulting in no activation NF-κB (Figure III-26, lane 4). Treatment of the CEM cells with taxol concentrations ranging from 1 $\mu$M to 0.01 nM (diluted 10 folds, Figure III-26, lanes 5, 7-11) did not activate NF-κB.

Activation of NF-κB signaling cascade requires an intact nucleus and topoisomerase-1 mediated nuclear damage in CEM cells.[19c] This suggests that the drug-induced apoptotic and anti-apoptotic signaling pathways related to DNA damage are crucial to the enhancement of efficacy by non-cytotoxic imidazoline **III-4**. This is in contrast to

proteasome inhibitor bortezomib® (PS341), which inhibits the NF-κB pathway and potentiates the effect of tubulin-inhibitor taxol as well as DNA damaging agents, doxorubicin and cis-platin in pancreatic cell lines and pancreatic effusion lymphomas.[33, 98] Chiao and co-workers [98b] showed that mutated IκBα and PS-341 completely abolish the RelA constitutive activity in the pancreatic cancer cells. However, PS-341, but not expression of mutated IκBα, sensitized cells to apoptosis induced by Taxol. They suggested a reactivation of RelA induced by taxol in pancreatic cancer cells that express mutated IκBα occurred through phosphorylation and degradation of IκBβ. PS341, on the other hand, completely abrogates the degradation of phosphorylated IκB which is consistent with the role of NF-κB in apoptosis.

**k) Selective potentiation of apoptotic effect of DNA damaging agents by Imidazolines in cancer cells**

Encouraged by the finding that NF-κB modulation could potentiate the effect of DNA damaging agents in chemotherapy upon pre-treatment with imidazoline **III-4**, we now investigated various cancerous and non-cancerous cell lines for this effect. The effect of imidazoline **III-4** was evaluated on cancer cells; CEM, HT1080, HeLa and non-cancerous fibroblast cells (SL89). This was quantified by calculating the concentration at which CPT and CDDP induced 95% apoptosis ($CC_{95}$) with and without pretreatment of the cells with imidazoline **III-4** (100 nM for 1 hour, $CC_{95}$ combination).

Cells were incubated with varying concentrations of camptothecin or cis-platin in presence and absence of imidazoline (100 nM). Cells were monitored over 48h and the level of caspase-3 was measured. $CC_{95}$ were calculated in presence and absence of imidazoline **III-4**.

Table **III-4** and appendix 7 clearly indicate that only the cancerous cell lines exhibited strong enhancement of apoptosis. The $CC_{95}$ values of both CPT and CDDP dropped drastically (up to 74 fold for CPT and 14 fold for CDDP) upon pretreatment of cancerous cells with the imidazoline (Table III-3 and Figure III-27). No increase in apoptosis was seen in the non-cancerous fibroblasts (SL89) upon pretreatment with the non-cytotoxic imidazoline **III-4** (Table III-3 and Figure III-27).

| Cell Line | Drug | $CC_{95}$ | $CC_{95}$ Combination | Fold Enhancement |
|---|---|---|---|---|
| CEM | CPT | 44 nM | 0.6 nM | 74 |
| CEM | CDDP | 15 μM | 1.1 μM | 14 |
| HT1080 | CPT | 86 μM | 6.7 μM | 13 |
| HT1080 | CDDP | 53 μM | 5.2 μM | 10 |
| HeLa | CPT | 1.48 μM | 0.06 μM | 24 |
| HeLa | CDDP | 0.3 μM | 0.06 μM | 5 |
| SL89 | CPT | 15 μM | 16 μM | 1 |
| SL89 | CDDP | 0.5 μM | 0.6 μM | 1 |

**Table III-4.** Cancer cell specific enhancement of efficacy.

*Data reported as an average of two independent experiments after 48 hour drug exposure. The imidazoline itself induced no significant levels of apoptosis (tested up to 10 mM). CPT = camptothecin, CDDP = cis-platin.

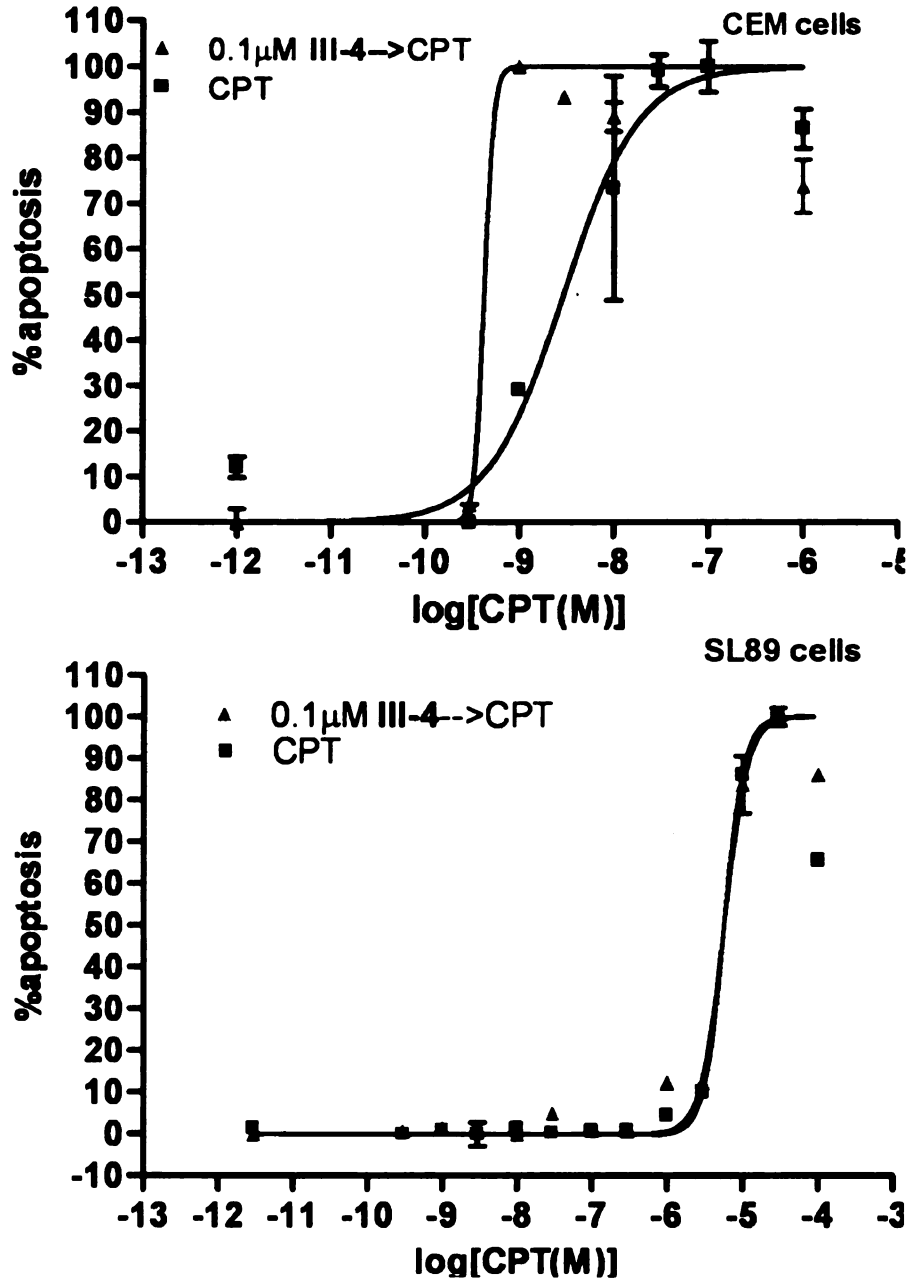



**Figure III-27A (top).** $CC_{95}$ comparison of CPT with and without imidazoline **III-4** in CEM leukemia cells. **27B (bottom).** $CC_{95}$ of CPT with and without imidazoline **III-4** in non-cancerous fibroblast cells. Data reported as an average of two independent experiments after 48 hour drug exposure.

**xii) Correlation between potentiation and NF-κB activation.**

Our data indicates that sensitization of cells to chemotherapeutic agents using imidazoline **III-4** exhibits a strong correlation with NF-κB activation. For example, CEM cells showed only moderate activation of NF-κB by CDDP (≥3 μM, Figure III-28)

resulting in moderate fold enhancement (14 fold) compared to the strong activation ($\geq$0.01 $\mu$M, Figure III-28) and enhancement (74 fold) with CPT. This observation was noticeable across the panel of cell lines (Figure III-28 and Table III-3). The ability of the imidazoline **III-4** to enhance the apoptotic levels caused by chemotherapeutic agents such as CPT and CDDP were found to be a function of sensitivity to NF-κB activation and directly proportional to it.

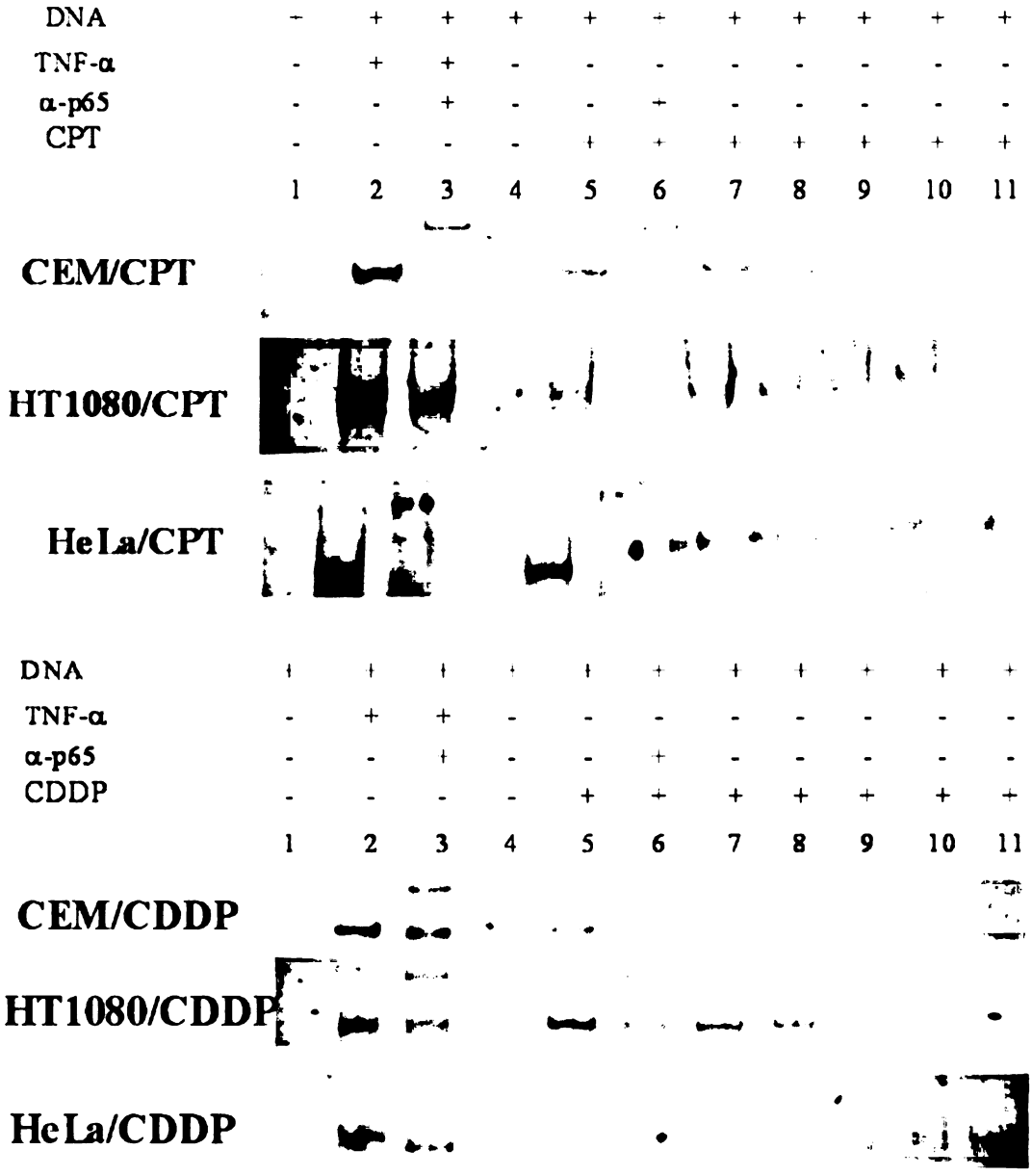



**Figure III-28a (top).** EMSA for NF-κB in CEM, HT1080, HeLa cells activated with various concentrations of CPT (10 μM, 1 μM, 0.1 μM, 0.01 μM, 1 nM, 0.1 nM). TNF-α was used as a positive control. **III-28b (bottom).** EMSA for NF-κB in CEM, HT1080, HeLa cells activated with various concentrations of CDDP (10 μM, 3 μM, 1μM, 0.3 μM, 0.1 μM, and 0. 03 μM). TNF-α was used as a positive control.

In particular SL89 cells, which showed no enhancement with the imidazoline treatment (Figure III-29, Table III-3) and were completely insensitive to NF-κB activation when treated with varying concentrations of either CPT, CDDP or TNF-α (Figure III-29).

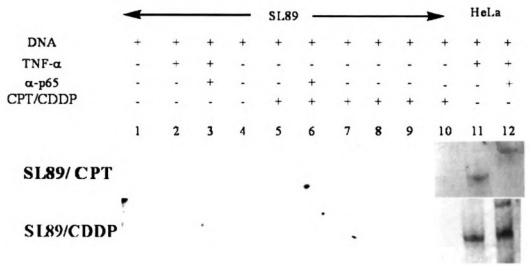



**Figure III-29.** EMSA for NF-κB activation in SL89 cells. Cells were activated with TNF-α and CPT (10μM, 1μM, 0.1μM, 0.01μM, 1 nM, 0.1 nM) and CDDP (10 μM, 3 μM, 1 μM, 0.3 μM, 0.1 μM). HeLa cells activated with TNF-α were used as a positive control. The supershift with antibody specific for p65 unit is shown in lane 12.

This observation was further supported by the NF-κB driven transcription studied via luciferase based reporter assay in HeLa and SL89 cells. Both TNF-α as well as CPT activated NF-κB and enhanced NF-κB based transcription in HeLa cells (Figure III-30). SL89 cells were devoid of any significant NF-κB transcriptional activity (Figure III-30) correlating well with the inability to show potentiation of DNA damage in presence of imidazoline **III-4**.

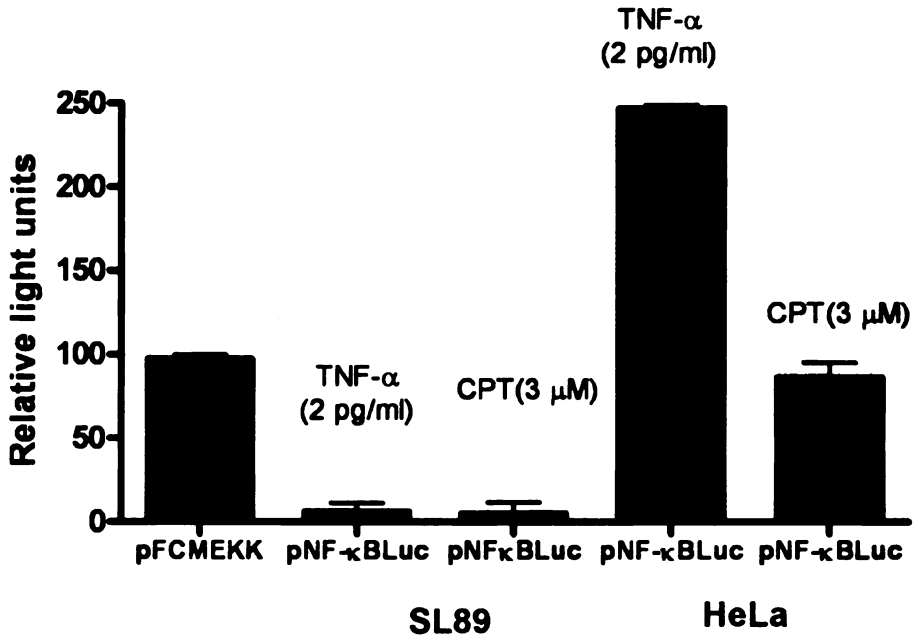
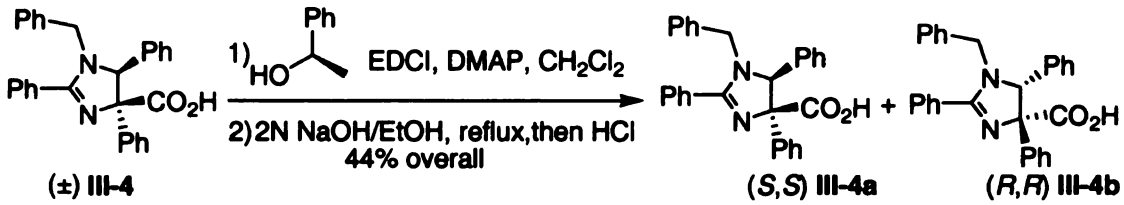



**Figure III-30.** SL89 cells do not show NF-κB mediated transcriptional activity with either TNF-α or CPT. pFCMEKK causes constitutive activation of NF-κB. pCIS-CK, does not contain the enhancer element for NF-κB and serves as a negative control. TNF-α and CPT are known to activate NF-κB in a variety of cell lines.

**xiii. Chemical resolution of III-4 and biological activity of the enantiomers.**

Encouraged by the ability of the racemic **III-4** in potentiating the effect of DNA damaging agents such as camptothecin and cis-platin, we further investigated the effects of the pre-treatment of enantiomers followed by exposure to camptothecin on cancerous cells. Racemic **III-4** was chemically resolved using an EDCI-mediated esterification with R(+)-1-phenylethanol yielding two diastereomeric esters, **III-9** and **III-10**, which on hydrolysis afforded the (*S,S*) enantiomer **III-4a** and (*R,R*) enantiomer **III-4b** in overall 44% yield (Scheme III-3).




**Scheme III-3.** Chemical resolution of racemic **III-4** into enantiomers.

The enantiomers were crystallized and their absolute configuration was determined by X-ray crystallography (Figure III-31).

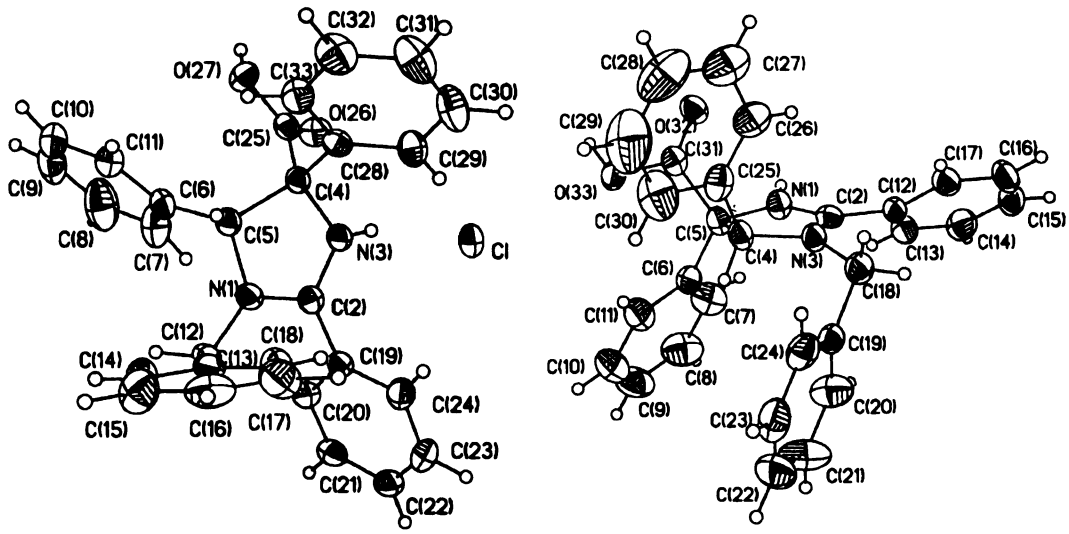
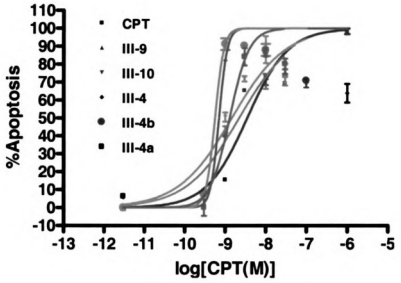



**Figure III-31.** X-ray crystal structures of III-**4b** (left, *R,R*) and **III-4a** (right, *S,S*).

The diastereomeric esters, **III-9**, **III-10** and the enantiomers **III-4a** and **III-4b** were tested for enhancement of apoptosis for a period of 48 h with CPT (Figure III-32). None of the compounds induced any significant cell death over 48 hours, however the (*R,R*) enantiomer **III-4b** potentiated the apoptotic effect of CPT ($CC_{95}$ combination = 0.53 nM, 83 fold enhancement) nearly 5 times more than the (*S,S*) enantiomer **III-4a** ($CC_{95}$ combination = 2.4 nM, 18 fold enhancement). The esters did not show a significant enhancement of apoptosis as determined by the caspase based Apo-One assay™ ($CC_{95}$ **III-9**=77.2 nM, CC95 **III-10**=54.3 nM). Figure III-32 illustrates the effects of pre-treatment of the diastereomeric esters and the enantiomers.




| Entry | Conditions | $CC_{95}$ | Fold Enhancement |
|---|---|---|---|
| 1 | CPT | 44 nM | - |
| 2 | III-9--> CPT | 77.2 nM | 0.6 |
| 3 | III-10--> CPT | 54.3 nM | 0.8 |
| 4 | III-4---> CPT | 0.6 nM | 75.1 |
| 5 | III-4a---> CPT | 2.4 nM | 18.4 |
| 6 | III-4b---> CPT | 0.5 nM | 83.6 |

**Figure III-32.** Caspase 3/7 activity measured in CEM cells. $CC_{95}$ comparison for CPT in CEM leukemia cells in presence and absence of racemic **III-4**, the diastereomers **III-9** and **III-10** and the enantiomers **III-4a** and **III-4b**. Data reported as an average of two separate experiments.

**xiv. FACS analysis.**

Since apoptosis can't be described by one assay, CEM cells were subjected to FACS analysis. Briefly, cells were pre-incubated with the **III-4**, **III-4a** and **III-4b** for 40' followed by treatment with CPT (0.01 $\mu$M) for 12h and 48 h to mark the early and late phases of apoptosis. Cells were harvested at the desired time point and washed with 1XPBS. Further, cells were stained, and sorted on a flow cytometer.

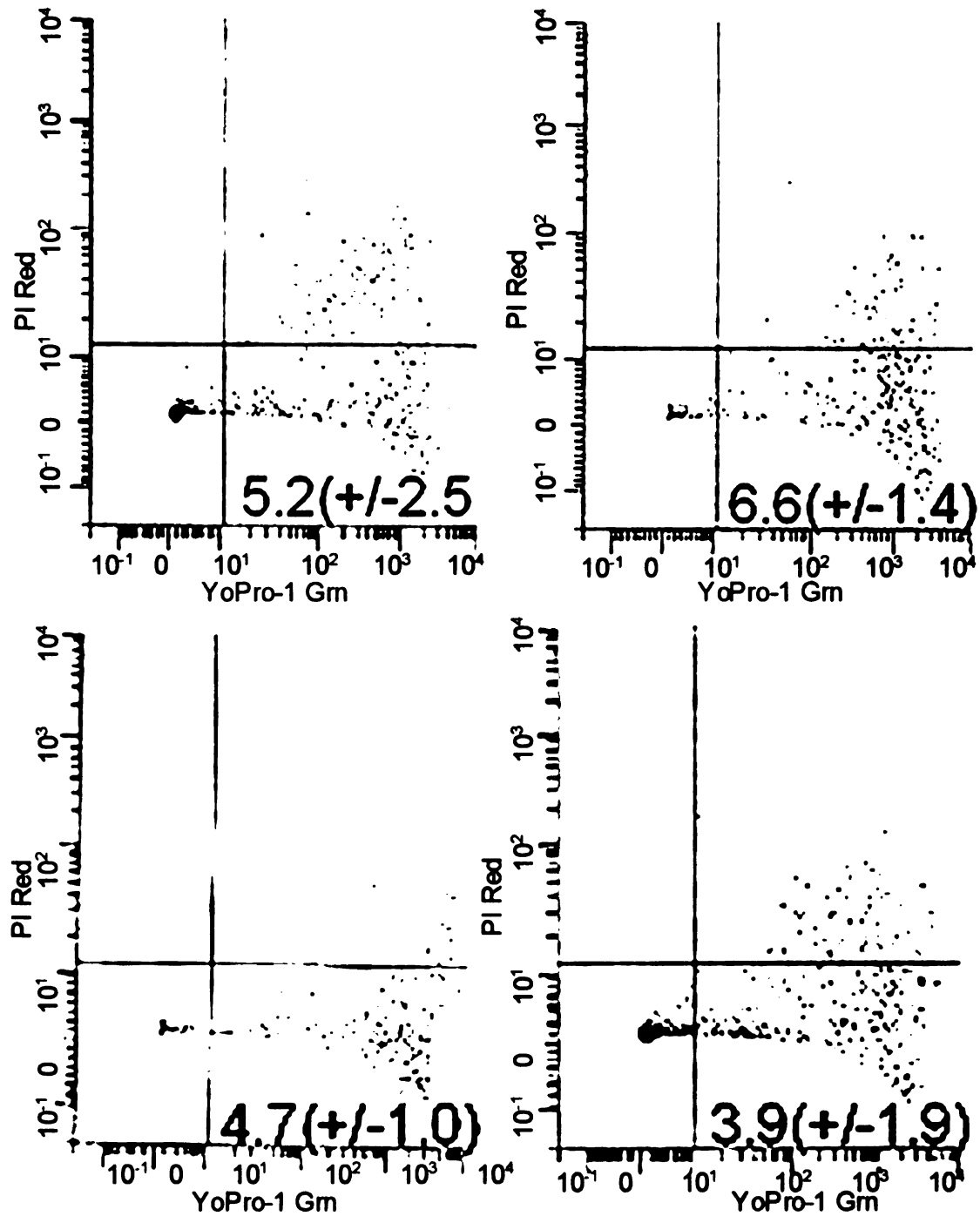



**Figure III-33a.** FACS analysis (TOP) cells alone, racemic **III-4** (0.1 $\mu$M); (BOTTOM) **III-4b** (0.1 $\mu$M), **III-4a** (0.1 $\mu$M); The lower right quadrant indicates the % apoptosis (+/- standard deviation) for the sample.

As confirmed by the flow cytometry experiments, compounds **III-4**, **III-4a** or **III-4b** did not induce apoptosis themselves (Figure III-33a) as compared to the cells by themselves.

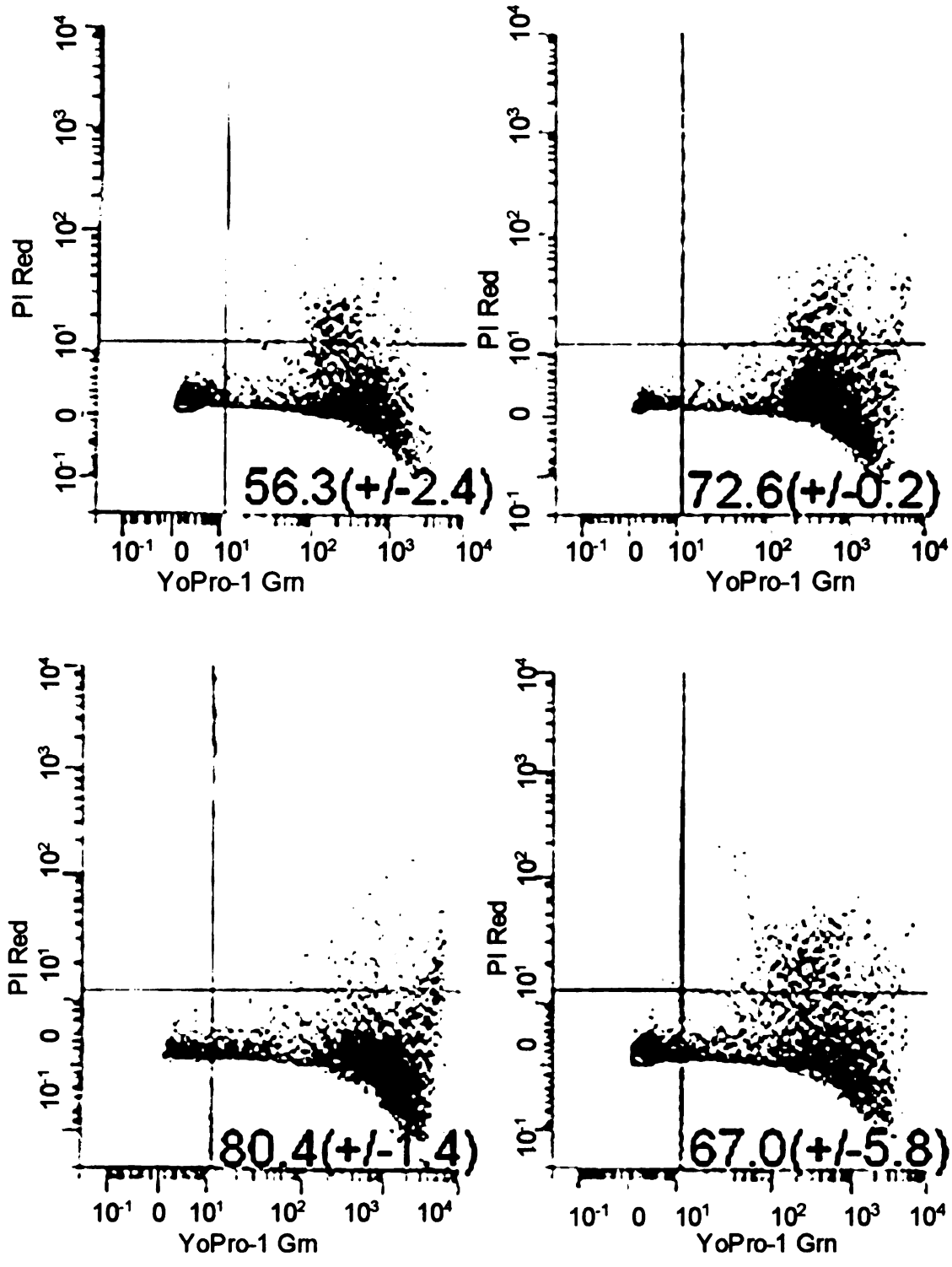



**Figure III-33b**. FACS analysis (TOP) CPT (0.01 $\mu$M), **III-4**→CPT (0.01$\mu$M); (BOTTOM) **III-4b**→CPT (0.01$\mu$M), **III-4a**→CPT (0.01$\mu$M). The lower right quadrant indicates the % apoptosis (+/- standard deviation) for the sample.

However, after CPT treatment, the percent of cells that became YoPro-1 positive increased. CPT alone induced apoptosis in CEM cells upto 58%. This effect was further pronounced upon pre-treatment with racemic **III-4** (72%) as well as its enantiomers **III-4a** (*R,R*) (80%) and **III-4b** (*S,S*)(67%). Further, concomitant to the increase in the dye's ability to enter the cells, there was a decrease in the cell size (mean forward scatter) decreased from viable cells (529) for viable CEM cells to 509 upon CPT treatment and 457 for pre-treatment with **III-4a** (highly fluorescent population), a common feature of apoptotic cells.

**xv. NF-κB-p53 crosstalk**

Webster and Perkin[40] have demonstrated the effect on transcription upon DNA damage. Many cellular stimuli result in the induction of both the tumor suppressor p53 and NF-kobo. In contrast to activation of p53, which is associated with the induction of apoptosis,[99] stimulation of NF-kobo has been shown to promote resistance to programmed cell death.[38] This suggests a regulatory mechanism must to integrate these opposing outcomes and a tightly coordinated critical cellular decision-making event. Further, Collins *et al* showed that CREB binding protein is the common nuclear integrator and that p65 and p53 suppress each other and compete for the CBP/p300 which is essential to form DNA-transcriptional complex.[100]

Encouraged by these facts, we wanted to determine if the inhibition of NF-κB can correlate down to a pro-apoptotic protein p53. Briefly, HT1080 with *wt* p53 were transfected with p53Luc. pRLTK was used as a co-transfecting agent. Transfected cells were treated with CPT (0.01 $\mu$M, activates NF-κB, 2h; activates p53, 8h) in presence and absence of **III-4**. Figure III-33 clearly indicates accumulation of p53. This result tallies

well with the fact that **III-4** inhibited NF-κB *via* degradation of phosphorylated I-κB-α. Since, there is ample evidence that NF-κB and p53 crosstalk and transcriptional activity of one outweighs the other, [99, 100] inhibition of NF-κB would lead to more transcriptional activity of p53.

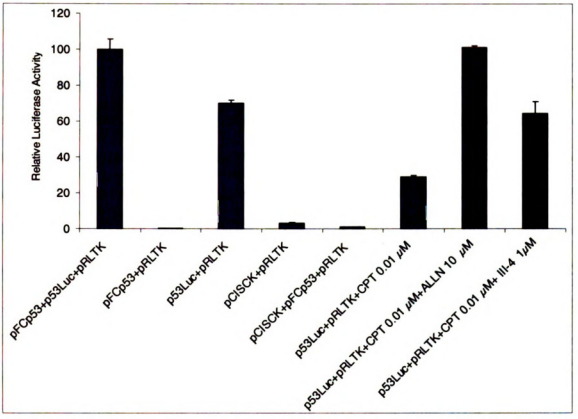



**Figure III-34.** Enhanced p53 transcriptional activity in HT1080 due to inhibition of NF-κB by **III-4**

Figure III-34 shows the enhancement of transcriptional activity due to p53. We have shown previously that imidazoline **III-4** modulate the levels of NF-κB and inhibit its translocation via blocking the degradation of I-κB by the proteosome. This inhibition of translocation reduces the levels of NF-κB in the nucleus for NF-κB-DNA binding. It has been well documented that chemotherapeutic agents activate both the p53 (pro-apoptotic) [103] and the NF-κB (anti-apoptotic) [19c] pathways. Under normal conditions, the p53 tumor

suppressor protein is rapidly degraded by the 26S proteasome[101] in a process mediated by MDM2.[102]After cellular stresses, such as exposure to DNA-damaging agents, the half-life of the p53 protein is significantly increased, and p53 accumulates in the nucleus of treated cells.[103] The mechanism by which the stability of p53 is increased after cellular stress is not fully understood, but it is likely that modifications such as phosphorylation of the p53 protein itself are involved. Inhibition of the 26S proteasome results in the rapid accumulation of p53[101] and of p53-inducible gene products such as p21WAF1, MDM2, and Bax.[104] Since, p65 and p53 compete for the same CREB-protein to initiate the transcription process, low levels of p65 ( in presence of a proteasomal inhibitor) would allow p53 to bind to CREB binding protein and start its transcription. ALLN (column 7, Figure III-34) showed a similar system as described above. Previous studies confirmed that the proteasomal inhibitor ALLN inhibits the transcriptional activity of NF-κB by preventing its translocation across the nuclear membrane. Compound **III-4** (column 8, Figure III-34) also indicates similar accumulation of p53 and subsequent enhancement in its transcriptional activity when compared to CPT treatment alone (column 6, Figure III-34). These studies indicate that pre-treatment with imidazoline **III-4** enhances the apoptotic induction due to DNA damaging agents such as camptothecin by modulation of NF-κB which further translates into accumulation of p53 leading to an increase in the transcriptional activity of p53.

**xvi. CPT-III-4 stacking interactions.**

Self-aggregation of some CPT drugs is regarded as an additional factor affecting the biological activity of the medications. [105] The tendency to self-aggregation has provided a diminished tendency to other non-specific interactions and therefore increases the rate of

accumulation within the cells and, consequently, the availability at the receptor site. Based on such studies, we wanted to ascertain if **III-4** and CPT might have such interactions to form ion pair. We allowed CPT and **III-4** to incubate for 3h in DMSO at room temperature and an NMR was recorded for the mixture (Figure III-35). No change was seen in the NMR values of either CPT or imidazoline indicating no chemical change in either or them.

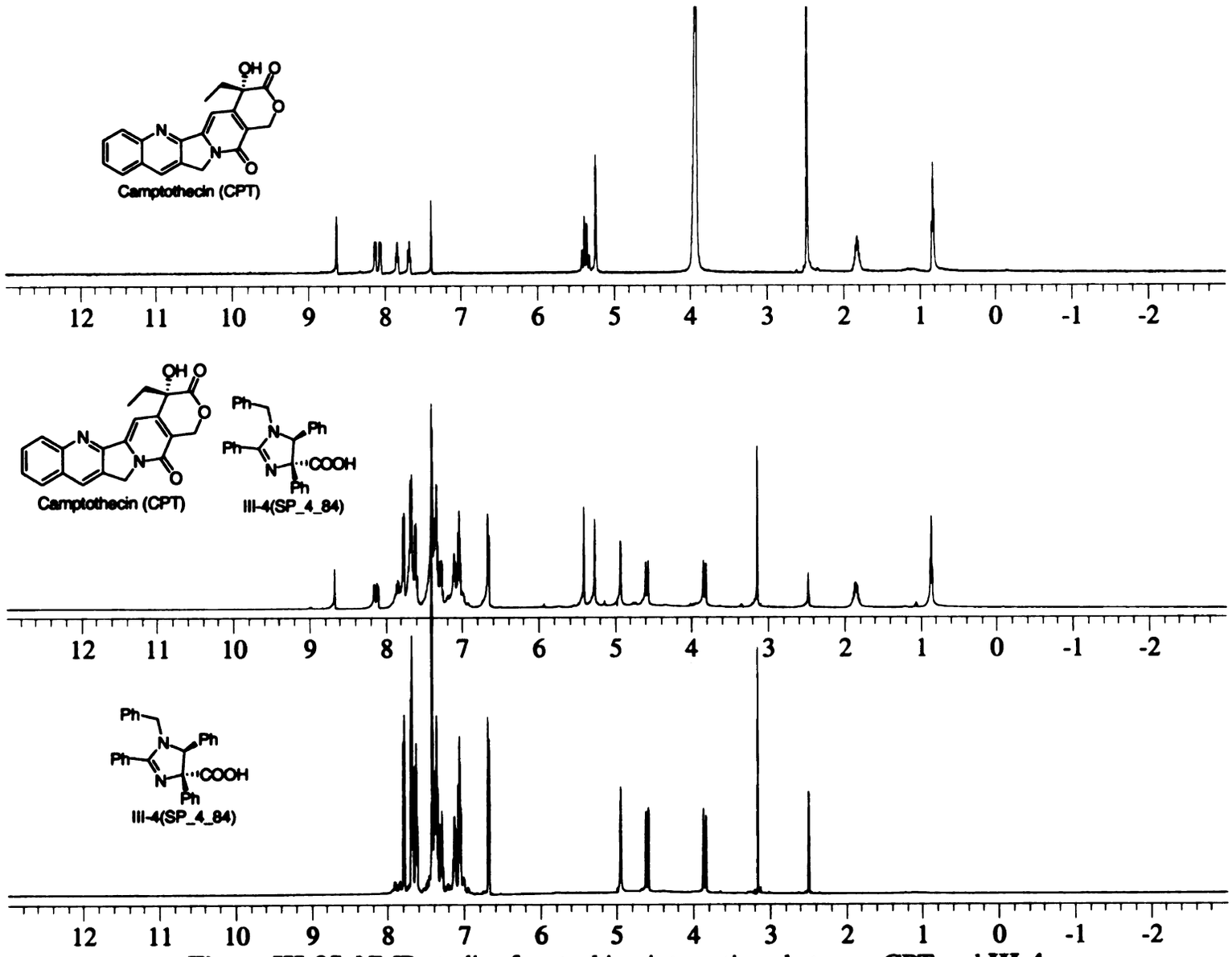


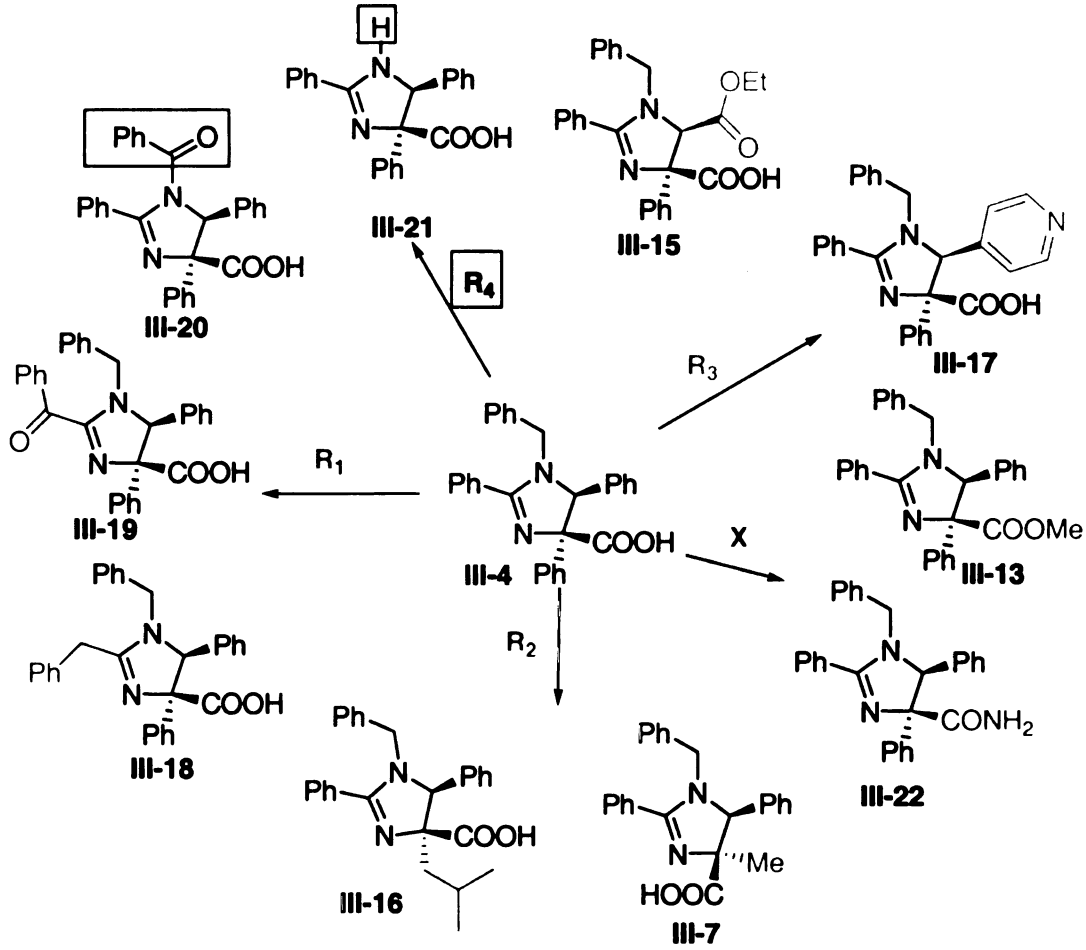

**Figure III-35.** NMR studies for stacking interactions between CPT and **III-4**.

**G. Future directions.**

**i) Structure-Activity Relationship based on III-4 as the parent scaffold.**

Compound **III-4** was used as parent scaffold to build a 10-member library for structure –activity relationship. This collection of compounds comprised of some trans-imidazolines synthesized previously by other researchers in our group.[84]

The four point diversity was altered on the parent scaffold in a mutually exclusive manner as shown in Scheme III-4.




**Scheme III-4.** Structure-Activity Relationship based on **III-4.**

These imidazolines were then subjected to a preliminary screen to find out the chemopotentiation index. Table III-5 shows the importance of a phenyl group at $R_2$ position. We have seen from table III-1 that a Me could not be tolerated at $R_2$,

when the phenotype under investigation is enhancement of apoptosis induced by CPT.

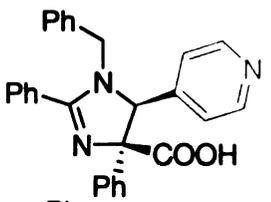
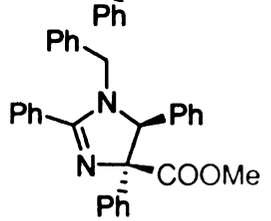
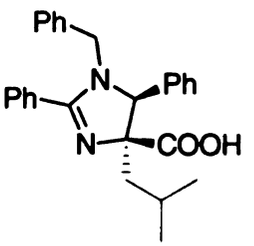
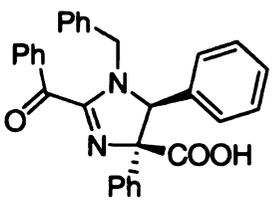
| Entry | *trans*-Imidazoline | Chemopotentiation Index @1μM, 48h |
|---|---|---|
| III-8 |  | 1.4(+/-0.03) |
| III-13 |  | 1.2(+/-0.05) |
| III-16 |  | 1.0 (+/- 0.01) |
| III-19 |  | 0.9(+/-0.0) |

**Table III-5.** Structure-activity profiling based on chemo-potentiation indices.

Based on the chemo-potentiation indices, we are attempting to quantify the enhancement of apoptosis of this SAR model.

**ii)Target validation of combination therapy with camptothecin and the imidazoline III-4(SP_4_84).**

**a) Microchip analysis for combination studies with camptothecin and III-4**

The relevance of a target in a disease process needs 'validation'. The question whether the desired effect on a diseased state can be achieved by influencing a

biological system or process requires definitive proof most clearly answered by clinical studies. However, early stages of development require involvement of target validation via biochemical experimentation, studying differential gene expression in healthy and diseased tissue using gene chips, functional studies of proteins and animal models.

DNA microarrays offer tremendous advantages over traditional 'single-gene' methods such as northern hybridization, reverse transcriptase- PCR etc. These have been developed to simultaneously analyze the expression patterns of thousands of genes.

**b) The process of gene chip array**

Typically, high density cDNAs are delivered in a robotic system on coated surfaces such as a microscopic glass slide and immobilized to the surface. Each DNA fragment representing a gene is assigned a specific location on the array, usually a glass slide, and then microscopically spotted (< 1 mm) to that

location.[106]

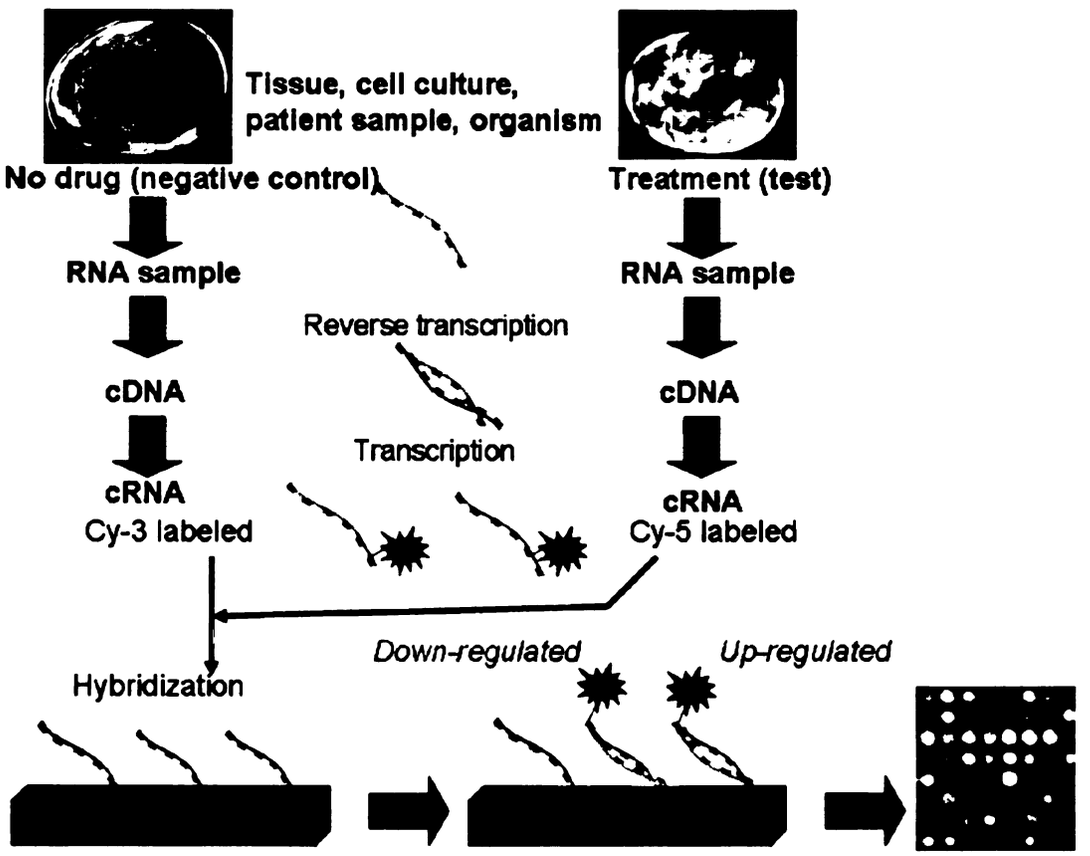



**Figure III-36.** General scheme for a microchip array assay

This microarray is then hybridized with a complex mixture of fluorescently labeled nucleotides derived from a source such as cell culture, patient derived samples, etc. The chips are then washed and then scanned under a laser. Typically this process could be carried out as a oligonucleotide array or a cDNA array system. While the former require probe design, they have greater hydridization specificity. cDNA microarrays on the other hand represent the whole genome on a chip, hence analyze at a 'global' level. [107]

The ability to analyze the activity of every gene in a cell is a powerful tool. It helps in answering typical questions such as: what is different in the brain of a patient with Alzheimer disease? Can we understand why cancer occurs? Or

perhaps, what is happening in a blood vessel that is developing a cardiovascular plaque? Microarray technology can help in identification of genes and help in bringing out the potential of a targeted therapy.[107, 109] Differential gene expression in-between tissue types also uncovers facts about development and differentiation to warn us which genes would be harmful to target for disease therapy. For example it would not be wise to target a gene that is found to promote cancer but is also important to heart function. Some genes could be regularly assayed in order to determine a person's risk of developing a particular disease or determine the complexity of the same.[109, 110] Besides the ability to determine the efficacy in mode of action of a small molecule, microarrays also determine the therapeutic index of a compound by helping to determine the potential toxic burden. The aim of this field – toxicogenomics- is to provide an effective screen for toxicity based on changes in gene expression. It has also been speculated that array technology could diagnose diseases at very early stages, so that therapy could be commenced before the disease has the chance cause detrimental effects.[110]

**c) Genechip array and Cancer**

Cancer is known to be a result as well as a cause for numerous changes in gene expressions.[106] For example, more than 50% cancers undergo a mutation in p53, a pro-apoptotic protein. Comparative studies between healthy and diseased cells have helped in rapid identification of gene profiles for a particular type of cancer.[106, 108] Finally, there is evidence of use of microarray analysis in drug

design and screening, to determine the expression of important genes with compounds.[105]

Christman *et al* studied the genes and pathways regulated by them in Hela cells upon treatment with camptothecin.[28a] They found an up-regulation in NF-κB and EGFR pathways upon camptothecin treatment and further showed that adjuvant therapy with EGFR inhibitor AG1478 increased the apoptotic response of camptothecin in HeLa cells.

Microarrays still need to undergo some optimization before they can be used in such clinical screens, due to the problem of false positive results caused by genes reacting with the array in an inappropriate manner. Current microarray technology at its best still incorrectly report the expression of about 1% of genes it screens. A combination with biochemical experiments would almost always be required to prove any screening results. Improvements in the technology are therefore required before such screens are feasible.

**d) Animal models: *in vivo* studies**

Earlier, in an active collaboration with Chemgenics Therapeutics Incorporation, Menlo Park, CA we studied the effect of these drugs in mouse models. RIF-1 tumor cells were injected sub-cutaneously and allowed to grow to approximately 100 $mm^3$. Administration of drugs was then started and the number of days that the tumor took to reach 'four times' its original size, were counted (Figure III-37). Now, *in vivo* models are being studies in collaboration with the Carcinogenesis Lab at Michigan State University including the pharmacological properties of **III-**

4. Preliminary studies involved sub-cutaneous injection and growth of tumors in nude mice.

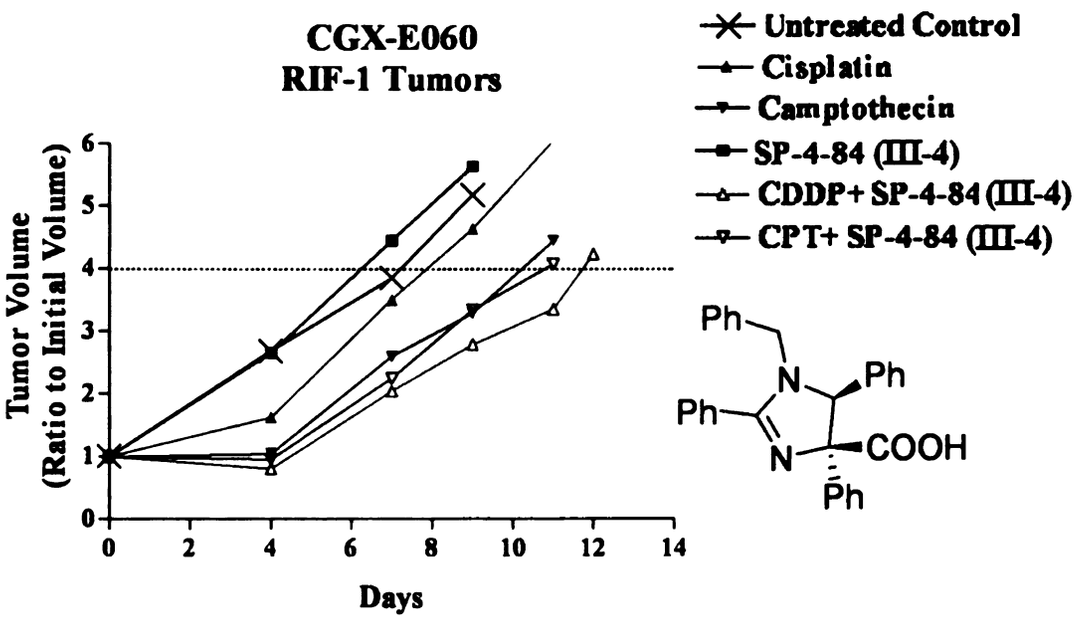



**Figure III-37.** Decrease in the tumor size in RIF-1 tumors. Tumor volume (y-axis) vs. Days (x-axis) to 'four times' volume. Chemotherapeutic drug: 8 mg/ 1kg; Imidazolines: 4 mg/ 1kg.[36b]

These however, caused too much tumor burden for the animals. Cells are now injected intraperitoneally and allowed to develop ascites. Earlier optimization studies reveal that 40 mg/kg of **III-4** does not cause cytotoxicity and was able to show potentiative activity with cis-platin. Concentration higher than 60 mg/kg cause sever liver cytotoxicity and those lower than 20 mg/kg do not have a potentiative effect. Further optimization in generation of tumors and probing potentiative effects of racemic **III-4** and enantiomers **III-4a** and **b** are being investigated in animal models by the carcinogenesis lab.

**H. Significance**

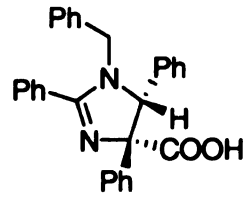
We have described a novel class of NF-κB inhibitors, imidazoline **III-4**, that is unique with respect to its insignificant toxicity and remarkable ability to enhance the anticancer efficacy of the chemotherapeutic agent camptothecin.

Antitumor drugs exert their antitumor activity *via* the initiation of apoptotic signaling pathways ultimately resulting in cell death. Conventional chemotherapy suffers a compromised clinical efficacy due to the induction of anti-apoptotic factors, such as NF-κB, concurrent with the desired induction of programmed cell death. Inhibition of this anti-apoptotic transcription factor retards this cellular resistance. The imidazoline **III-4** is the lead compound from a novel class of non-toxic small molecules that enhance the efficacy of the DNA damaging agents at low nanomolar concentrations. Combination therapy of CEM cells with camptothecin and the imidazoline **III-4**, provided ~74 fold enhancement of efficacy after 48 hours. This is postulated to proceed *via* the inhibition of the pro-survival signaling pathways, such as NF-κB, in a highly cell-specific manner. Inhibition of the NF-κB pathway was found to occur via the inhibition of degradation of the inhibitory protein I-κBα. In particular, imidazoline **III-4** selectively sensitized cancer cells to genotoxic stress at low nanomolar concentrations. No such effect was seen in non-cancerous fibroblasts. These studies indicate the potential of small molecule NF-κB inhibitors to sensitize tumor cells towards classical anticancer agents in order to target this chemoresistance and enhance the overall efficacy of anticancer treatment. Work towards identifying the target, detailed mechanism of action and clinical potential of these agents is currently under investigation in our laboratories.

## I. Experimental Section

Reactions were carried out in oven-dried glassware under nitrogen atmosphere, unless otherwise noted. All commercial reagents were used without further purification. All solvents were reagent grade. THF was freshly distilled from sodium/benzophenone under nitrogen. Toluene, $CH_2Cl_2$, and TMSCl were freshly distilled from $CaH_2$ under nitrogen. All reactions were magnetically stirred and monitored by TLC with Analtech 0.25 mm pre-coated silica gel plates. Column chromatography was carried out on silica gel 60 (230–400 mesh) supplied by EM Science. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Melting points were determined on a Mel-Temp (Laboratory devices) apparatus with a microscope attachment. Infrared spectra were recorded on a Nicolet IR/42 spectrometer. $^1H$ and $^{13}C$ NMR spectra were recorded on a Varian Gemini-300 spectrometer or a Varian VXR-500 spectrometer. Chemical shifts are reported relative to the residue peaks of the solvent $CDCl_3$ (δ 7.24 for $^1H$ and δ77.0 for $^{13}C$), DMSO-*d*6 (δ 2.49(5) for $^1H$ and δ39.5 (7) for $^{13}C$) and $CD_3OD$ (δ 3.3 (5), 4.78 (1) for $^1H$ and δ49.0 (7) for $^{13}C$). High resolution mass spectra and FAB mass spectra were obtained at the Mass Spectrometry Laboratory of the Department of Biochemistry, Michigan State University, with a JEOL AX-505H and a JEOL HX-110 double focusing mass spectrometer (JEOL USA, Peabody, MA), respectively. Gas chromatography/low resolution mass spectra were recorded on a Hewlett–Packard 5890 Series II gas chromatograph connected to a TRIO-1 EI mass spectrometer. All chemicals were obtained from Sigma-Aldrich Chemical Co. and used as received.

**Compound III-4. dl-(3S, 4S)-1-Benzyl-2,4,5-triphenyl-4,5-dihydro-1*H*-imidazole-4-carboxylic acid**



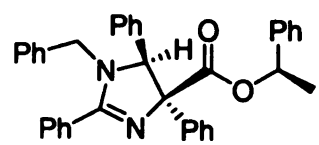

A solution of benzaldehyde (0.6 g, 5.7 mmol), benzylamine (0.61 g, 5.7 mmol) in dry dichloromethane (120 mL) was refluxed under nitrogen for 2 h. 2,4-Diphenyl-4*H*-oxazolin-5-one (1.35 g, 5.7 mmol) and chlorotrimethylsilane (0.8 g, 7.4 mmol) were added and the mixture was refluxed under nitrogen for 6 h and then stirred overnight at room temperature. The product was purified by silica-gel column chromatography with 1:5 ethanol/ ethyl acetate to afford 2.1 g of the product in 65 % yield as an off-white solid. M. P.: decomposes at 153-155 °C. $^1$H NMR (300 MHz) ($CDCl_3$): d 3.8 (1H, d, $J$ = 15.6Hz), 4.62 (1H, d, $J$ = 15.6Hz), 4.98 (1H, s), 6.58 (2H, d, $J$ = 8.1Hz), 7.05-7.65 (16H, m), 7.9 (2H, d, $J$ = 7.2 Hz); $^{13}$C NMR (75 MHz) ($CDCl_3$): d 48.3, 75.6, 79.1, 123.1, 125.7, 126.7, 127.3, 127.4, 127.9, 128.1, 128.2, 128.8, 128.9, 129, 129.3, 132.9, 133.8, 136, 143.1, 164.8, 168.1; IR (neat): 3400cm-1 (very broad), 1738 cm$^{-1}$; HRMS (EI): calculated for $C_{29}H_{24}N_2O_2$ $[(M-H)-CO_2]^+$ 387.1526 and observed $[(M-H)-CO_2]^+$ 387.1539.

**Compound III-4a and III-4b.**

In a flame dried flask, under nitrogen, a well-stirred suspension of 1-Benzyl-2, 4, 5-triphenyl-4, 5-dihydro-1H-imidazole-4-carboxylic acid (0.1g, 0.18 mmol) in dry methylene chloride (20 ml) was maintained at 0 °C. To this mixture was added EDCI.HCl (36 mg, 0.18 mmol), followed by DMAP (22 mg, 0.18 mmol) and stirred for 20 minutes.

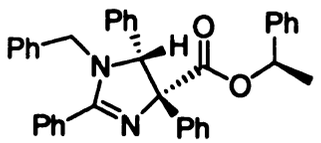
(R)-(+)-1-Phenyl-ethanol (43.5 mg, 2 eq., 0.36 mmol) was added and mixture stirred overnight at room temperature. The reaction mixtures was washed, 2N HCl (2 x 20 ml), saturated sodium bicarbonate (2 x 20 ml), and then with brine (20ml). The organic layer dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by column silica-gel chromatography using 40 % ether / hexane mixture. These were characterized and subjected to hydrolysis under reflux with 2N NaOH/EtOH separately. The reaction mixtures were cooled and acidified to pH=3 and extracted with $CHCl_3$. The organic layer was dried and evaporated *in vacuo* to obtain the two enantiomers in 44%yield.

**S, S, R-1-Benzyl-2, 4, 5-triphenyl-4, 5-dihydro-1H-imidazole-4-carboxylic acid 1-phenyl-ethyl ester, III-9**



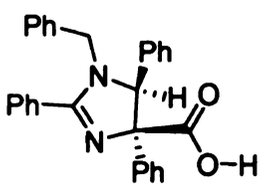

$[\alpha]_D$= +19.45°, c = 1, $CHCl_3$; $^1$H NMR (300MHz), $CDCl_3$: δ: 0.873 (d, *J* = 6.6, 3H), 3.73 (d, *J* = 15 Hz, 1H), 4.56 (d, *J* =15 Hz,1H), 4.93 (s, 1H), 5.23 (q, *J*= 6.5, 1H), 6.73-7.72 (m, 25H); $^{13}$C NMR (74.4 MHz) $CDCl_3$: 21.80, 48.53, 72.77, 73.26, 82.32, 125.73, 126.75, 127.23, 127.30, 127.38, 127.94, 128.26, 128.37, 128.54, 128.58, 128.74, 130.29, 130.34, 136.47, 137.69, 141.37, 143.58, 165.38, 169.79; IR($CHCl_3$): 3584.2, 3063.4, 3032.5, 2926.4, 2855.0, 1736.1, 1595.3, 1495.0, 1412.1, 1064.8 $cm^{-1}$ EIMS: m/z = 537.2 (M+H), 387.3 (M-COOCH(Me)Ph). HRMS (FAB+) ($C_{37}H_{33}O_2N_2$)(M+H) 537.2542, found, 537.2542.

**R, R, R -1-Benzyl-2, 4, 5-triphenyl-4, 5-dihydro-1H-imidazole-4-carboxylic acid 1-phenyl-ethyl ester, III-10**



$[\alpha]_D$= -7.77 °, c = 1, $CHCl_3$; $^1H$ NMR (300MHz), $CDCl_3$: δ 1.205 (d, *J* = 6.6, 3H), 3.73 (d, *J* = 15 Hz, 1H), 4.56 (d, *J* =15 Hz,1H), 4.95 (s, 1H), 5.23 (q, *J*= 6.5, 1H), 6.80-7.81 (m, 25H) $^{13}C$ NMR (74.4 MHz) $CDCl_3$: 21.75, 48.50, 73.42, 73.49, 82.87, 126.01, 126.87, 127.14,127.29, 127.35, 127.95,127.99, 128.13, 128.20, 128.40, 128.57, 128.75, 130.23, 36.72, 137.28, 141.00, 143.95,165.50, 169.94; IR($CHCl_3$): 3584.2, 3063.4, 3032.5, 2924.5, 2855.0, 1736.2, 1595.3, 1495.0, 1412.1, 1064.8 $cm^{-1}$ EIMS: m/z = 537.3(M+H), 387.3 (M-COOCH (Me) Ph) HRMS (FAB+) ($C_{37}H_{33}O_2N_2$) (M+H)537.2542, found, 537.2542.

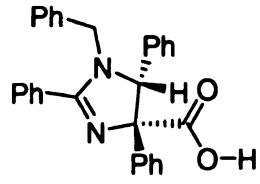
**(4S, 5S) -1-benzyl-4, 5-dihydro-2, 4, 5-triphenyl-1H-imidazole-4-carboxylic acid, III-4a**



M. P. decomposed at 148-149 °C. $[\alpha]_D$= -47.58 °, c = 1, $CHCl_3$; $^1H$ NMR (300MHz), $CDCl_3$: δ 3.96 (d, *J* = 15 Hz, 1H), 4.81 (d, *J* =15 Hz,1H), 5.05 (s, 1H), 6.61 (2H, d, *J* = 8.1Hz), 7.09-7.92 (16H, m), 7.90 (2H, d, *J* = 7.2 Hz), 11.42(s, 1H) $^{13}C$ NMR (74.4 MHz) $CDCl_3$: 49.37, 75.33, 76.06, 121.29, 125.93, 127.65, 129.24, 129.40, 129.42, 129.86, 130.02, 130.14, 130.76, 131.69, 131.82, 132.18, 134.62, 138.54, 166.69, 167.71;

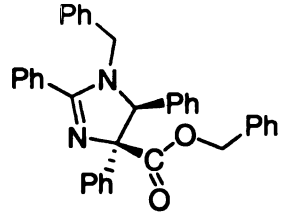
IR($CHCl_3$): 3584.2, 3063.4, 2924.5, 1743.9, 1558.9, 1456.4, 1253.9 $cm^{-1}$ EIMS: m/z = 387.3 (M-COOH) HRMS (FAB+) ($C_{29}H_{25}O_2N_2$) (M+H) 433.1916, found, 433.1914.

**(4R, 5R) -1-benzyl-4, 5-dihydro-2, 4, 5-triphenyl-1H-imidazole-4-carboxylic acid (III-4b.HCl)**




M.P. decomposed at 147-148°C. $[\alpha]_D$= +47.87°, c = 1, $CHCl_3$; M. P. decomposed at 147-149 °C. $^1$H NMR (300MHz), $CDCl_3$: δ 4.02 (d, *J* = 15 Hz, 1H), 4.84 (d, *J* =15 Hz,1H), 5.09 (s, 1H), 6.61 (2H, d, *J* = 8.1Hz), 7.12-7.77 (16H, m), 7.97 (2H, d, *J* = 7.2 Hz) $^{13}$C NMR (74.4 MHz) $CDCl_3$: 48.76, 74.96, 77.03, 121.45, 125.90, 126.57, 127.15, 128.29, 128.48, 128.57, 128.81, 129.12, 129.39, 129.56, 129.65, 130.09, 132.47, 132.84, 133.01, 134.14, 139.96, 165.84, 168.34; IR($CHCl_3$): 3584.2, 3063.4, 2924.5, 1741.9, 1556.7, 1456.4, 1221.1 $cm^{-1}$ EI MS :m/z = 386.3 (M-COOH-H) HRMS (FAB+) ($C_{29}H_{25}O_2N_2$) (M+H) 433.1916, found, 433.1915. LRMS (FAB -) 35, 37.

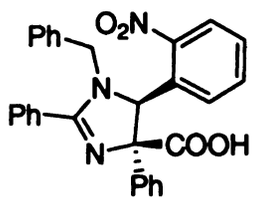
**(4S, 5S)-benzyl 1-benzyl-4,5-dihydro-2,4,5-triphenyl-1H-imidazole-4-carboxylate, III-11**




In a flame dried flask, under nitrogen, a well-stirred suspension of III-4 (0.056g, 0.13 mmol) in dry methylene chloride (20 ml) was maintained at 0 °C. To this mixture was added EDCI.HCl (27.5 mg, 0.14 mmol), followed by DMAP (17.5 mg, 0.14 mmol) and

stirred for 20 minutes. Benzyl alcohol (15.4 mg, 0.14 mmol) was added and mixture stirred overnight at room temperature. The reaction mixtures was washed, 2N HCl (2 x 20 ml), saturated sodium bicarbonate (2 x 20 ml), and then with brine (20ml). The organic layer dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by column silica-gel chromatography using 40 % ether / hexane mixture. A low melting waxy solid was obtained in 70% yield (47.1 mg); ( $^{1}H$ NMR (300MHz), $CDCl_3$: δ: 3.59(m, 2H), 3.91(d, 1H, *J*=9.3 Hz), 4.26(s, br, 1H), 4.66(s, 1H), 4.78(d, 1H, *J*=9.3 Hz), 6.74-7.91(m, 20H); $^{13}C$ NMR (74.4 MHz) $CDCl_3$: 29.69, 49.00, 66.76, 73.93, 125.68, 127.06, 127.55, 127.72, 127.82, 128.53, 128.59, 128.74, 128.85, 129.08, 130.91, 166.74; IR($CHCl_3$): 3245.3, 2924.5, 1593.4, 1390.9 $cm^{-1}$. EIMS: m/z =387.2(M-$CH_2OH$). HRMS (EI): *m/z* calcd. for $C_{29}H_{26}N_2O$ [M + H], 522.2307; found, 522.2386.

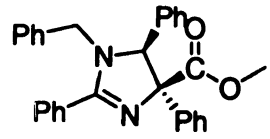
**(4S, 5S)-1-benzyl-4, 5-dihydro-5-(2-nitrophenyl)-2,4-diphenyl-1H-imidazole-4-carboxylic acid, III-12**




A solution of o-nitrobenzaldehyde (1 g, 6.6 mmol), benzylamine (0.7 g, 6.6 mmol) in benzene (100 ml) was refluxed under nitrogen for 3 h. Benzene was removed *in vacuo* and $CH_2Cl_2$ (anhydrous) was added 2,4- diphenyl-4*H* oxazolin-5-one (1.3 g, 5.48 mmol) and chlorotrimethylsilane (0.8 g, 7.12 mmol) were added and the mixture was refluxed under nitrogen for 8 h and then stirred at r.t for 4 h. The reaction mixture was evaporated to dryness under vacuum. The product was purified on a silica plug under 30% MeOH in

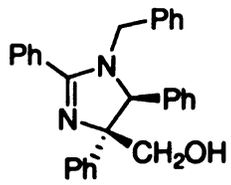
EtOAc; yield: 15% (0.39 g) $^1$H NMR (300MHz), $CDCl_3$: δ: 3.95(d, 1H, *J*=9.0 Hz), 4.73(d, 1H, *J*=9.0 Hz), 6.15(s, 1H), 6.63-8.00(m, 19H); IR($CHCl_3$): 2924.5, 1740.01, 1635.8, 1527.3, 1352.3 cm$^{-1}$. EIMS: m/z =387.2(M-COOMe). HRMS (EI): *m/z* calcd. for $C_{29}H_{23}N_3O_4$ [M+H],(FAB+) 478.1770; found, 478.1770. (FAB-) no 35, 37. Anal. Calcd. ($C_{29}H_{23}N_3O_4$) C, H, N.

**(4S, 5S)-methyl 1-benzyl-4, 5-dihydro-2,4,5-triphenyl-1H-imidazole-4-carboxylate, III-13**



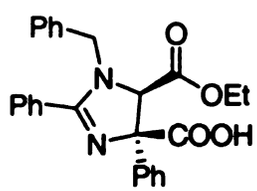

A solution of III-4 (0.48 g, 1.11 mmol) in MeOH (8 ml) was maintained at 0°C. $TMSCHN_2$ (2 M solution in ether) was added dropwise (0.87 ml, 1.73 mmol) and the reaction mixture was stirred for 5h. The reaction mixture was concentrated *in vacuo* and the product was subjected to column chromatography (gradient, first run 1:1 $Et_2O$: hexane) and then by eluting with ether. The only product ($R_f$ = 0.55) was isolated. The fractions were collected and and dried *in vacuo* to give a low melting solid in 70% yield (0.36 g); $^1$H NMR (300MHz), $CDCl_3$: δ: 3.18(s, 3H), 3.84(d, 1H, *J*=9.6 Hz), 4.62(d, 1H, *J*=9.6 Hz), 4.93(s, 1H), 6.70-7.75(m, 20H); $^{13}$C NMR (125 MHz) $CDCl_3$: 48.69, 51.86, 73.83, 82.51, 126.73, 127.09, 127.35, 127.45, 128.03, 128.33, 128.38, 128.45, 128.59, 128.87, 130.43, 136.43, 137.72, 143.85, 165.58, 171.30; IR($CHCl_3$): 3063.4, 2947.6, 1734.2, 1595.3, 1448.73 cm$^{-1}$. EIMS: m/z =387.2(M-COOMe). HRMS (EI): *m/z* calcd. for $C_{30}H_{27}N_2O_2$ [M +], 446.2073; found, 446.2072.

**(4S,5S)-1-benzyl-4,5-dihydro-2,4,5-triphenyl-1H-imidazol-4-yl)methanol, III-14**



A well stirred solution of $LiAlH_4$ (51.3 mg, 1.38 mmol) in THF (6 ml) was maintained at 0°C for 10'. Added a solution of III-13 (0.42 g, 1.16 mmol) in THF (2 ml) dropwise and allowed to sitr for 30'. The reaction was followed up with TLC. The reaction mixture was diluted with an excess of EtOAc (50 ml) and quenched with ice cold sat. $NH_4Cl$ (**caution**: $NH_4Cl$ solution kept at 0 °C for about 30 min.; and should be added with extreme care; highly exothermic reaction and the reaction mixture should be at 0 °C). Extracted the product from the organic layer and evaporated the organic layer *in vacuo.* Purified the product on column chromatography by eluting with ether. Colorless low melting solid was obtained in 62% yield (0.24 g); $^1H$ NMR (300MHz), $CDCl_3$: δ: 3.59 (m, 2H), 3.91 (d, 1H, *J*=9.3 Hz), 4.26 (s, br, 1H), 4.66 (s, 1H), 4.78 (d, 1H, *J*=9.3 Hz), 6.74-7.91 (m, 20H); $^{13}C$ NMR (74.4 MHz) $CDCl_3$: 29.69, 49.00, 66.76, 73.93, 125.68, 127.06, 127.55, 127.72, 127.82, 128.53, 128.59, 128.74, 128.85, 129.08, 130.91, 166.74; IR($CHCl_3$): 3245.3, 2924.5, 1593.4, 1390.9 $cm^{-1}$. EIMS: m/z =387.2(M-$CH_2OH$). HRMS (EI): *m/z* calcd. for $C_{29}H_{26}N_2O$ [M + H], 419.2122; found, 419.2122.

**(4S, 5R)-5-(ethoxycarbonyl)-1-benzyl-4,5-dihydro-2,4-diphenyl-1H-imidazole-4-carboxylic acid, III-15**



A solution of ethyl glyoxalate (0.95 g, 9.34 mmol) as a 50% solution in toluene (1.03 g $ml^{-1}$), benzylamine (1 g, 9.34 mmol) in anhydrous $CH_2Cl_2$ (50 ml) was stirred over $Na_2SO_4$ (anhydrous, 5g) under nitrogen for 5 h. 2,4- diphenyl-4*H* oxazolin-5-one (0.5 g, 2.11 mmol) and chlorotrimethylsilane (0.29 g, 2.74 mmol) were added and the mixture was refluxed under nitrogen for 8 h and then stirred at r.t for 4 h. The reaction mixture was evaporated to dryness under vacuum. The product was precipitated by MeOH–ether (1:7); yield: 65% $^1H$ NMR (300MHz), $CDCl_3$ + 5 drops CD3OD: δ: 0.74(m, 3H), 3.66(m, 2H), 4.32(d, 1H, *J* = 11.7 Hz), 4.81(d, 1H, *J* = 11.7 Hz), 5.73(s, 1H), 7.13-7.69(m, 15H); $^{13}C$ NMR (125 MHz) $CDCl_3$: 13.15, 50.49, 61.88, 70.76, 77.2, 122.1, 126.9, 127.6, 128.13, 128.53, 128.9, 129.24, 129.46, 132.07, 133.53, 134.23, 165.64, 166.79, 170.08 IR($CHCl_3$): 2924.5, 1738.1.01, 1639.7 $cm^{-1}$. EIMS: m/z =383.3(M-COOH).

**Chemicals and antibodies**

Camptothecin and TNF-α were purchased from Sigma. ALLN was purchased from Calbiochem. Parthenolide and monoclonal antibody for supershifts for p65 were purchased from Santacruz Biotechnology. HeLa cells were a kind gift from Prof. John LaPres, Dept. of Biochemistry and Molecular Biology, Michigan State University.

**Cell culture.** CEM cells ((CCRF-CEM); Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 Media (Gibco-BRL, Rockville, MD) supplemented with 10 % fetal bovine serum, penicillin (614 ηg/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. HT1080 and Hela cells were cultured in DMEM supplemented with 10 % fetal bovine serum, penicillin (614 ηg/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. SL89 cells were cultured in MEM supplemented

with 10 % fetal bovine serum, penicillin (614 ηg/mL), streptomycin (10 μg/mL) at 37°C, 5% $CO_2$.

**Optimization of concentrations of chemotherapeutic agents for apoptosis.**

Cells were cultured as described above. DMSO was used as the vector for all drugs and added in the control experiments. Cell cultures were treated with various concentrations of the Camptothecin or cis-platin and allowed to incubate at 37°C, 5% $CO_2$ At time points 0, 6, 12, 24, 48 h, an aliquote of the cell culture was transferred to a 96-well plate and mixed with an equal volume of Apo-ONE™ Homogenous Caspase- 3/7 assay reagent (Promega Corporation). The contents of the plate were gently mixed and allowed to incubate for 30 minutes at room temperature. The fluorescence of each well was then measured on a Molecular Imager FXPro at 532 nm. All reported data is the average of two independent experiments unless otherwise indicated.

Cells were cultured as described above. DMSO was used as the vector for all drugs and added in the control experiments. Cell cultures were treated with various concentrations of the cytotoxic agent (taxol, vincristine, vinblastine, camptothecin, topotecan, etoposide, daunomycin, doxorubicin or cis-platin) and allowed to incubate at 37°C, 5% $CO_2$ At time points 0, 6, 12, 24, 48 h, an aliquote (100 μl) of the cell culture was transferred to a 96-well plate and mixed with 20 μl of MTS assay reagent (Promega Corporation). The contents of the plate were gently mixed and allowed to incubate for 60 minutes at 37 °C. The fluorescence of each well was then measured on a Biorad microplate reader at 490 nm. All reported data is the average of two independent experiments unless otherwise indicated.

**Measurement of apoptosis using caspase 3/7 assay**

CEM cells (CCRF-CEM); Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 Media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ηg/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. DMSO was used as the vector for all drugs and was added in the control experiments. Cell cultures were treated with 0.1 μM, 0.01 μM or 1 nM of the imidazoline **III-4** and allowed to incubate at 37°C, 5% $CO_2$ atmosphere for 30 minutes. Camptothecin was added at concentrations 50 μM, 1μM, 0.3 μM, 0.1 μM, 0.03 μM, 0.01 μM, 3 nM, 1 nM or 0.3 nM and further incubated. An aliquote was transferred to a 96-well plate at various time points 0, 6, 12, 24, 48 hours and mixed with an equal volume of Apo-ONE™ Homogenous Caspase- 3/7 assay (Promega Corporation) reagent. The contents of the plate were gently mixed and allowed to incubate for 1 hour. The fluorescence of each well was then measured on a Molecular Imager FX Pro at 532 nm. All reported data is the average of two independent experiments unless otherwise indicated. These were then put into GraphPad prism and $CC_{95}$ were calculated using the equation for the sigmoidal curve for variable slope. Data was normalized between maximum and minimum cell death relative to the respective control sets.

**EMSA assay for NF-κB – DNA binding**

EMSA assay for NF-κB-DNA binding: CEM cells (CCRF-CEM; Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ng/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. Cells (1.6 X $10^6$ cells/mL) were subsequently treated with various concentrations of the compound **III-4** for 30 min. at

37°C and 5% $CO_2$ followed by TNF-α (0.4 pg) or camptothecin (Sigma-Aldrich)(1.0 μM) stimulation for an additional 30 minutes or 2 hours, respectively. The cells were harvested by centrifugation, washed in ice cold PBS and the nuclear extracts were prepared as previously described.[97] The protein concentration of the extracts was determined according to the Bradford method (1976) with BioRad reagents. Nuclear extracts (20 μg total protein) were incubated for 20 minutes at room temperature with a double stranded Cy3 labeled NF-κB consensus oligonucleotide, 5'-AGTTGAGGGGACTTTC CCAGGC-3' (0.16 pmol). The binding mixture (25 μL) contained 10 mM HEPES-NaOH pH 7.9, 4 mM tris-HCl, pH 7.9, 6.0 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.3 mg/mL bovine serum albumin and 1 μg of poly (dI.dC). The mixture was loaded on a 4% polyacrylamide gel prepared in 1X tris borate/EDTA buffer and was electrophoresed at 200 V for 20 minutes. After electrophoresis the gel was analyzed using a phosphorimager (Biorad FX plus) for detection of the NF-κB-DNA binding.

**Inhibition of translocation with p65-ELISA assay**

The quantity of p65/p50 heterodimer that has translocated into the nucleus was measured using a NF-κB p65 sandwich ELISA assay (Imgenex Corp.). CEM cells were grown to 1.6 X $10^6$ cells/mL and were subsequently treated with various concentrations of the compound **III-4** for 30 min. at 37°C and 5% $CO_2$ followed by TNF-α (0.4 pg) or camptothecin (1.0 μM) stimulation for an additional 30 minutes or 2h, respectively. The cells were harvested after 30 minutes and nuclear extracts were prepared as previously described by Dignam and coworkers.[97] The NF-κB p65 sandwich ELISA assay was used to monitor and quantify p65 translocation into the nucleus according to the

manufacturer's protocol. Results were read on Biorad's Microplate Benchmark reader at 405 nM. PDTC is reported to be an inhibitor of NF-κB translocation and our data confirmed that it inhibited NF-κB translocation at concentrations ranging from 100 nM to 5.0 μM (data not shown).

**Inhibition of I-κBα degradation in CEM cells**

CEM cells (CCRF-CEM; Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 Media (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum, penicillin (614 ηg/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. Cells (1.6 X $10^6$ cells/mL) were subsequently treated with various concentrations of the compound **III-4**, ALLN (50 μM) , parthenolide (50 μM) or vehicle (DMSO) for 1h at 37°C and 5% $CO_2$ followed by TNF-α (0.4 pg) or camptothecin (Sigma-Aldrich)(2.0 μM) stimulation for an additional 4 minutes or 1 h, respectively. The cells were harvested by centrifugation, washed in ice cold PBS and the cytoplasmic extracts were prepared as described by the manufacturer's protocol. The protein concentration of the extracts was determined according to the Method of Bradford (1976) with BioRad's Bradford reagent. Extracts were diluted and subjected to the I-κBα (pS32) and I-κBα (total) ELISA (Biosource International, CA) to measure and quantify the effect of imidazoline on the phosphorylation status of I-κBα. Results were read on Biorad's Microplate Benchmark reader at 450 nM.

**Inhibition of NF-κB mediated transcription in HeLa cells**

Transient transfections were performed using lipofectamine 2000. Briefly, 0.8 μg of plasmid DNA, was combined with lipofectamine 2000 (Invitrogen) (1.5 μL). The mixture was incubated at room temperature for 20' and mixed with the cells without

serum. Cells were transfected for 5 hours at 37°C in 5% $CO_2$. Cells were allowed to grow in complete medium for 15 hours in 5% $CO_2$. Various treatments with various concentrations of imidazoline **III-4**, parthenolide (50 μM) or ALLN (50 μM) were performed for 1 hour followed by a treatment with TNF-α (10 ng/μL) or camptothecin (3 μM) for 5 hours or 10.5 hours, respectively. Cells were washed with 1 X PBS. Washed cells were assayed for luciferase production using the manufacturer's protocol for both luciferase as well as renilla luciferase (co-reporter) using the Dual-Glo reporter assay (Promega). The results were read on a Veritas microplate luminometer (Turner Biosystems, CA) as relative light units. Values from the luciferase activity were normalized to those with renilla luciferase.

**Luciferase enzyme assay.**

Purified Luciferase, isolated from *Photinus pyralis* (Promega,WI), was resuspended in BSA (1 mg/mL). The enzyme was incubated with various concentrations of the imidazoline or DMSO for 1 hour at 0°C on wet ice. An aliquote of the samples was then assayed on the luminometer as described above.

***In vitro* kinase Assay**: Compound **III-4** was tested in vitro against the kinases indicated by Upstate, UK using a Kinase Profiler Assay according to the manufacturer's protocol. Briefly, in a final volume of 25 μl, the kinase was incubated with the desired buffer and the required polypeptide substrate, in presence of 10 mM magnesium acetate and $\gamma$-$^{33}$P-ATP (10 μM). After incubation for 40 minutes at room temperature, the reaction was stopped by the addition of 3% $H_3PO_4$ (5 μl). A 10 μl of the reaction was then spotted on a P30 filtermat and washed 3X in 75 mM $H_3PO_4$ and finally in methanol. Samples were then dried and signals counted on a scintillation counter.

**FACS analysis.**

CEM cells were cultured as described above. Cell cultures were treated with 0.1 $\mu$M of the imidazoline **III-4**, **III-4a** or **III-4b** and allowed to incubate at 37°C, 5% $CO_2$ atmosphere for 30 minutes. Camptothecin was added at 0.01 $\mu$M and further incubated for 12 and 48 h to mark early and late apoptotic events. Cells were then harvested, washed in 1XPBS. Cells were stained with Yo-Pro1 and propidium iodide to mark the apoptotic cells. These were then analyzed on a BD Biosciences Vantage SE (San Jose, CA) flow cytometer using a 530/30 bandpass filter for Yo-Pro1 and 660/20 (bandpass filter) for PI respectively.

**Fluorescence microscopy.**

CEM cells (CCRF-CEM) were cultured as described above. Cell cultures were treated with 0.1 $\mu$M of the imidazoline **III-4**, **III-4a** or **III-4b** and allowed to incubate at 37°C, 5% $CO_2$ atmosphere for 30 minutes. Camptothecin was added at 0.01 $\mu$M and further incubated for 12 and 48 h to mark early and late apoptotic events. Cells were then harvested, stained with Yo-Pro-1 and visualized under a fluorescence microscope Olympus CK2 using a BP480-550C filter.

## J. References.


1. (a) Green, D. R. *Cell* **1998**, *94*, 695-698. (b) Hengartner, M.O. *Nature* **2000**, *407*, 770-776.

2. Tomaselli, K. J. *Drug Development Res.* **2001**, *52*, 505-507.

3. Schmitt, C. A.; Lowe, S. W. *J. Pathol.* **1999**, *187*, 127-37. (b) Fesik, S.W. *Cell*, **2000**, *103*, 273-282.

4. Thompson, C. B. *Science* 1995, *267*, 1456-1462.

5. Hanahan, D.; Weinberg, R. A. *Cell* **2000**, *100*, 57-70.

6. Johnstone, R. W.; Ruefli, A. A.; Lowe, S. W. *Cell* **2002**, *108*, 153-164.

7. Lowe, S. W.; Lin, A. W. *Carcinogenesis* **2000**, 21, 485-495.

8. Foye, W. O. *Cancer Chemotherapeutic Agents* (Washington: ACS) **1995**.

9. Pommier, Y.; Pourquier, P.; Fan, Y.; Strumberg, D. *Biochim. Biophys. Acta* **1998**, *1400*, 83-105.

10. (a) Wall, M. E. *Med. Res. Rev.* **1998**, *18*, 299-314.(b) Thomas, C. J., Rahier, N. J. ; Hecht, S. M. *Bioorg. Med. Chem.* **2004**, *12*, 1585-604. (b) Macdonald, T. L.; Labroli, M. A.; Tepe, J. J. In *Comprehensive natural products chemistry* **1999**, Volume 7, E.T. Kool, ed. (Amsterdam: Elsevier-North Holland), pp. 593-614. (d) Hsiang, Y. H.; Hertzberg, R.; Hecht, S.; Liu, L. F. *J. Biol. Chem.* **1985**, *260*, 14873–14878.

11. Pourquier, P.; Pommier, Y. *Adv. Cancer Res.* **2001**, *80*, 189-216.

12. (a) Wang, D.; Lippard, S. J. *Nat. Rev. Drug Discov.* **2005**, *4*, 307-20. (b) Barnes, K. R. ; Lippard, S. J. *Met. Ions. Biol. Syst.* **2004**, *42*, 143-77.

13. (a) Cohen, S. M. ; Lippard, S. J. *Prog. Nucleic Acid Res. Mol. Biol.* **2001**, *67*, 93-130. (b) Jamieson, E. R.; Lippard, S. J. *Chem. Rev.* **1999**, *99*, 2467-2498. (c) Sedletska, Y., Giraud-Panis, M. J.; Malinge, J. M. *Curr. Med. Chem. Anti-Canc. Agents* **2005**, *5*, 251-265.

14. Wong, E.; Giandomenico, C. M. *Chem. Rev.* **1999**, *99*, 2451-2466.

15. Rixe, O.; Ortuzar, W.; Alvarez, M.; Parker, R.; Reed, E.; Paull, K.; Fojo, T. *Biochem. Pharmacol.* **1996**, *52*, 1855-1865.

16. (a) Orrenius, S.; Zhivotovsky, B.; Nicotera, P. *Nat. Rev. Mol. Cell Biol.*

**2003**, *4*, 552-65. (b) Yuan, Z.- M.; Utsugisawa, T.; Huang, Y.; Ishiko, T.; Nakada, S.; Kharbanda, S.; Weichselbaum, R.; Kufe, D. *J. Biol. Chem.* **1997**, *272*, 23485-8. (c) Vivanco, I.; Sawyers, C. L. *Nat. Rev. Cancer* **2002**, *2*, 489-501.

17. Avendaño, C.; Menéndez, J. C. *Curr. Med. Chem.* **2002**, *9*, 159-193.

18. Lowe, S. W.; Lin, A. W. *Carcinogenesis* **2000**, *21*, 485-95.

19. (a) Wang, C. Y.; Mayo, M. W.; Korneluk, R. G.; Goeddel, D. V. ; Baldwin A, S., Jr. *Science* **1998**, *281*, 1680-1683. (b) Wang, C. Y.; Mayo, M. W.; Baldwin, A. S., Jr. *Science* **1996**, *274*, 784-7. (c) Huang, T. T.; Wuerzberger-Davis, S. M.; Seufzer, B. J.; Shumway, S. D.; Kurama, T.; Boothman, D. A.; Miyamoto, S. *J. Biol. Chem.* **2000**, *275*, 9501-9509. (d) Piret, B.; Piette, J. *Nucleic Acids Res.* **1996**, *24*, 4242-4248. (e)Xu, Y.; Villalona-Calero, M. A. *Ann. Oncol.* **2002**, *13*, 1841-1851. (f) Bottero, V.; Busuttil, V.; Loubat, A.; Magne, N.; Fischel, J. L.; Milano, G.; Peyron, J. F. *Cancer Res.* **2001**, *61*, 7785-7791.

20. (a) Bellamy, William T. *Ann. Rev. Pharmacol. Toxicol.* **1996**, *36*, 161-83. (b) Teicher, B.A.; Ara, G.; Herbst, R.; Palombella, V. J.; Adams, J. *Clin. Cancer Res.* **1999**, *5*,2638-2645.

21. (a) Drewe, J.; Gutmann, H.; Fricker, G.; Torok, M.; Beglinger, C.; Huwyler, J. *Biochem. Pharmacol.* **1999**, *57*,1147-1152.(b) Schmidtke, G.; Holzhutter, H. G.; Bogyo, M.; Kairies, N.; Groll, M.; de Giuli, R.; Emch, S.; Groettrup, M. *J. Biol. Chem.* **1999**, *274*, 35734-35740.

22. Fekete, M. R.; McBride, W. H.; Pajonk, F. *BMC cancer* **2005**, *5*, 114.

23. (a) Amsterdam, A.; Pitzer, F.; Baumeister, W. *Proc. Natl. Acad. Sci. U S A* **1993**, *90*, 99-103.(b) Kumatori, A.; Tanaka, K.; Inamura, N.; Sone, S.; Ogura, T.; Matsumoto, T; Tachikawa, T.; Shin, S.; Ichihara, A. *Proc. Natl. Acad. Sci. U S A* **1990**, *87*, 7071-7075.

24. (a) Qiu, J. H.; Asai, A.; Chi, S.; Saito, N.; Hamada, H.; Kirino, T. *J. Neurosci.* **2000**, *20*, 259-265.(b) Pasquini, L. A.; Besio, M. M.; Adamo, A. M.; Pasquini, J. M.; Soto, E. F. *J. Neurosci. Res.* **2000**, *59*, 601-611.

25. Bold, R. J.; Virudachalam, S.; McConkey, D. J. *J. Surg. Res.* **2001**, *100*, 11-17.

26. Pajonk, F.; Pajonk, K.; McBride, W. *Int. J. Radiat. Oncol. Biol. Phys.* **2000**, *47*, 1025-1032.

27. (a) Cusack, J. C.; Liu, R.; Baldwin, A. S. *Drug Resist. Update* **1999**, *2*, 271-273. (b) Weldon, C. B.; Burow, M. E.; Rolfe, K. W.; Clayton, J. L. Jaffe; B. M.; Beckman, B. S. *Surgery* **2001**, *130*, 143-50. (c) Pomerantz, J. L. ; Baltimore, D. *Nature* **2000**, *406*, 26-29. (d) Boland, M. P. *Biochem. Soc. Trans.* **2001**, *29*, 674-8. (e) Pommier, Y.; Sordet, O.; Antony, S.; Hayward, R. L.; Kohn, K. W. *Oncogene* **2004**, *23*, 2934-49. (f) Mayo, M.W.; Baldwin, A. S. *Biochim. Biophys. Acta* **2000**, *1470*, M55-62.

28. (a) Carson, J. P.; Zhang, N.; Frampton, G. M.; Gerry, N. P.; Lenburg, M. E.; Christman, M. F. *Cancer Res.* **2004**, *64*, 2096-2104. (b) Zhou, Y.; Gwadry, F. G.; Reinhold, W. C.; Miller, L. D.; Smith, L. H.; Scherf, U.; Liu, E. T.; Kohn, K. W.; Pommier, Y.; Weinstein, J. N. *Cancer Res.* **2002**, *62*, 1688-1695.

29. Makin, G.; Hickman, J. A. *Cell Tissue Res.* **2000**, *301*, 143-152.

30. Nesterenko, V.; Putt, K. S.; Hergenrother, P. J. *J. Am. Chem. Soc.* **2003**, *125*, 14672-14673.

31. Nicholson, D. W. *Nature* **2000**, *407*, 810-816.

32. Hayakawa, Y.; Kim, J. W.; Adachi, H.; Shin-ya, K.; Fujita, K. -I.; Seto, H. *J. Am. Chem. Soc.* **1998**, *120*, 3524-3525.

33. Mitsiades, N.; Mitsiades, C. S.; Richardson, P. G.; Poulaki, V.; Tai, Y. T.; Chauhan, D.; Fanourakis, G.; Gu, X.; Bailey, C.; Joseph, M.; Libermann, T. A.; Schlossman, R.; Munshi, N. C.; Hideshima, T.; Anderson, K. C. *Blood* **2003**, *101*, 2377-2380.

34. Weaver K. D.; Yeyeodu, S.; Cusack J. C.; Jr.; Baldwin A. S., Jr.; Ewend M. G. *J. Neuro-Oncol.* **2003**, *61*, 187-196.

35. Chen, L.; Agrawal, S.; Zhou, W.; Zhang, R.; Chen, J. *Proc. Natl .Acad. Sci. USA* **1998**, *95*, 195-200.

36. (a) Sharma, V.; Lansdell, T. A.; Peddibhotla, S.; Tepe, J. J. *Chem. Biol.* **2004**, *11*, 1689-1699. (b) Tepe, J. J.; Sharma, V.; Lansdell, T.; Peddibhotla, S.; Michaels, S.; Brown, D. M. **2003**, Abstracts of Papers, 225th ACS National Meeting, New Orleans, LA, United States, March 23-27, 2003.

37. Baeuerle, P. A.; Henkel, T. *Annu. Rev. Immunol.* **1994**, *12*, 141-179.

38. Baldwin, A. S., Jr. *Annu. Rev. Immunol.* **1996**, *14*, 649-683.

39. Perkins, N. D. *Int. J. Biochem. Cell Biol.* **1997**, *29*, 1433-1448.

40. Webster, G. A.; Perkins, N. D. *Mol. Cell Biol.* **1999**, *19*, 3485-3495.

41. Das, K. C.; White, C. W. *J. Biol. Chem.* **1997**, *272*, 14914-14920.

42. Baldwin, A. S. *J. Clin. Invest.* **2001**, *107*, 241-246.

43. Hideshima, T.; Chauhan, D.; Richardson, P.; Mitsiades, C.; Mitsiades, N.; Hayashi, T.; Munshi, N.; Dang, L.; Castro, A.; Palombella, V.; Adams, J.; Anderson, K. C. *J. Biol. Chem.* **2002**, *277*, 16639-16647.

44. Um, J. H.; Kang, C. D.; Lee, B. G.; Kim, D. W.; Chung, B. S.; Kim, S. H. *Oncogene* **2001**, *20*, 6048-6056.

45. Arlt, A.; Vorndamm, J.; Breitenbroich, M.; Folsch, U. R.; Kalthoff, H.; Schmidt, W. E.; Schafer, H. *Oncogene* **2001**, *20*, 859-868.

46. Liu, S.; Yu, Y.; Zhang, M.; Wang, W.; Cao, X. *J. Immunol.* **2001**, *166*, 5407-5415.

47. Kim, J. Y.; Lee, S.; Hwangbo, B.; Lee, C. T.; Kim, Y. W.; Han, S. K.; Shim, Y. S.; Yoo, C. G. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 140-146.

48. Lin, Y. C.; Brown, K.; Siebenlist, U. *Proc. Natl. Acad. Sci. U S A* **1995**, *92*, 552-556.

49. Karin, M.; Lin, A. *Nat. Immunol.* **2002**, *3*, 221-227.

50. Zhong, H.; SuYang, H.; Erdjument-Bromage, H.; Tempst, P.; Ghosh, S. *Cell* **1997**, *89*, 413-424.

51. Sizemore, N.; Leung, S.; Stark, G. R. *Mol. Cell Biol.* **1999**, *19*, 4798-4805.

52. Madrid, L. V.; Wang, C. Y.; Guttridge, D. C.; Schottelius, A. J.; Baldwin, A. S.; Jr.; Mayo, M. W. *Mol. Cell Biol.* **2000**, *20*, 1626-1638.

53. Wu, M. X.; Ao, Z.; Prasad, K.V.; Wu, R.; Schlossman, S. F. *Science* **1988**, *281*, 998-1001.

54. Levine, A. J. *Cell*, **1997**, *88*, 323-331.

55. Boland, M. P. *Biochem. Soc. Trans.* **2001**, *29*, 674-678.

56. Cusack, J. C., Jr.; Liu, R.; Baldwin, A. S., Jr. *Cancer Res.* **2000**, *60*, 2323-2330.

57. Guttridge, D. C.; Albanese, C.; Reuther, J. Y.; Pestell, R.G., Baldwin, A. S., Jr. *Mol. Cell Biol.* **1999**, *19*, 5785-5799.

58. Jones, D. R.; Broad, R. M.; Madrid, L. V.; Baldwin, A. S., Jr.; Mayo, M. W. *Ann. Thorac Surg.* **2000**, *70*, 930-936; discussion 936-937.

59. Cusack, J. C., Jr.; Liu, R.; Houston, M.; Abendroth, K.; Elliott, P. J.; Adams, J.; Baldwin, A. S., Jr. *Cancer Res.* **2001**, *61*, 3535-3540.

60. Balkwill, F.; Mantovani, A. *Lancet* **2001**, *357*, 539-545.

61. Slaviero, K. A.; Clarke, S. J.; Rivory, L. P. *Lancet Oncol.* **2003**, *4*, 224-32.

62. Karin, M; Greten, F. R. *Nature Rev. Immunol.* **2005**, *5*, 749-759.

63. Bharti, A. C.; Aggarwal, B. B. *Ann. NY Acad. Sci.* **2002**, *973*, 392–395.

64. Greten, F.; Eckmann, L.; Greten, T.; Park, J. ; Li, Z.; Egan, L.; Kagnoff, M.; Karin M. *Cell*, **2004**, *118*, 285-296.

65. Bonizzi, G. ; Karin, M. *Trends Immunol.* **2004**, *25*, 280–288.

66. Maeda, S.; Kamata, H.; Luo, J. L.; Leffert, H. ; Karin, M. *Cell* **2005**, *121*, 977–990.

67. Moore,R. J.; Owens, D .M.; Stamp, G.; Arnott, C.; Burke, F. ; East, N.; Holdsworth, H.; Turner, L.; Rollins, B.; Pasparakis, M.; Kollias, G. ; Balkwill, F. *Nature Med.* **1999**, *5*, 828–831.

68. (a) Coussens, L. M. ; Werb, Z. *Nature* **2002**, *420*, 860–867. (b) Pollard, J. W. *Nature Rev. Cancer* **2004**, *4*, 71–78. (c) Karin, M.; Cao, Y.; Greten, F. R. ; Li, Z. W. *Nature Rev. Cancer* **2002**, *2*, 301–310.

69. Dunn, G. P.; Old, L. J. ;Schreiber, R. D. *Immunity* **2004**, *21*, 137–148.

70. Dranoff, G. *Nature Rev. Cancer* **2004**, *4*, 11–22.

71. Koehne, C. H.; Dubois, R. N. *Semin. Oncol.* **2004**, *31*, 12-21.

72. Bresalier, R. S.; Sandler, R. S.; Quan, H.; Bolognese, J. A.; Oxenius, B.; Horgan, K.; Lines, C.; Riddell, R.; Morton, D.; Lanas, A.; Konstam, M.A.; Baron, J. A. *N. Engl. J. Med.* **2005**, *352*, 1092–1102.

73. Habraken, Y.; Piret, B.; Piette, J. *Biochemical Pharmacol.* **2001**, *62*, 603-616.

74. Epinat, J. C.; Gilmore, T. D. *Oncogene* **1999**, *18*, 6896-6909.

75. Cuzzocrea, S.; Chatterjee, P. K.; Mazzon, E.; Dugo, L.; Serraino, I.; Britti, D.; Mazzullo, G.; Caputi, A. P.; Thiemermann, C. *Br. J. Pharmacol.* **2002**, *135*, 496-510.

76. (a) Roshak, A.; Jackson, J. R.; Chabot-Fletcher, M.; Marshall, L. A. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 955-961.(b) Breton, J. J.; Chabot-Fletcher, M. C. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 459-466. (c) Sharma, S.; Tepe, J. J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4319-4321.(d) Meijer, L.; Thunnissen, A. M.; White, A. W.; Garnier, M.; Nikolic, M.; Tsai, L. H.; Walter, J.; Cleverley, K. E.; Salinas, P. C.;Wu, Y. Z.; Biernat, J.; Mandelkow, E. M.; Kim, S. H.; Pettit, G. R. *Chem. Biol.* **2000**, *7*, 51-63.(e)Wan, Y.; Hur, W.; Cho, C. Y.; Liu, Y.; Adrian, F. J.; Lozach, O.; Bach, S.; Mayer, T.; Fabbro, D.; Meijer, L.; Gray, N. S. *Chem. Biol.* **2004**, *11*, 247-259.

77. Kishore, N.; Sommers, C.; Mathialagan, S.; Guzova, J.; Yao, M.; Hauser, S.; Huynh, K.; Bonar, S.; Mielke, C.; Albee, L.; Weier, R.; Graneto, M.; Hanau, C.; Perry, T.; Tripp, C.S. *J. Biol. Chem.* **2003**, *278*, 32861-32871.

78. (a)Grisham, M. B.; Palombella, V. J.; Elliott, P. J.; Conner, E. M.; Brand, S.; Wong, H. L.; Pien, C.; Mazzola, L. M.; Destree, A.; Parent, L.; Adams, J. *Methods Enzymol.* **1999**, *300*, 345-363. (b)Nemeth, Z. H.; Wong, H. R.; Odoms, K.; Deitch, E. A.; Szabo, C.; Vizi, E. S.; Hasko, G. *Mol. Pharmacol.* **2004**, *65*, 342-349.(c) Fenteany, G.; Standaert, R. F.; Soongyu Choi, W. S.; Corey, E. J.; Schreiber, S. L. *Science* **1995**, *268*, 726.

79. Teicher, B. A.; Ara, G.; Herbst, R.; Palombella, V. J.; Adams, J. *Clin. Cancer Res.***1999**, *5*, 2638-2645.

80. (a)Kwok, B. H.; Koh, B.; Ndubuisi, M. I.; Elofsson, M.; Crews, C. M. *Chem Biol* **2001**, *8*, 759-766.(b) Hehner, S. P.; Hofmann, T. G.; Droge, W.; Schmitz, M. L. *J. Immunol.* **1999**, *163*, 5617-5623.

81. Kane, R. C.; Bross, P. F.; Farrell, A. T.; Pazdur, R. *The Oncologist* **2003**, *8*, 508-513.

82. Shah, S. A.; Potter, M. W.; McDade, T. P.;, Ricciardi, R.; Perugini, R. A.; Elliott, P. J.; Adams, J.; Callery, M. P. *J. Cell. Biochem.* **2001**, *82*, 110-122.

83. Ma, M. H.; Yang, H. H.; Parker, K.; Manyak, S.; Friedman, J. M.; Altamirano, C.; Wu, Z. Q.; Borad, M. J.; Frantzen, M.; Roussos, E.; Neeser, J.; Mikail, A.; Adams, J.; Sjak-Shie, N.; Vescio, R. A.; Berenson, J. R. *Clin. Cancer Res.* **2003**, 9, 1136-1144.

84. (a) Peddibhotla, S.; Tepe, J. J. *Synthesis* **2003**, *9*, 1433-1440.(b) Peddibhotla, S.; Jayakumar, S.; Tepe, J. J. *Org. Lett.* **2002**, *4*, 3533-3535.(c)

85. (a) Rutgeerts, P.; D'Haens, G.; Targan, S.; Vasiliauskas, E.; Hanauer, S. B.; Present, D. H.; Mayer, L.; Van Hogezand, R. A.;Braakman, T.; DeWoody, K. L.; Schaible, T. F.; Van Deventer, S. J. H. *Gastroenterology* **1999**, *117*, 761-769.(b) Lee, J. C.; Laydon, J. T.; McDonnell, P. C.; Gallagher, T. F.;Kumar, S.; Green, D.; McNulty, D.; Blumenthal, M. J.; Heys, J. R.; Landvatter, S. W.; Strickler, J. E.; McLaughlin, M. M.;Siemens, I. R.; Fisher, S. M.; Livi, G. P.; White, J. R.; Adams, J. L.; Young, P. R. *Nature* **1994**, *372*, 739-745.(c) Boehm, J. C.; Adams, J. L. *Exp. Opin. Ther. Pat.* **2000**, *10*, 25-37. (d) Tong, L.; Pav, S.; White, D. M.; Rogers, S.; Crane, K. M.; Cywin, C. L.; Brown, M. L.; Pargellis, C. A. *Nat. Struct.Biol.* **1997**, *4*, 311-316.

86. (a) Regunathan, S.; Feinstein, D. L.; Reis, D. J. *Ann. N.Y. Acad. Sci.* **1999**, *881*, 410-419. (b) Houghton, A. K.; Westlund, K.N. *NeuroReport* **1996**, *7*, 1497-1501.(c)Laufer, S. A; Wagner, G. K. *J. Med. Chem.***2002**, *45*, 2733-2740.

87. Lansiaux, A.; Facompre, M.; Wattez, N.; Hildebrand, M. P.; Bal, C.; Demarquay, D.; Lavergne, O.; Bigg, D. C.; Bailly, C. *Mol. Pharmacol.* **2001**, *60*, 450-461.

88. (a)Nicholson, D. W.; Thornberry, N. A. *Science* **2003**, *299*, 214-215. (b) Nicholson, D. W.; Thornberry, N. A. *Trends Biochem. Sci.* **1997**, *22*, 299-306.(c) Thornberry, N. A. *Chem. Biol.* **1998**, *5*, R97-103.

89. (a) Luo, J. L.; Kamata, H.; Karin, M. *J. Clin. Invest.* **2005**, *115*, 2625-32. (b) Levkau, B.; Scatena, M.; Giachelli, C. M.; Ross, R.; Raines, E.W. *Nat. Cell Biol.* **1999**, *1*, 227–233. (c) Tang, G.; Yang, J.; Minemoto, Y.; Lin, A. *Mol. Cell.* **2001**, *8*, 1005–1016. (d) Reuther, J. Y.; Baldwin, A. S., Jr. *J. Biol. Chem.* **1999**, *274*, 20664–20670.

90. Johnson, N.; Ng, T. T. C.; Parkin, J. M. *Leukemia Res.* **1997**, *21*, 961-972.

91. Delhase, M.; Hayakawa, M.; Chen, Y.; Karin, M. *Science* **1999**, *284*, 309-313.

92. Karin, M. *Oncogene* **1999**, *18*, 6867-6874.

93. Sakurai, H.; Chiba, H.; Miyoshi, H. Sugita, T.; Toriumi, W. *J. Biol. Chem.* **1999**, *274*, 30353-30356.

94. Thanos, D.; Maniatis, T. *Cell* **1995**, *80*, 529-532.

95. Tozawa, K.; Okamoto, T.; Hayashi, Y.; Sasaki, S.; Kawai, N.; Kohri, K. *Urological Res.* **2002**, *30*, 53-58.

96. Schow, S. R.; Joly, A. *Cell Immunol.* **1997**, *175*, 199-202.

97. Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. *Nucleic Acids Res.***1983**, *11*, 1475-1489.

98. (a) An, J.; Sun, Y.; Fisher, M.; Rettig, M. B. *Leukemia* **2004**, *18*, 1699-704. (b) Dong, Q. G.; Sclabas, G. M.; Fujioka, S.; Schmidt, C.; Peng, B.; Wu, T.; Tsao, M.- S.; Evans, D. B; Abbruzzese; J. L.; McDonnell, T. J.; Chiao, P. J. *Oncogene*, **2002**, *21*, 6510-6519.

99. (a) Haupt, S.; Berger, M.; Goldberg, Z.; Haupt, Y. *J. Cell. Sci.* **2003**, *116*, 4077-85. (b)Haupt, S.; Louria-Hayon, I.;Haupt, Y. *J. Cell. Biochem.* **2003**, *88*, 76-82.

100. Wadgaonkar, R.; Phelps, K. M.; Haque, Z.; Williams, A. J.; Silverman, E. S.; Collins, T. *J. Biol .Chem.* **1999**, *274*, 1879-1882.

101. Maki, C. G.; Huibregtse, J. M.; Howley, P. M. *Cancer Res.* **1996**, *56*, 2649–2654.

102. Haupt, Y.; Maya, R.; Kazaz, A.; Oren, M. *Nature* **1997**, *387*, 296–299.

103. Kastan, M.; Onyekwere, O.; Sidransky, D.; Vogelstein, B.; Craig, R. *Cancer Res.* **1991**, *51*, 6304–6311.

104. (a) Gazos Lopes, U.; Erhardt, P., Yao, R.; Cooper, G. *J. Biol. Chem.* **1997**, *272*,12893–12896. (b) Chang, Y. C.; Lee, Y. S.; Tejima, T.; Tanaka, K.; Omura, S.; Heintz, N. H.; Mitsui, Y.; Magae, J. *Cell Growth Differ.* **1998**, *9*, 79–84.

105. Nabiev,I.; Fleury, F.; Kudelina, I.; Pommier,Y.; Charton, F.; Riou,J. –F.; Alix, A. J. P.; Manfait, M. *Biochemical Pharmacol.* **1998**, *55*, 1163–1174.

106. Schena M., **2003**. Microarray Analysis. Wiley-Liss. (Hoboken, NJ), 630.

107. Cheung V.G.; Morley, M.; Aguilar, F.; Massimi, A.; Kucherlapati R.; Childs, G. *Nat. Genet.* **1999**, *21*(1 Suppl), 15-9.

108. Heller M. J. *Annu. Rev. Biomed. Eng.* **2002**, *4*, 129-53.

109. Jain K. K. *Pharmacogenomics* **2000**, *1*, 289-307.

110. Crowther D. J. *Curr. Opin. Pharmacol.* **2002**, *2*, 551-4.

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# STRATEGIC IMPROVEMENTS IN EFFICACY OF DNA DAMAGING CHEMOTHERAPEUTIC DRUGS THROUGH MODULATION OF SURVIVAL FACTORS.

VOLUME II

By

Vasudha Sharma

A DISSERTATION

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Michigan State University
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## CHAPTER IV

## APPLICATION OF *TRANS*-IMIDAZOLINES IN CHEMISTRY AND BIOLOGY

*There doesn't exist a category of science to which we can give the name applied science. There are science and the application of science, bound together as the fruit to the tree which bears it..... Louis Pasteur*

### A. Introduction

Present day organic and medicinal chemistry aim at the design and small scale synthesis of drug candidates and large scale production of molecules valuable as human therapeutic agents. With the rapid exploration of potential drug candidates via high throughput screening, the current challenge is the increasing demand for novel small molecules to satisfy and sustain the screening efforts. The search for molecules of therapeutic value depends on new leads discovered via a rationally designed synthesis or screening of libraries generated via combinatorial synthesis. With the progress achieved in the field of functional genomics and proteomics, more information about the structures and environment of the biomolecules is available. To keep pace with such formidable advance in biological research, the identification and optimization of new scaffolds with specificity towards their target constitutes the bottleneck in biological and medicinal chemistry. Thus, there is tremendous need to develop more methods for ligand optimization to illustrate the functions of various biomolecules.[1]

The completion of the Human Genome project has outlined a network of cellular processes. The nature and amount of proteins, and their interplay has created paramount interest in investigating intervention of these cellular processes by small molecules. While combinatorial chemistry was considered to be firmly integrated to the drug

discovery enterprise for lead identification and optimization, unfortunately, the predicted scope of combinatorial synthesis in terms of accessing 'drug-like molecules' did not reach its pinnacle and in fact certain libraries did not yield even a single target molecule.[2] Hence, the challenge in designing a focused library of 'drug-like' compounds still persists.

**B. Alternative approaches to the same problem**

Upon comparison between combinatorial libraries, natural products and drug molecules, Feher and Schmidt suggested that the properties of combinatorial libraries could be improved by incorporation of natural product like character.[3] Similar studies were also performed by Henkel *et al* and Schneider *et al* independently.[4] Hansske *et al* reported an approach biased on natural-product library called 'diversity modified natural scaffolds' (DYMONS) in which diverse substituents are introduced to natural product cores.[5] Bajorath described 'natural/synthetic hybrid libraries' known as 'MetaFocus (Metabolite Focused)' libraries.[6] Once a biologically active natural compound is identified, available similar compounds are examined for similar activity and all could be placed under one library. In the second phase, compound arrays similar to the initially identified natural product are prepared.

**i. Nature sets an example – natural-product guided synthesis**

Nature has already identified natural products as structures with biological relevance in a 'universe' of chemical structures.[7] Historically, 60% of cancer drugs and 75% of those for infectious diseases are provided by Nature.[8] This provides ample reason to make efforts to design libraries based on such 'natural-product like' scaffolds. However, Nature is fairly restrictive in exploiting the innumerable proteins and allows access to specific folds

and domains. This forms a matrix like grid with variations of scaffolds for individual sites on proteins, and deciphering all the matches still remains a challenge. One of the categorical hallmarks of natural products is their exquisite level of three dimensional sophistication. The diversity of ring skeletons, the presentation of the functionalities etc. are beyond the capacity of medicinal chemistry.

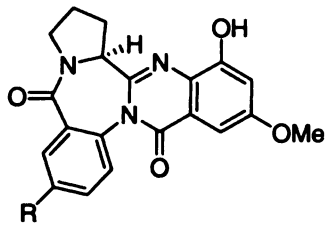
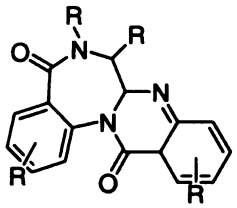
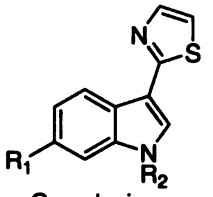
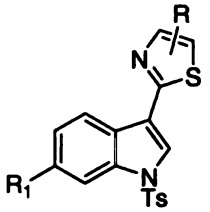
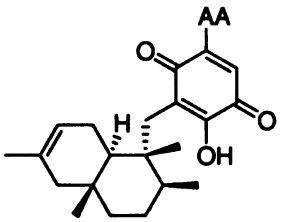
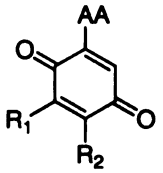
| Representative Natural products | Natural Product Library | Reference |
|---|---|---|
|  Circumdatin D, R=OMe Circumdatin E, R=H |  | 9 |
|  Camalexin |  | 10 |
|  Nakijiquinone A, AA=Gly |  | 11 |

**Figure IV-1.** Natural product like scaffolds.

With the plethora of emerging targets for drug discovery, natural product-like libraries serve as vehicles to probe biological activity. Figure IV-1 shows a few examples of

natural-product like scaffolds being pursued by various research groups. [9-11] Structure-activity relationships can be better understood on a pharmacologically rich platform which would enable us to identify new therapeutic targets.[8]

**ii) Diversity oriented synthesis.**

A promising method begins by synthesizing structurally complex and diverse small molecules in an efficient manner using diversity oriented synthesis. Diversity oriented synthesis as coined by Schreiber *et al* for chemical genetics aims at moderate sized libraries of compounds with huge three dimensional complexity, functionality, and diversity and thus these scaffolds can be regarded as 'natural-product like'.[12c, 14] Such scaffolds usually comprise of cyclic structures and a high degree of stereochemical content.

Diversity-oriented synthesis (DOS) strategically constructs libraries with stereochemical, skeletal and functional group diversity with the aim to create a broad distribution of compounds in chemical space.[12] Very often, modern drug discovery involves screening molecules for a known protein target or modulation of a biological pathway. While target oriented syntheses of small molecules are considered as focused libraries, sometimes, drug discovery process can also involve screening of compounds and their ability to modulate pathways, without a particular protein target. This latter case would be beneficial in evolving forward analysis to synthesize complex building blocks and diverse small molecules for a particular phenotype. An important aim of diversity oriented synthesis is to build a collection of molecules able to perturb a biological pathway, leading to target identification being modulated by small molecules. This is analogous to genetics where random mutations could be generated and then screened for

a phenotype. From the perspective of drug discovery, this approach can help in simultaneous identification of proteins which could serve as targets for therapeutic intervention (therapeutic target validation) and molecules that modulate functions of those therapeutic targets (chemical target validation).[13]

**iii) Where do small molecules fit in ?**

In contrast to one synthesis-one skeleton approach, DOS targets accessing both skeletal and stereochemical diversity to create a wide display of functionality as well as 3D space. Achieving such aims could facilitate the search for specific small molecule modulators for diverse biomolecules. Small-molecule scaffolds offer a handle to dissect biological pathways, identify targets and modulate their functions for potential therapeutic use.[14] Small molecules posses numerous advantages in the drug discovery process as compared with peptides (metabolically stable and bioavailable) and natural products (easier access, greater solubility and support for structure activity relationships). Accessing small molecules via DOS is beneficial in particular since one compound can be converted into multiple structurally unique scaffolds. Brummond *et al* have shown access to three different types of scaffolds starting from alkynyl allenes.[15]

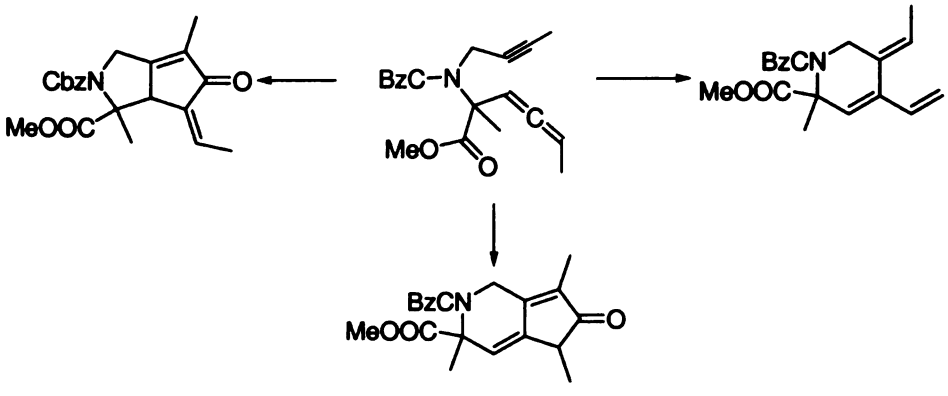



**Figure IV-2.** Accessing diverse scaffolds

Besides the scaffold and R-generating diversity, stereochemistry offers a third dimension for diversity in DOS. Control over stereochemical diversity in particular, increases the number of relative orientations of potential macromolecule-interacting elements in small molecules.

Hence, in order to access small molecules in chemical space that can overlap with the biological space with targets, suitably functionalized 'drug-like' privileged structures (which can bind to one or more targets) are required that have three dimensional rigidity and are accessed through high yielding, reliable clean reactions.

**C. Azlactones: similarity, diversity and complexity.**

One of the very important features in DOS is to use a substrate having the potential of diverse reactivity with different reagents and produce different skeletons. In our effort to synthesize biologically active small molecule heterocycles, we focused on azlactone (5-oxazolone) on account of its versatile nature. Azlactones were first discovered by Plöcl(1883), however these were named by Erlenmeyer in 1893.[16] Azlactones gained eminence and have been used as important synthons due to the ability to undergo racemization when exposed to nucleophiles such as alcohols, and other amino acids in peptide synthesis.[17, 18] Traditionally, the azlactone is synthesized by a dehydration reaction after activation of the carboxyl group of the N-acyl amino acid. The N-acyl amino acids are conveniently prepared via a Schotten-Baumann reaction on the commercially available amino acid or acylation of amino acid esters followed by hydrolysis under mild alkaline conditions.[18,19]

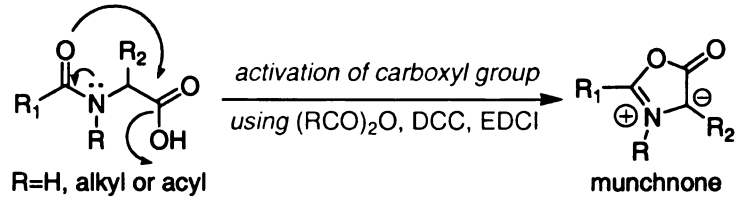



**Figure IV-3.** Cyclodehydration of *N*-acyl-α-aminoacids to azlactones/ münchnones.

The choice of the carboxyl group activator depends on the amino acid substrate and stability of the resulting azlactone. Use of $(CH_3CO)_2O$, $(CF_3CO)_2O$ often suffer of side reactions and instability of the azlactone to the acid generated in the reaction.[20, 21] Use of $Ac_2O$ and other acid anhydrides gives rise to münchnones (1,3 oxazolium oxides).[23] While DCC usually gives high yielding reaction, removal of the urea and isolation of pure azlactone from the reaction mixture may not be simple.[22] EDCI (water soluble carbodiimide salt) is free of either of these problems and affords the azlactone in very high yields and purity.[22]

The azlactone is an activated cyclic ester of the corresponding amino acid that can provide a variety of products via a nucleophilic attack or an electrophilic attack to give a $C_4$-alkylated product.

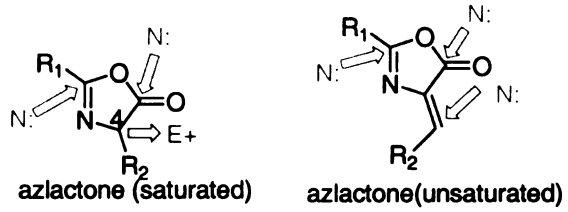



**Figure IV-4.** Nucleophilic and electrophilic sites on saturated and unsaturated azlactone.
N: Nucleophile; E+ Electrophile.

For example, due to acidic nature of $C_4$-H bond, monosubstituted azlactones can react with electrophiles. Reactions such as alkylation, arylation, acylation, Claisen rearrangement, Michael addition, addition of diazonium salts etc. have been well

documented.[23] Condensation between carbonyl groups and azlactone yields unsaturated azlactones where the exocyclic bond can undergo additional reactions with nucleophiles.[24] Further, reaction of alcohols or *N*-terminated peptides, with the ketene tautomer (less stable) provides access to α-amino acid and peptide derivatives, [25] and a [2+2] cycloaddition with imines yields peptide-based β-lactams.[26] Figure IV-5 illustrates a wide range of skeletal diversity that can be accessed via azlactone template as the substrate material.

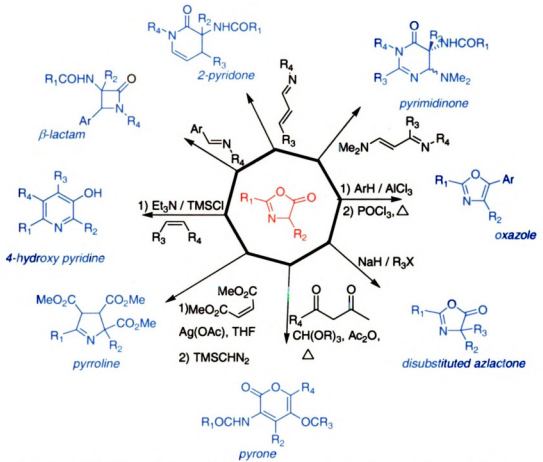



**Figure IV-5.** Generation of diverse heterocycles from azlactone template.

**D. 1, 3 dipolar cycloaddition on azlactone template**

Other reactions of azlactones include the highly versatile 1, 3 dipolar cycloadditions of *N*-alkylated azlactones (münchnones) with various dipolarophiles provide a general method for the synthesis of pharmacologically relevant aromatic heterocycles like pyrroles,[27] imidazoles,[28] oxazoles,[23c] pyrrolines,[29] and indole derivatives etc.[30] The azlactone, (which is in equilibrium with tautomeric ketene intermediate), undergoes a [2+2] cycloaddition reaction when heated with the imines (Figure IV-5) to yield β-lactams via a Staudinger-type reaction. It is now well known that under such conditions, 1, 3 dipolar cycloaddition does not occur. This could be attributed to two reasons, either mesoionic oxazolium oxide, which has the nitrogen atom unsubstituted, is not in reasonable concentration and/or the imine nitrogen is not nucleophilic enough. Hence formation of ketene takes predominance, which reacts readily with the imines to afford the β-lactam.[18, 31] In 1970, Huisgen had reported that a moderate equilibrium concentration of the mesoionic tautomer is essential for the 1, 3-dipolar cycloaddition leading to heterocycle formation (Figure IV-6).[23a]

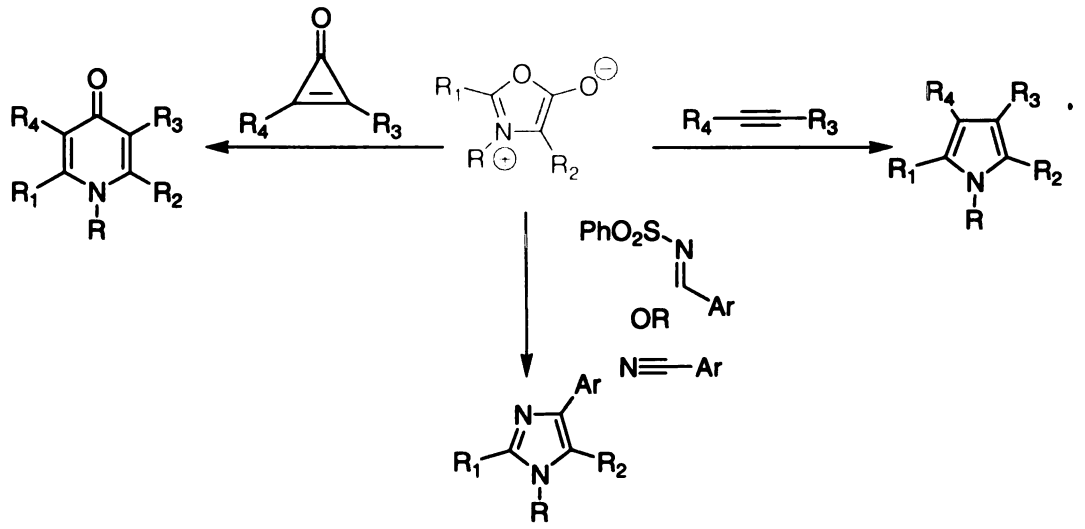



**Figure IV-6.** 1, 3 dipolar cycloaddition reaction on münchnones.

The azlactones are locked in the mesoionic form by N-methylation and are extremely reactive dipoles, which afford the heterocycles in very high yields. However, due to the harsh reaction conditions and the ease of aromatization under these standard conditions, the stereochemical information generated in the cycloaddition reaction is lost.

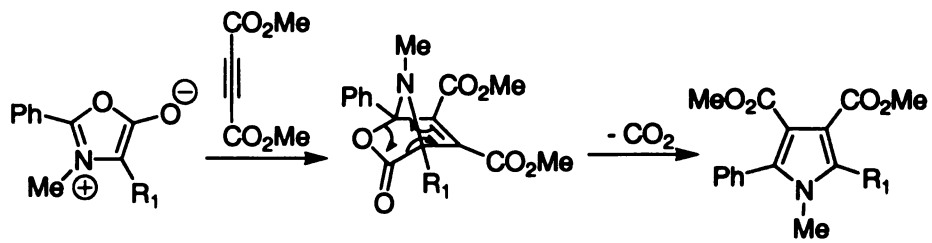
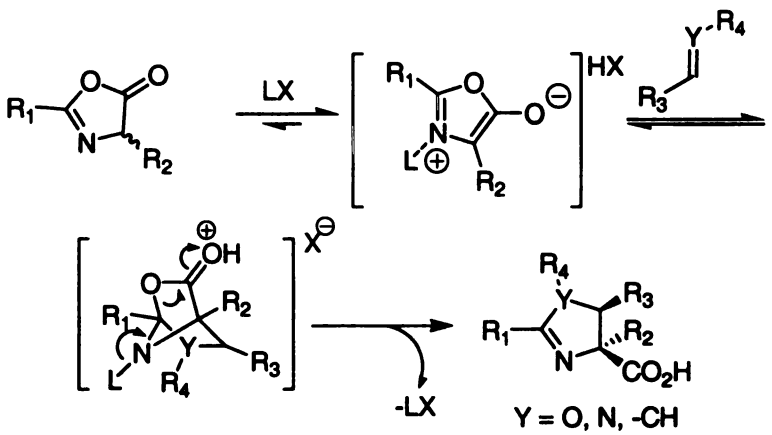



**Figure IV-7.** Synthesis of pyrroles from münchnones.

**E. Synthesis of novel dihydroheterocyclic scaffolds: harnessing the potential of Lewis acid mediated generation of münchnones**

As part of our program focused on generating structurally and stereochemically diverse heterocyclic scaffolds, other members in our laboratory (Satyamaheshwar Peddibhotla) have reported the use of a Lewis acid mediated generation of münchnones as a mild and stereoselective process to access highly substituted dihydroheterocyclic scaffolds such as imidazolines and $\Delta^1$-pyrrolines.[32-34]




**Scheme IV-1.** Synthesis of dihydroheterocycles from münchnones.

Control of the stereo-, regio-, and enantioselectivity of 1, 3-dipolar cycloadditions with Lewis acids is a relatively new area of research and has not been fully explored. Competition between an electrophilic dipolarophile and the dipole for the Lewis acid for instance can result in a slower reaction. Such binding interactions of 1, 3-dipoles to a Lewis acid are based on the typical presence of Lewis basic sites on the 1, 3-dipoles. Accordingly, Lewis acidic reagents may combine with the basic sites on certain 1, 3-dipoles, making relatively stable salt-like complexes. Optimization of the co-ordinating power of Lewis acids, the lability and exchangeability of these ligands must be done in order to increase rates of reactions.

**F. Chemical significance of imidazolines**

The most direct chemical utility of imidazolines has been their conversion to chiral diamines, [35] which are widely preferred group of ligands for asymmetric synthesis. MacMillan *et al* have reported a very impressive use of imidazolidinones, [36] as organocatalysts for highly enantioselective indole alkylations and Diels-Alder reactions. Furthermore, chiral 2-imidazolines have attracted considerable interest as templates for asymmetric synthesis, [37a] and as chiral ligands for asymmetric catalysis[37b, 37c] and have found wide application as potent N-heterocyclic carbene ligands that unlike the phosphines, do not dissociate from the transition metals and hence provide sturdy catalysts.[37d]

Efficient stereoselective syntheses of imidazolines has been very limited with respect to control of diastereochemical diversity and has primarily provided the *anti*-products.[38] Orru *et al* developed a novel multicomponent reaction for the combinatorial synthesis of 2-imidazolines presumably via a stepwise aldol synthesis of the isocyanide to the *in situ*

generated imine followed by ring closure[38a] They further increased the scope of their reaction to study the influence of more sterically demanding groups on amines and carbonyl compounds (including ketones). They also showed the catalytic use of AgOAc in facilitating the formation of 2-imidazolines from the isocyanides.[38b] Recently, Arndtsen *et al* developed a catalytic synthesis of münchnones, using Pd(0) metal catalyzed carbonylation of simple building blocks such as acid chlorides and imines.[38c] Casey *et al* have described a novel modular approach for the preparation of enantiopure 2-imidazolines from enantiopure β-amino alcohols.[38d]

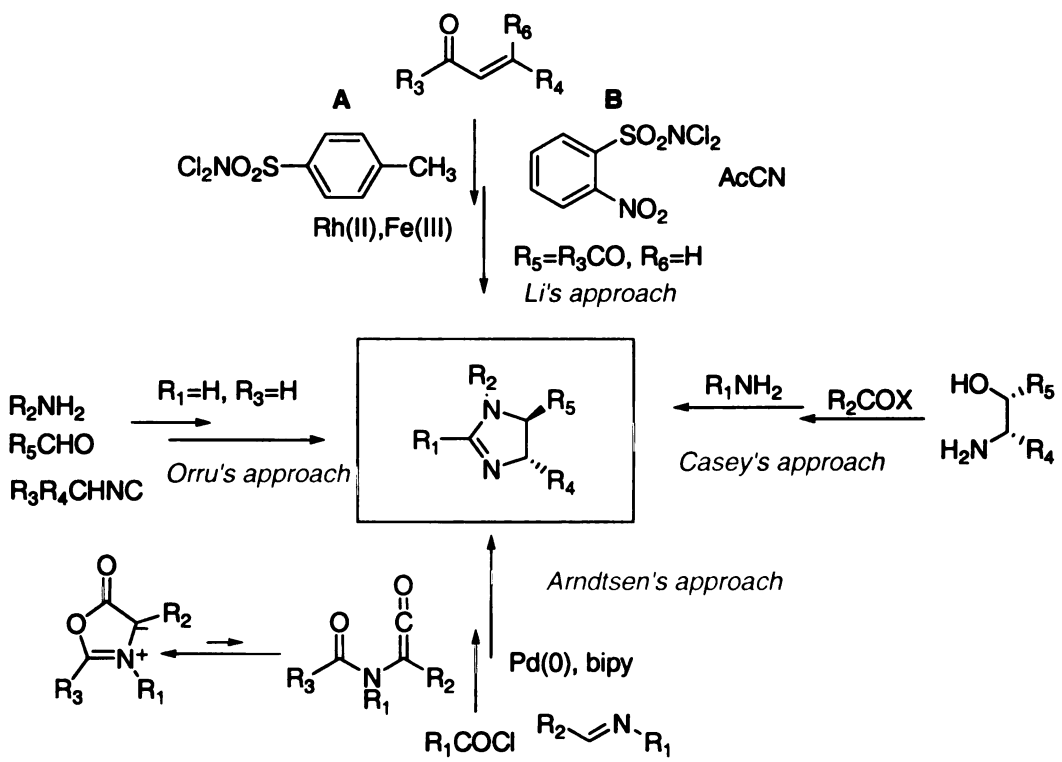
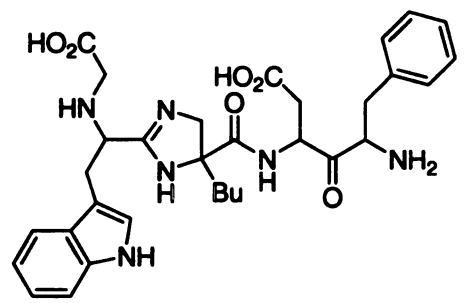



**Figure IV-8.** Stereoselective synthesis of anti-imidazolines.

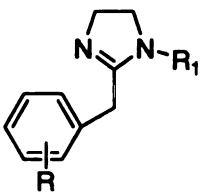
## G. Biological importance of imidazolines

The stereochemical diversity of small dihydroheterocyclic scaffolds presents novel territory in terms of both structure and biological activity. The imidazoline and their aromatic counter parts –imidazole scaffold, have attracted considerable interest owing to

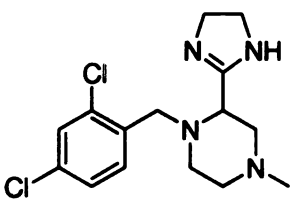
their biological activities.[38] Imidazolines exhibit important pharmacological activities[39a] in the inflammation pathways, including α-receptor stimulation, α-adrenergic inhibition, vasodepressor activity, anti-histaminic, and cholinomimetic activity. These exhibit anti-inflammatory,[39b] anti-nociceptive, antioxidant, immuno-modulating activities[39c] and are useful intermediates for the synthesis of molecules with pharmacological activities such as anti-hypercholesterolemic, anti-diabetic, anti-hypertensive, or anti-cancer activity.[39b,d] Cyclic ureas and thioureas are inhibit human immunodeficiency virus (HIV) protease and HIV replication.[39e] Further, 2-imidazolines serve as building blocks for the synthesis of pharmaceutically relevant molecules such as azapenams, dioxocyclams, and diazapinones.[35a] Also, 2-imidazolines (suitably functionalized) can be converted to 2, 3-diamino acids, which could be used in antibiotic synthesis.[8]



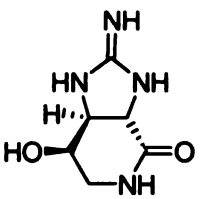

rodent CNS cholecystokinin inhibitor




seratonin receptor agonists




*in vivo* anti-hyperglycemic activity in rats




streptolidine lactam
core of streptothricin antibiotics

**Figure IV-9.** Biological activity of 2-imidazoline nucleus.

Imidazoline derivatives, such as midaglizole and deriglidole have been identified as promising anti-hyperglycemic agents[39c] and clonidine is a known antihypertensive agent, clonidine (Figure IV-10).[40]

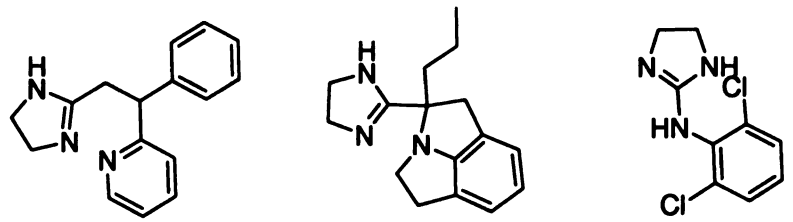



**Figure IV-10**. Examples of antihyperglycemic and anti-hypertensive agents.

Since the 1990s, presence of imidazoline (I) receptors in various tissues has gained consensus.[41] Studying the role of imidazolines in various physiological processes could provide invaluable information for treating efficaciously a variety of disorders such as hypertension, diabetes mellitus, gastric ulcer, endogenous depression, and stroke. Imidazoline derivatives have been studied as estrogen receptor modulators.[40] Imidazoline rings can substitute for an amino acid in a peptide, [42] making it less susceptible to proteolytic cleavage and hence improve the stability and therapeutic utility peptides.

Development of small-molecule inhibitors of non-enzyme protein-protein interactions has been a herculean task because binding pockets on protein surfaces involved are shallow and dynamic as compared to enzymes which have well defined and rigid binding pockets or active sites.[44] Moreover, the area of expanse for protein-protein interactions is large. As an exception to this idea, the crystal structure of MDM2 bound to a peptide from the transactivation domain of p53 has revealed that MDM2 possesses a relatively deep hydrophobic pocket that is filled primarily by three side chains from the helical region of the peptide.[45] This well-defined pocket on the MDM2 highlights the fact that small

molecules might block the interaction of MDM2 with p53. Cis-imidazoline analogs named Nutlins[46] are one such class of molecules where the imidazoline scaffold replaces the helical backbone of the p53 peptide and projects the three groups into the pockets normally occupied by $Phe^{19}$, $Trp^{23}$, and $Leu^{26}$ of p53.

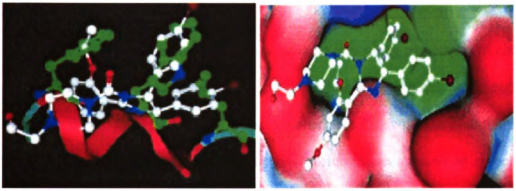


**Figure IV-11**. (A) Overlay of p53 and nutlin. (B) Nutlin in the deep pocket of MDM2.[46]

This binding of cis-imidazolines to MDM2 in the p53 binding pocket, leads to accumulation of p53 leading to cell cycle arrest, apoptosis, and growth inhibition of human tumor xenografts in nude mice.[46] This example has served as an archetype for development of other small molecule inhibitors of protein-protein interactions (SMPPI's).

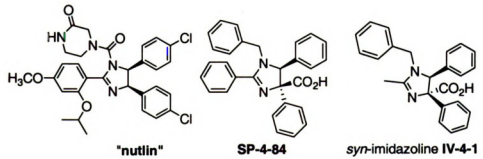


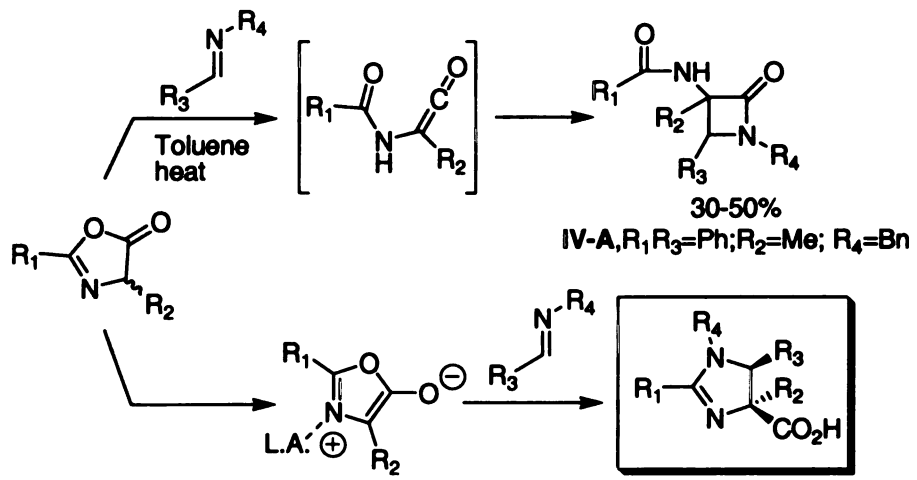

**Figure IV-12.** Structure of nutlin (*syn*-imidazoline), SP-4-84 (*anti*-imidazoline), **IV-4-1.**

The diverse bio-activity of anti-imidazolines is exemplified by the unique anticancer properties of *anti*-imidazolines (SP-4-84, described as **III-4** in this chapter III of this

thesis).[47] The *anti*-imidazoline SP-4-84 was found to be a drastic enhancer of the chemotherapeutic efficacy of anticancer agents through a possible modulation of the anti-apoptotic NF-κB signaling pathway.[47]

**H. Lewis acid mediated synthesis of *anti*-imidazolines via the generation of münchnones**

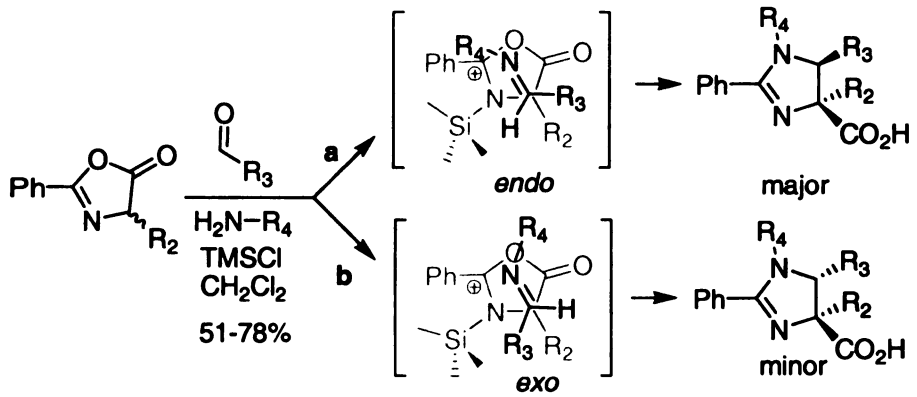
We previously reported the preparation of *anti*-imidazolines using trimethylsilyl chloride mediated 1, 3 dipolar cycloaddition on the azlactone template in good yields and high diastereoselectivity (Scheme IV-2).[32-34] We envisioned that a Lewis acid coordination to nitrogen of the azlactone would increase the equilibrium concentration of münchnone intermediate and mediate the azomethine ylide-imine cycloaddition reaction. The choice of the imine was important as unactivated or electron rich imines have rarely been used with münchnones before (tosylimines lead to enhanced aromatization).




**Scheme IV-2.** Lewis acid mediated generation of münchnone and cycloaddition

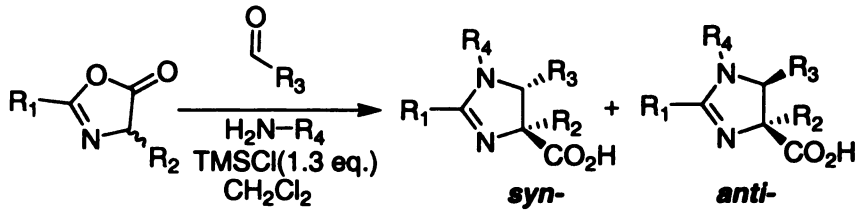
In case of 2-phenyl imidazolines, high diastereoselectivity was attributed to A (1, 3) strain, in the azlactone dipole, which prevents co-planarity of Ph, TMS and $R_2$ groups. In order to prevent steric interaction between the bulky silyl group of the azlactone and $R_3$

group of imine, the *endo* approach of the imine was favored resulting in *anti*-isomer as the major or sole product (Scheme IV-3, path a).[33]




**Scheme IV-3.** Proposed transition states for diastereoselective synthesis of *anti*-imidazolines.

However, by reducing the resonance stabilization of the carbocation in the dipole by changing the electronic nature of the $R_1$ substituent, a significant change in diastereoselectivity was observed (entries **1-4**, Table IV-1). Replacement of the phenyl group by a benzyl or a methyl moiety significantly eroded the stereoselectivity (entries **1-3**, Table IV-1). Further, switching the Ph and Me groups at $R_1$ and $R_2$ positions favored the formation of *syn*- imidazoline (with respect to $R_2$ and $R_3$) as the major product in 90:10 ratio (compare entry **1** and **4**, Table IV-1).



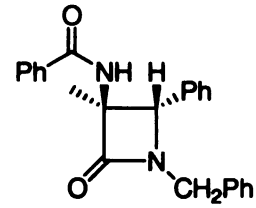

| Entry | Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Yield | syn:anti |
|---|---|---|---|---|---|---|---|
| 1 | IV-1-1 | Ph | Me | Ph | Bn | 75 | >5:95 |
| 2 | IV-2-1 | Bn | Me | Ph | Bn | 76 | 33:67 |
| 3 | IV-3-1 | Me | Me | Ph | Bn | 12* | 50:50 |
| 4 | IV-4-1 | Me | Ph | Ph | Bn | 72 | 90:10 |

*The azlactone for entry 2 is volatile resulting in low isolated yields

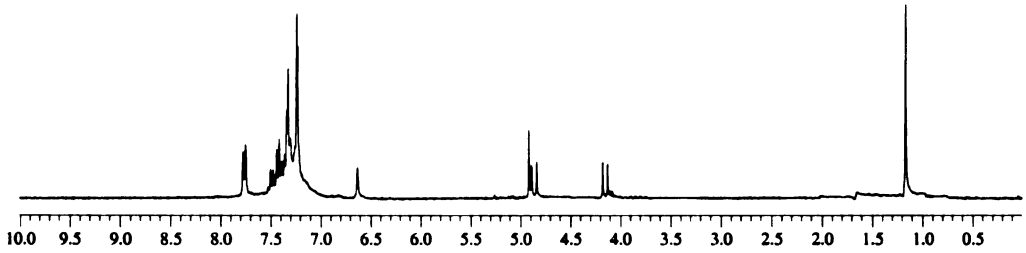
**Table IV-1.** Influence of $R_1$ group on diastereoselectivity.

Intrigued by the clear reversal of diastereoselectivity, we investigated the role of the $R_1$ and $R_2$ substituents on the azlactone template. In view of a potential diverse biological response resulting from the stereochemical diversity of these imidazoline scaffolds, we identified a trimethylsilyl chloride mediated substrate controlled 1,3-dipolar cycloaddition for the diastereoselective formation of *syn*- or *anti*-imidazolines.

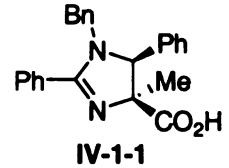
β-lactam



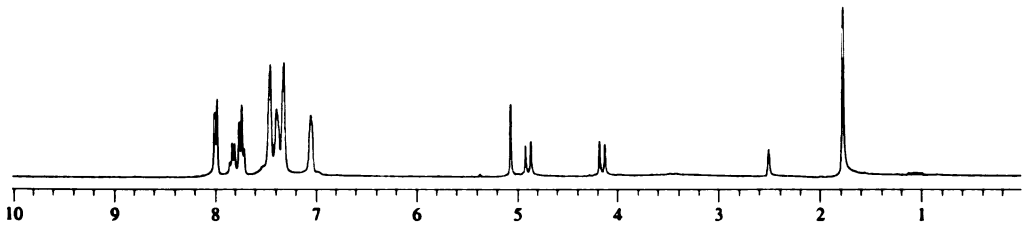

IV-A



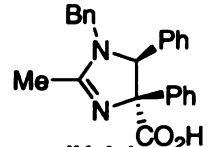

Trans-Imidazoline




IV-1-1




Cis-Imidazoline




IV-4-1

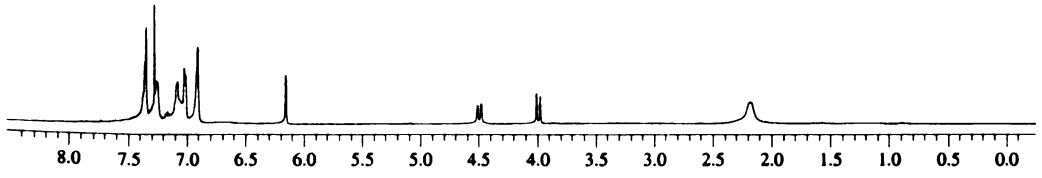
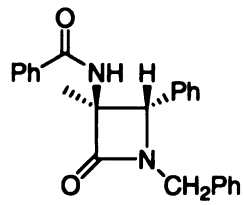
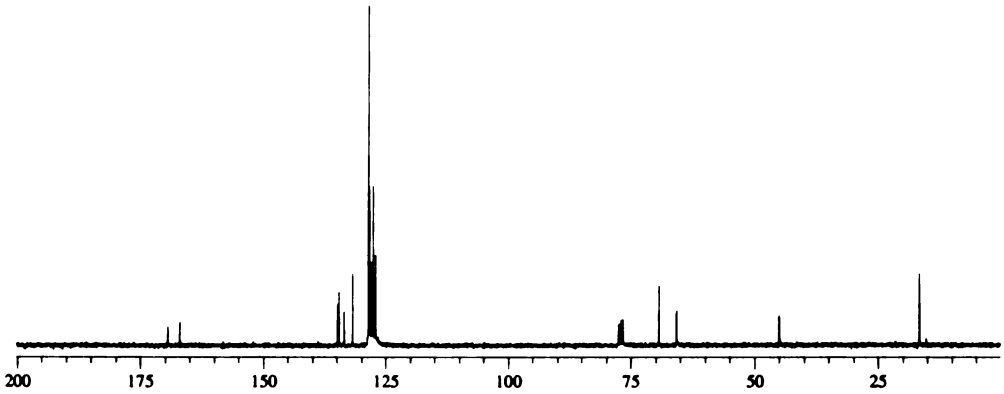



**Figure IV-13.** (a) $^{1}H$ spectra for a representative β-lactam (**IV-A**), trans-imidazoline (**IV-1-1**) and cis-imidazoline (**IV-4-1**).

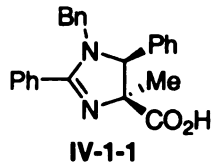
β-lactam



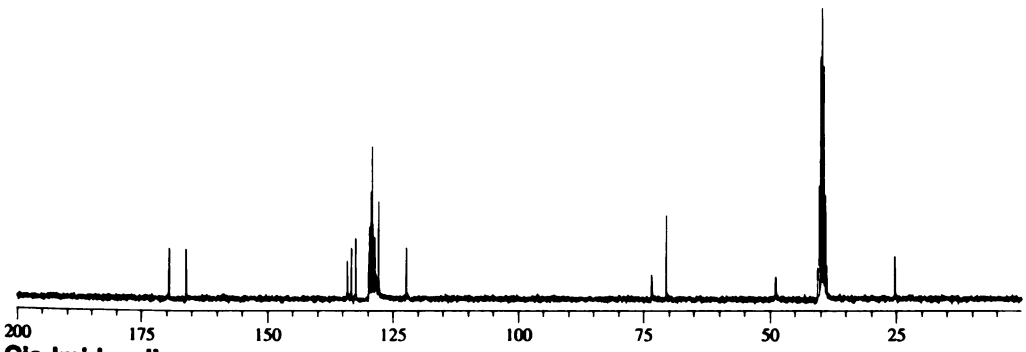

IV-A



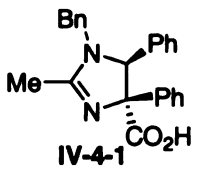

Trans-Imidazoline




IV-1-1




Cis-Imidazoline




IV-4-1

**Figure IV-13b.** $^{13}C$ spectra for a representative β-lactam (**IV-A**), trans-imidazoline (**IV-1-1**) and cis-imidazoline (**IV-4-1**).

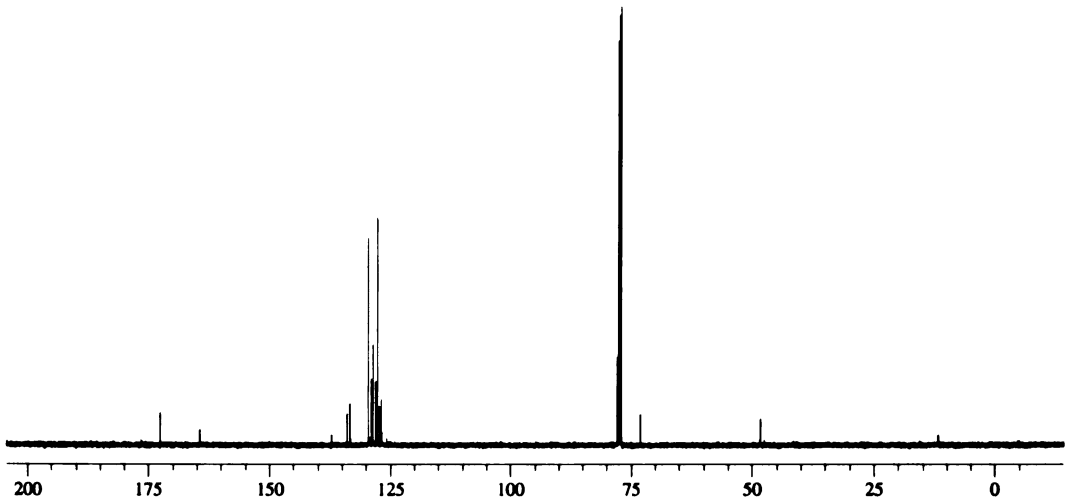



**Figure IV-13 b.** (contd.') $^{13}C$ spectra for a representative β-lactam (**IV-A**), trans-imidazoline (**IV-1-1**) and cis-imidazoline (**IV-4-1**).

The azlactones were prepared from the *N*-acetyl amino acids *via* an EDCI-mediated dehydration process as previously reported.[32, 18, 22] The *N*-acyl *p*-methoxy phenylglycine (aminoacids for entries **10** and **11**, Table IV-2) was synthesized by employing a modified procedure of Wasserman *et al.*[48]

The cycloaddition reactions were conducted under mild reflux conditions in dichloromethane or THF in presence of 1.3 equivalents of TMSCl. The imidazoline products were isolated by precipitation. *Syn*- imidazolines were re-precipitated from the mixture in modest to good yields and the *syn*-configuration (relative to $R_2$ and $R_3$) was confirmed unambiguously by X-ray crystallography (Figure IV-14).

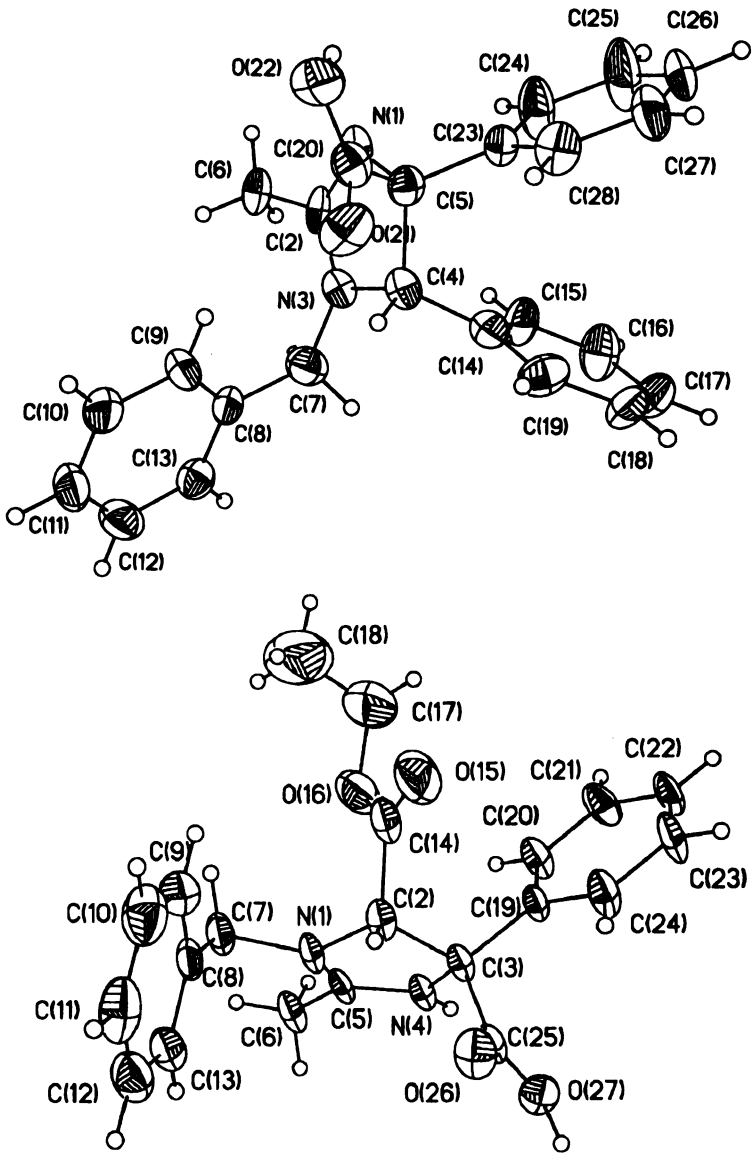



**Figure IV-14.** X-ray crystal structure of compound **IV-4-1** (top), **IV-4-6** (middle), **IV-5-1** (bottom).

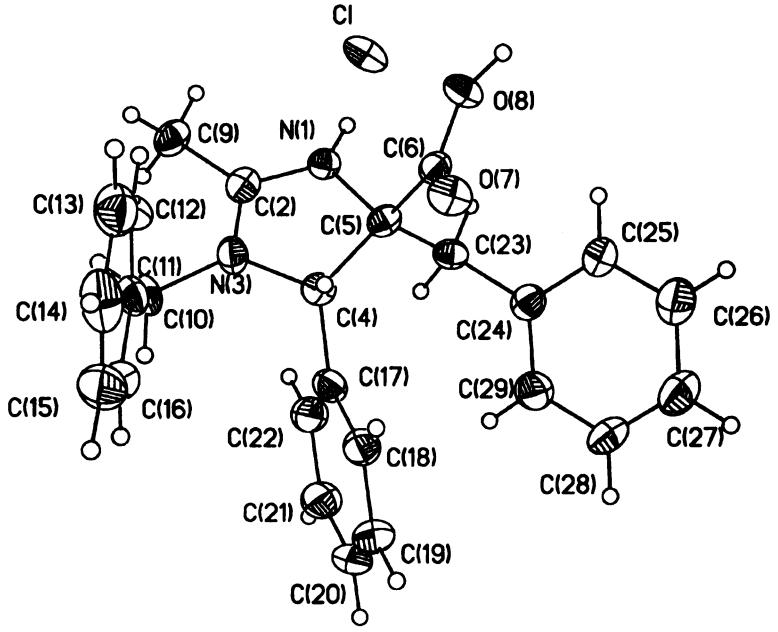



**Figure IV-14. (contd.')** X-ray crystal structure of compound **IV-4-1** (top), **IV-4-6** (middle), **IV-5-1** (bottom).

In order to understand the role of the $R_2$ group in the diastereochemical outcome of the reaction, the $R_2$ group was varied while $R_1$ was maintained as methyl or phenyl.

| Entry | Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Yield | syn:anti |
|---|---|---|---|---|---|---|---|
| 5 | IV-5-1 | Me | Bn | Ph | Bn | 55 | 75:25 |
| 6 | IV-6-1 | Ph | Bn | Ph | Bn | 45 | >5:95 |
| 7 | IV-7-1 | Me | $^i$Pr | Ph | Bn | 79 | 50:50 |
| 8 | IV-8-1 | Ph | $^i$Pr | Ph | Bn | 71 | >5:95 |
| 9 | IV-9-1 | Ph | Ph | Ph | Bn | 65 | >5:95 |
| 10 | IV-10-2 | Me | p-OMePh | p-$NO_2$Ph | Bn | 66 | >95:5 |
| 11 | IV-11-2 | Ph | p-OMePh | p-$NO_2$Ph | Bn | 53 | >5:95 |

**Table IV-2.** Influence of $R_2$ favoring *syn*-imidazolines.

The *anti*-diastereomer was obtained with $R_1$ as a phenyl group and $R_2$ an alkyl or aryl group (entries **6**, **8** and **9**, Table IV-2).

However, when $R_1$ is methyl, and $R_2$ is methyl, benzyl or *iso*-propyl (entry **3**, Table IV-1; entries **5** and **7**, Table IV-2) a mixture of diastereomers was obtained. *Syn*-imidazolines were obtained as the major or sole products with the $R_2$ substituent as an aryl group (entry **4**, Table IV-1; entry **10**, Table IV-2). These results suggest a possible role of $\pi$-stacking interactions between neighboring $\pi$-donor $R_2$ and $\pi$ -acceptor $R_3$ (the centroid-centroid distance between $R_2$ and $R_3$ in **IV-4-1** was found to be 3.98 Å, corresponding to a van der Waals contact).[49]

**J. Variation of $R_3$ and $R_4$**

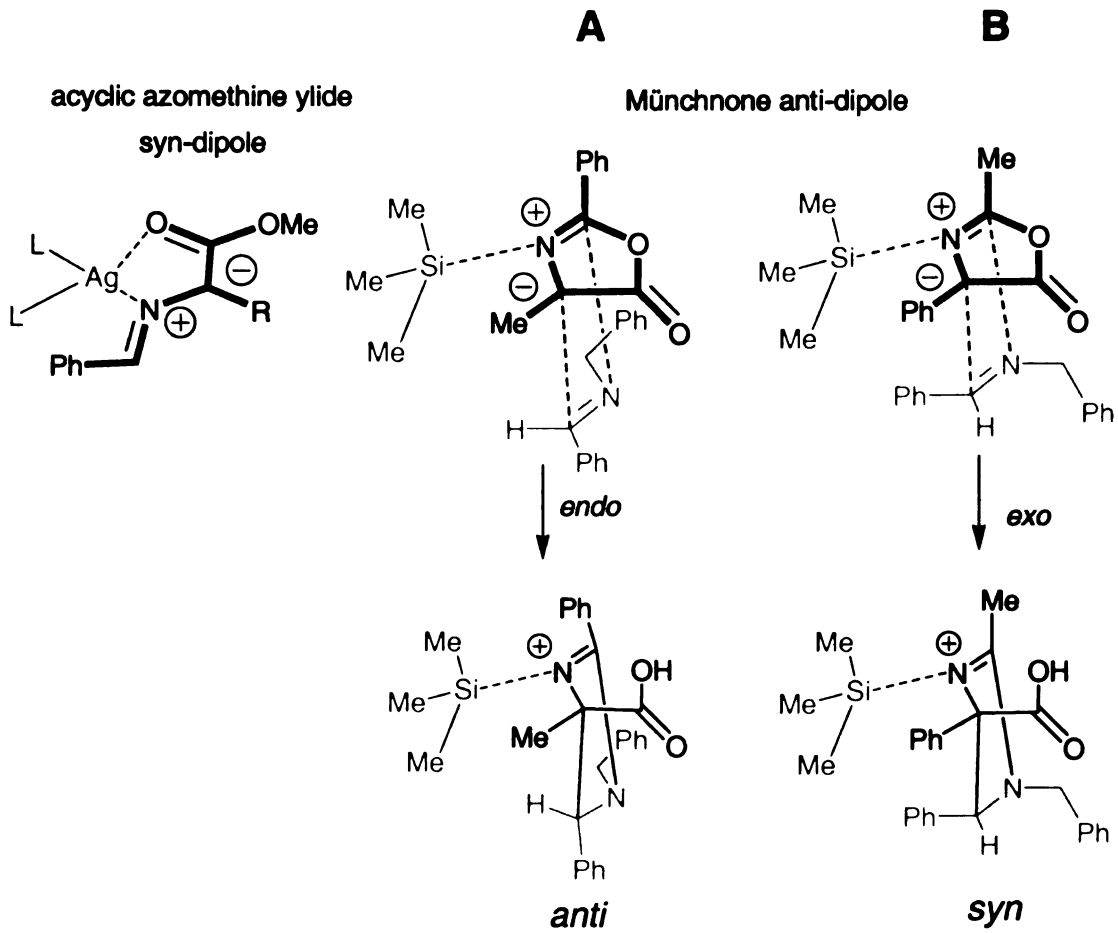
Some control on the diastereoselectivity was observed based on the electronic nature of the incoming dipolarophile. Electron donating groups at $R_3$ resulted in reduced *syn*-diastereoselectivity (entry **14**, Table IV-3), while electron-withdrawing groups enhanced *syn*-diastereoselectivity (entry **16**, Table IV-3).

Not surprisingly, when $R_1$ is phenyl, the nature of $R_2$, $R_3$ or $R_4$ did not compromise the *anti*-diastereoselectivity (entries **6**, **8**, **9**, **11**, Table IV-2; entries **13**, **15**, **17**, **19**, **21**, Table IV-3). A bulky group such as *iso*-propyl at the $R_3$ position did not afford any imidazoline and only yielded β-lactam, presumably via a traditional [2+2] cycloaddition reaction (entry **12**, Table IV-3). Both aryl as well as substituted alkyl groups are tolerated at $R_4$ position (entry **4**, Table IV-1; entries **20** and **22**, Table IV-3).

| Entry | Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Yield | syn:anti |
|---|---|---|---|---|---|---|---|
| 12 | IV-4-3 | Me | Ph | $^i$Pr | Bn | 0* | - |
| 13 | IV-1-4 | Ph | Me | 4-methoxy phenyl | Bn | 78 | >5:95 |
| 14 | IV-4-4 | Me | Ph | 4-methoxy phenyl | Bn | 60 | 60:40 |
| 15 | IV-1-5 | Ph | Me | 4-nitrophenyl | Bn | 51 | >5:95 |
| 16 | IV-4-5 | Me | Ph | 4-nitrophenyl | Bn | 71 | >95:5 |
| 17 | IV-1-6 | Ph | Me | -COOEt | Bn | 65 | >5:95 |
| 18 | IV-4-6 | Me | Ph | -COOEt | Bn | 32 | >95:5 |
| 19 | IV-1-7 | Ph | Me | Ph | $-CH_2COOMe$ | 70 | >5:95 |
| 20 | IV-4-7 | Me | Ph | Ph | $-CH_2COOMe$ | 44 | >95:5 |
| 21 | IV-1-8 | Ph | Me | Ph | 4-fluorophenyl | 74 | >5:95 |
| 22 | IV-4-8 | Me | Ph | Ph | 4-fluorophenyl | 20 | >95:5 |
| 23 | IV-1-9 | Ph | Me | Ph | n-Bu | 5* | >95:5 |
| 24 | IV-4-9 | Me | Ph | Ph | n-Bu | 43 | >5:95 |

**Table IV-3.** Variation of $R_3$ and $R_4$. *Main product is β-lactam.

The above observations indicate that $R_1$ controls the branch point of the stereochemical diversity. When $R_1$ is aryl, the *anti-* diastereomer is exclusively formed via the *endo* approach (Figure IV-15). Upon coordination with the TMS group, the münchnone gets locked in an anti-dipole orientation. Acyclic azomethine ylides derived from α-iminoesters have been proposed to adopt a preferred *syn* orientation in presence of Lewis acids due to chelation with the carboxyl oxygen.[50] *Endo-* approach favored due to steric interaction between TMS and Ph ($R_2$) group (Scheme IV-4).




**Scheme IV-4.** Proposed mechanistic rationale for the formation of *syn*- imidazolines.

However, when $R_1$ is alkyl, $R_2$ needs to be aryl to obtain *syn*-imidazolines with high diastereoselectivity. A possible explanation for this switch in diastereoselectivity is the stabilization of the *exo* approach due to favorable π-stacking interactions between $R_2$ (Ph) and $R_3$ (Ph).

## K. Conclusion

We have identified an efficient branch point for enhancing the stereochemical diversity of imidazoline scaffolds by manipulation of the substituents on the general azlactone template. The previously reported high *anti*-diastereoselectivity could be completely reversed favoring the *syn*-imidazolines in synthetically useful yields.

**L. Experimental Section**

Reactions were carried out in oven-dried glassware under nitrogen atmosphere, unless otherwise noted. All commercial reagents were used without further purification. All solvents were reagent grade. THF was freshly distilled from sodium/benzophenone under nitrogen. Toluene, $CH_2Cl_2$, and TMSCl were freshly distilled from $CaH_2$ under nitrogen. All reactions were magnetically stirred and monitored by TLC with Analtech 0.25 mm pre-coated silica gel plates. Column chromatography was carried out on silica gel 60 (230–400 mesh) supplied by EM Science. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Melting points were determined on a Mel-Temp (Laboratory devices) apparatus with a microscope attachment. Infrared spectra were recorded on a Nicolet IR/42 spectrometer. $^1H$ and $^{13}C$ NMR spectra were recorded on a Varian Gemini-300 spectrometer or a Varian VXR-500 spectrometer. Chemical shifts are reported relative to the residue peaks of the solvent $CDCl_3$ ($\delta$ 7.24 for $^1H$ and $\delta$77.0 for $^{13}C$), DMSO-*d*6 ($\delta$ 2.49(5) for $^1H$ and $\delta$39.5 (7) for $^{13}C$) and $CD_3OD$ ($\delta$ 3.3 (5), 4.78 (1) for $^1H$ and $\delta$49.0 (7) for $^{13}C$). High resolution mass spectra and FAB mass spectra were obtained at the Mass Spectrometry Laboratory of the Department of Biochemistry, Michigan State University, with a JEOL AX-505H and a JEOL HX-110 double focusing mass spectrometer (JEOL USA, Peabody, MA), respectively. Gas chromatography/low resolution mass spectra were recorded on a Hewlett–Packard 5890 Series II gas chromatograph connected to a TRIO-1 EI mass spectrometer. All chemicals were

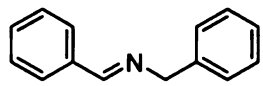
obtained from Sigma-Aldrich Chemical Co. and used as received. N-acetyl phenyl glycine was obtained from Bachem Co.

**General procedure for synthesis of imines**

A solution of aldehyde (1mmol) in benzene/ toluene (20 ml) was refluxed with the amine (1 mmol) for 2h The solvent was evaporated under vacuum to afford the imine in good yields. These imines were used without further purification.

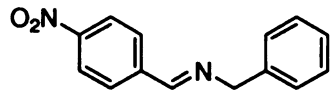
*Note:* IV-*I*-6 ((E)-ethyl 2-(benzylimino)acetate) was synthesized on by stirring ethylglyoxalate(1 eq.) and benzylamine(1.1 eq.) over $Na_2SO_4$ in $CH_2Cl_2$. Due to its instability, the imine was not isolated or characterized. IV-*I*-3 was synthesized by stirring isopropanal (1 eq.) and benzylamine(1.01 eq.) over alumina for 7h at room temperature.

**(E)-N-benzylidene(phenyl)methanamine, IV-*I*-1**



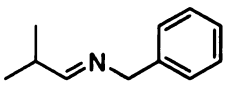
$^1$H NMR (300MHz), $CDCl_3$: δ: 4.83(s, 2H), 7.22-7.96(m, 10H), 8.39(s, 1H); $^{13}$C NMR (74.4 MHz) $CDCl_3$: 64.99, 126.97, 127.99, 128.26, 128.48, 128.58, 130.77, 136.06, 139.21, 162.05; IR($CHCl_3$): 3063.3, 3028.6, 2843.4, 1643.5, 1452.6 cm$^{-1}$. EIMS: m/z = 195.3(M+).

**(E)-N-(4-nitrobenzylidene)(phenyl)methanamine, IV-*I*-2**



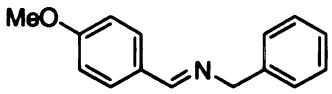
$^1$H NMR (300MHz), $CDCl_3$: δ: 4.87(s, 2H), 7.23-7.35(m, 5H), 7.91(d, 2H, *J*=8.4 Hz), 8.23(d, 2H, *J*=8.4 Hz), 8.44(s, 1H) ; $^{13}$C NMR (74.4 MHz) $CDCl_3$: 65.19, 123.87, 127.31, 128.06, 128.65, 128.92, 138.41, 141.56, 159.44; IR($CHCl_3$): 3030.6, 2851.1, 1647.4, 1645.5, 1603.1, 1522.4, 1344.6 $cm^{-1}$. EIMS: m/z = 239.8M+).

**(E)-N-(2-methylpropylidene)(phenyl)methanamine, IV-*I*-3**



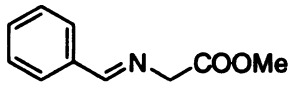

$^1$H NMR (300MHz), $CDCl_3$: δ: 1.09(d, 6H, *J*=6.9 Hz), 2.5(m, 1H), 4.54(s, 2H), 7.25-7.31(m, 5H), 7.62(d, 1H, *J*=4.2 Hz); $^{13}$C NMR (74.4 MHz) $CDCl_3$: 19.25, 34.03, 64.71, 126.73, 127.68, 128.31, 139.38, 170.87; IR($CHCl_3$): 3028.6, 2964.1, 2872.4, 2828, 1670.6, 1452.6 $cm^{-1}$. EIMS: m/z = 160.7(M+).

**(E)-N-(4-methoxybenzylidene)(phenyl)methanamine, IV-*I*-4**



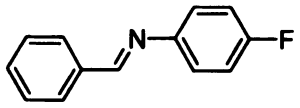

$^1$H NMR (300MHz), $CDCl_3$: δ: 3.82(s, 3H), 4.78(s, 2H), 6.90(d, 2H, *J*=8.1 Hz), 7.23-7.33(m, 5H), 7.71(d, 2H, *J*=8.1 Hz), 8.29(s, 1H) ; $^{13}$C NMR (74.4 MHz) $CDCl_3$: 55.33, 64.93, 113.95, 126.87, 127.92, 128.42, 129.80, 161.30; IR($CHCl_3$): 3028.6, 2928.2, 2837.7, 1647.4, 1600.9, 1512.4, 1251.9 $cm^{-1}$. EIMS: m/z = 225.3(M).

**(E)-methyl 2-(benzylideneamino)acetate, IV-*I*-7**



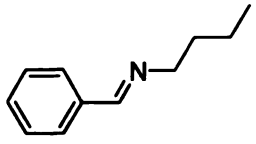

$^1$H NMR (300MHz), $CDCl_3$: δ: 3.76(s, 3H), 4.41(s, 2H), 7.40-7.77(m, 5H), 8.28(s, 1H); $CDCl_3$: 52.13, 61.98, 128.60, 131.24, 135.49, 165.45, 170.55; IR($CHCl_3$): 2924.1, 1743.9, 1647.4, 1199.9 $cm^{-1}$, EIMS: m/z = 178.0(M+).

**(E)-N-benzylidene-4-fluorobenzenamine, IV-*I*-8**




$^1$H NMR (300MHz), $CDCl_3$: δ: 7.06-7.90(m, 9H), 8.41(s, 1H); $^{13}$C NMR (74.4 MHz) $CDCl_3$: 115.48,115.97, 122.21, 128.76, 130.08, 135.99, 147.98, 159.58, 160.17, 162,83; IR($CHCl_3$): 3028.6, 2880.0, 1685.8, 1623.3 $cm^{-1}$. EIMS: m/z = 198.3 (M+H).

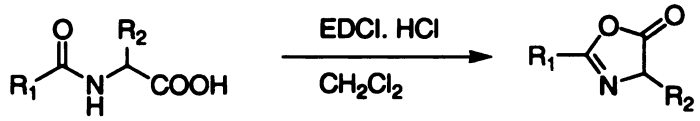
**(E)-N-benzylidenebutan-1-amine, IV-*I*-9**




$^1$H NMR (300MHz), $CDCl_3$: δ: 0.95(t, 3H, *J*=6.9 Hz), 1.40(m, 2H), 1.68(m, 2H), 3.61(q, 2H, *J*=6.9 Hz), 7.34-7.74(m, 5H), 8.25(s, 1H); $^{13}$C NMR (74.4 MHz) $CDCl_3$: 13.80, 20.36, 32.91, 61.37, 127.90, 128.22, 128.45, 130.32, 136.27,

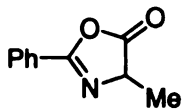
160.60, 171.93 IR($CHCl_3$): 3026.7, 2959.1, 2872.4, 1647.4, 1377.4 $cm^{-1}$. EIMS: m/z = 160.5 (M+).

**General Procedure for synthesizing azlactones**




A solution of benzoyl amino acid (2 mmol) and EDCI.HCl (2 mmol) in dichloromethane (20 ml) was stirred at 0°C for 1h until the starting material disappeared. The reaction mixture was washed successively with cold (containing ice) water, aqueous $NaHCO_3$ (10 ml) and water (10ml). The solution was dried over anhydrous magnesium sulfate, filtered and the solvent evaporated to dryness *in vacuo* providing the oxazolones as solids or oils.

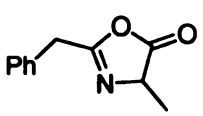
**2-phenyl-4-methyl-4H-oxazolin-5-one IV-1**




**IV-1**

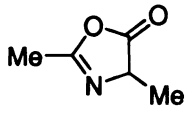
Cyclodehydration of N-benzoyl-L-alanine (2 g, 10 mmol) with EDCI.HCl (1.92 g 10 mmol.) gave 1.81g of **IV-1** in 90 % yield. $^1H$ NMR (300 MHz) ($CDCl_3$): δ 1.6 (3H, d, J = 7.4 Hz), 4.46 (1H, q, J= 7.5 Hz), 7.47 (3H, m), 8.0 (2H, d); $^{13}C$ NMR (75 MHz) ($CDCl_3$): δ16.1, 60.3, 125.3, 127.2, 128.2, 132, 160.7, 178.2; IR (neat): 1828 $cm^{-1}$, 1653 $cm^{-1}$.

**2-Benzyl-4-methyl-4H-oxazol-5-one, IV-2.**



Cyclodehydration of N-phenacetyl alanine (4 g, 19.3 mmol) with EDCI.HCl (3.71g, 19.3 mmol) gave 3.1g of **IV-2** as viscous oil in 85 % yield. **Partial data:** $^1$H NMR (300 MHz) ($CDCl_3$): δ 1.47 (3H, d, *J* = 6.9 Hz), 4.35-4.5 (2H, m), 4.77 (1H, q, *J* = 7.5 Hz), 7.38 (2H, t, *J* = 7.2 Hz), 7.48 (1H, t, *J* = 7.2 Hz, 7.72 (2H, d, *J* = 7.8 Hz); IR (neat) $cm^{-1}$, 1824 $cm^{-1}$, 1676 $cm^{-1}$.

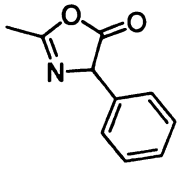
**2, 4-dimethyl-4*H*-oxazolin-5-one IV-3**




**IV-3**

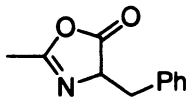
Cyclodehydration of N-acetyl-alanine (4 g, 30 mmol) with EDCI.HCl (6.3g, 33 mmol.) gave 2.0 g of **IV-3** in 60 % yield. $^1$H NMR (300 MHz) ($CDCl_3$): δ 1.37 (3H, t, J = 8 Hz), 2.05 (3H, s), 4.0-4.16 (1H, m); $^{13}$C NMR (75 MHz) ($CDCl_3$) δ 179.2, 162.6, 60.4, 16.4, 15.1; IR (neat) $cm^{-1}$, 1826 $cm^{-1}$, 1686 $cm^{-1}$.

**2-methyl-4-phenyloxazol-5(4H)-one, IV-4**




Cyclodehydration of N-acetyl-phenylglycine (2 g, 7.38 mmol) with EDCI.HCl (1.42 g, 10 mmol.) gave 1.77g of **IV-4** as a white solid in 95 % yield. $^1$H NMR (300MHz), $CDCl_3$: δ: 2.18(s, 3H), 5.16(s, 1H), 6.82-7.35(m, 5H), $^{13}$C NMR (74.4 MHz) $CDCl_3$: 67.20, 126.36, 128.30, 128.39, 128.60, 132.87,163.68,176.2; IR($CHCl_3$): 2924.5, 1826.8, 1632.1, 1257.8 $cm^{-1}$. EIMS: m/z = 175.0(M+).

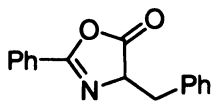
**4-benzyl-2-methyloxazol-5(4H)-one, IV-5.**




**IV-5**

Cyclodehydration of N-acetyl-phenylalanine (1 g, 4.82 mmol) with EDCI.HCl (1.01 g, 5.31 mmol.) gave 0.59g of **IV-5** as a white solid in 65 % yield. **Partial data:** IR($CHCl_3$): 1826.8, 1640.2 $cm^{-1}$ EIMS: m/z = 189.2(M+).

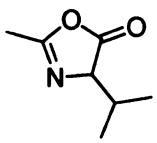
**2-phenyl-4-benzyl-4H-oxazolin-5-one IV-6**




**IV-6**

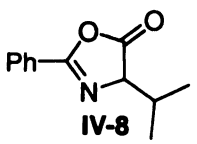
Cyclodehydration of N-benzoyl-L-phenylalanine (2 g, 7.38 mmol) with EDCI.HCl (1.42 g, 10 mmol.) gave 1.77g of **IV-6** as a white solid in 95 % yield. $^1H$ NMR (300 MHz) ($CDCl_3$): δ 3.17 (1H, dd, $J_1$ = 6.6 Hz, $J_2$ = 13.8 Hz), 3.35 (1H, dd, $J_1$ = 5.1 Hz, $J_2$ = 13.8 Hz), 4.67 (1H, dd, $J_1$ = 5.1 Hz, $J_2$ = 6.6 Hz), 7.17-7.25 (5H, m), 7.39-7.45 (2H, m), 7.5-7.55 (1H, m), 7.86-7.9 (2H, m); $^{13}C$ NMR (75 MHz) ($CDCl_3$): δ 37.3, 66.5, 125.7, 127.2, 127.8, 128.4, 128.7, 129.6, 132.7, 135.2, 161.7, 177.6; IR (neat): 1823 $cm^{-1}$, 1648 $cm^{-1}$.

**4-isopropyl-2-methyloxazol-5(4H)-one, IV-7**



Cyclodehydration of N-acetyl-L-valine (1 g, 7.09 mmol) with EDCI.HCl (1.49 g 7.8 mmol.) gave 0.8 g of **IV-8** as a low melting solid in 90 % yield. **Partial data:** 1822 $cm^{-1}$, 1651 $cm^{-1}$. EIMS: m/z = 141.1(M+).

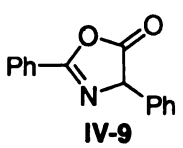
**2-phenyl-4-isopropyl-4H-oxazolin-5-one IV-8**



**IV-8**

Cyclodehydration of N-benzoyl-L-valine (6 g, 27 mmol) with EDCI.HCl (5.2 g 27 mmol.) gave 4.93 g of **IV-8** as a low melting solid in 90 % yield. **Partial data:** $^1$H NMR (300 MHz) ($CDCl_3$): δ 1.055 (3H, d, *J* = 6.9 Hz), 1.12 (3H, d, *J* = 6.9 Hz), 2.3-2.41 (1H, m), 4.29 (1H, d, *J*= 4.5 Hz), 7.47 (3H, t, *J* = 7 Hz), 7.56 (3H, t, *J* = 7.2 Hz), 7.99 (2H, d, *J* = 7.8 Hz); $^{13}$C NMR (75 MHz) ($CDCl_3$): δ 17.18, 18.33, 70.13, 125.38, 127.61, 128.11, 128.47, 132.5, 161.7, 177.13; IR (neat): 1826 $cm^{-1}$, 1657 $cm^{-1}$.

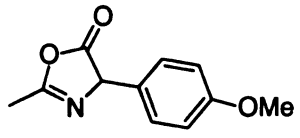
**2, 4-diphenyl-4H-oxazolin-5-one IV-9**



**IV-9**

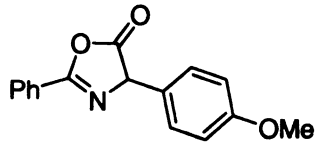
Cyclodehydration of N-benzoyl-L-phenylglycine (2 g, 7.8 mmol) with EDCI.HCl (1.5 g 7.8 mmol.) gave 1.3g of **IV-9** in 70 % yield. $^1$H NMR (300 MHz) ($CDCl_3$): δ 5.5 (1H, s), 7.35-7.65 (8H, m), 8.05-8.15 (2H, m); $^{13}$C NMR (75MHz) ($CDCl_3$): δ176.4, 162.8, 133.9, 133.2, 129.2, 129.18, 129.1,129.0, 128.99, 128.96 , 128.9, 128.7, 128.4,127.1, 126.1, 68.4; IR (neat) 1825 $cm^{-1}$, 1655 $cm^{-1}$. EI(LRMS): 237.2.

**4-(4-methoxyphenyl)-2-methyloxazol-5(4H)-one, IV-10**




Cyclodehydration of N-acetyl-(4-methoxy)phenylglycine **IV-17** (0.5 g, 2.24 mmol) with EDCI.HCl (0.47 g, 2.46 mmol.) gave 0.32 g of **IV-10** in 70 % yield. **Partial data:** IR($CHCl_3$): 2920.2, 1832.8, 1643.5, 1120.2 $cm^{-1}$. EIMS: m/z = 206.2(M+).

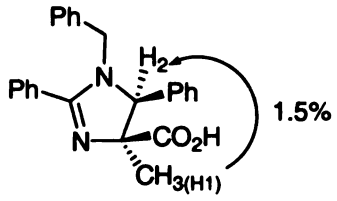
**4-(4-methoxyphenyl)-2-phenyloxazol-5(4H)-one, IV-11**




Cyclodehydration of N-benzoyl-(4-methoxy) Phenylglycine **IV-18** (0.5 g, 1.74 mmol) with EDCI.HCl (0.37 g, 1.92 mmol.) gave 0.3 g of **IV-11** in 65 % yield. **Partial data:** IR($CHCl_3$): 2929.2, 1830.8, 1645.5, 1118.2 $cm^{-1}$. EIMS: m/z = 267.2(M+).

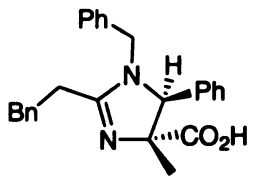
*NOTE: the stereochemical outcome of the reaction was confirmed by the NOESY1D for both the phenyl/methyl and methyl/phenyl substitutions at $R_1$ and $R_2$ .Compound IV-23 was isolated as the methyl ester.*

**Compound IV-1-1: *dl*- (4*S*, 5*S*)-1-benzyl-4-methyl 2, 5-diphenyl-4,5-dihydro-1*H*-imidazole- 4-carboxylic acid**




A solution of benzaldehyde (0.06 g, 0.57 mmol), benzylamine (0.061 g, 0.57 mmol) in anhydrous $CH_2Cl_2$ (15 ml) was refluxed under nitrogen for 2h 2-Phenyl-4-methyl-4*H*-oxazolin-5-one (0.1 g, 0.57 mmol) and chlorotrimethylsilane (0.08 g, 0.74 mmol) were added and the mixture was refluxed under nitrogen for 6 h and then stirred overnight at r.t. The solvent was evaporated under vacuum. The product was precipitated using $CH_2Cl_2$/hexanes (1:1); yield: 75% (0.155 g); white solid; $^1$H NMR (300MHz) DMSO-$d_6$: δ 1.8 (3H, s), 4.05 (1H, d, $J$ = 15Hz), 4.95 (1H, d, $J$ = 14.8 Hz), 5.05 (1H, s), 7.05 (2H, s), 7.25-7.54 (8H, m), 7.74 (2H, t, $J$ = 7.2Hz), 7.83 (1H, t, $J$ = 6.9Hz), 8.0 (2H, d, $J$ = 8.4Hz); NOESY1D: ($H_1 \rightarrow H_2$) 1.5%$^{13}$C NMR (75MHz) DMSO-$d_6$: δ 25.2, 48.8, 70.4, 73.3, 122.3, 127.8, 128.3, 128.5, 128.9, 129.1, 129.3, 129.6, 129.7, 132.3, 133.2, 134, 166.1, 169.5; IR (neat): 3350 cm$^{-1}$, 1738cm $^{-1}$; M.P. 185–190 °C; HRMS (EI): *m/z* calcd. for $C_{24}H_{21}N_2O_2$ [M – H], 369.1603; found, 369.1610.

**Compound IV-2-1: *dl*-(4*S*,5*S*)-1,2-dibenzyl-4-methyl 5-phenyl-4,5-dihydro-1*H*-imidazole- 4-carboxylic acid and *dl*-(4*S*,5*R*)-1,2 dibenzyl-4- methyl-5-phenyl-4,5-dihydro-1*H*-imidazole-4-carboxylic Acid:**



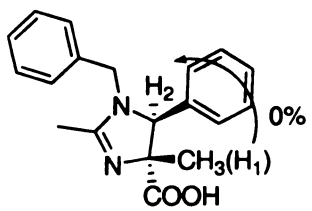

A solution of benzaldehyde (1.13g, 10.58 mmol), benzylamine (1.13g, 10.58 mmol) in anhydrous $CH_2Cl_2$ (250 ml) was refluxed under nitrogen for 2 h 2-Benzyl-4-methyl-4*H* oxazolin-5-one (2g, 10.58 mmol) and chlorotrimethylsilane (1.48 g, 10.58 mmol) were added and the mixture was refluxed under nitrogen for 6 h and then stirred overnight at r.t. The reaction mixture was evaporated to dryness under vacuum, *dr*=2:1. The product *cis* isomer was precipitated using $CH_2Cl_2$–$Et_2O$; yield: 30% (0.6 g); white solid. The *trans*-isomer was then precipitated out from the mother liquor. A small amount was then reprecipitated to remove traces of the cis-isomer. yield: 17% (0.15g).

**Cis** $^1$H NMR (300MHz) $CDCl_3$: δ 1.74 (3 H, s), 3.67 (1 H, d, *J* = 15.3 Hz), 4.11 (1 H, d, *J* = 14.7 Hz), 4.38 (1 H, s), 4.46 (1 H, d, *J* = 14.7 Hz) 4.59 (1 H, d, *J* = 15.3 Hz), 6.77 (2 H, d, *J* = 7 Hz), 7.00–7.60 (13 H, m); $^{13}$C NMR (75MHz) $CDCl_3$: □ 22.1, 32.4, 48.1, 70.6, 71.1, 127.6, 128.3, 128.6, 128.9, 129.1, 129.2, 129.7, 132.2, 132.8, 133.5, 164.8, 175.1; HRMS (FAB): *m/z* calcd for $C_{25}H_{25}N_2O_2$ [M + H], 385.1898; found, 385.1916; IR (neat): 3350 $cm^{-1}$, 1624 $cm^{-1}$.

**Trans:**$^1$H NMR (300MHz) $CDCl_3$: δ 1.14 (3 H, s), 3.94 (1 H, d, *J* = 15.6 Hz), 4.24 (2 H, q, *J* = 8.7 Hz), 4.56 (1 H, d, *J* = 15 Hz), 5.74 (1 H, s), 6.65 (2 H, d, *J* = 7.5 Hz), 7.00–7.40 (13 H, m) NOESY1D($H_1$->$H_2$) 0.9%; $^{13}$C NMR (75MHz) $CDCl_3$: □ 27.1, 38.7, 48.6, 71.5, 73.5, 127.9, 128.3, 128.5, 128.7, 128.9, 129, 129.3, 129.4, 129.5, 129.8, 120.9, 132.2, 133.5, 134.2, 165.6, 170.6 ; HRMS

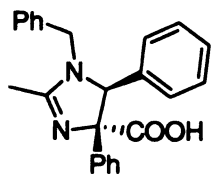
(FAB): *m/z* calcd for $C_{25}H_{25}N_2O_2$ [M + H], 385.1898; found, 385.1917; IR (neat): 3350 $cm^{-1}$, 1738 $cm^{-1}$.

**Compound IV-3-1: *dl* - (4R, 5S)-1-benzyl-4, 5-dihydro-2, 4-dimethyl-5-phenyl-1H-imidazole-4-carboxylic acid**



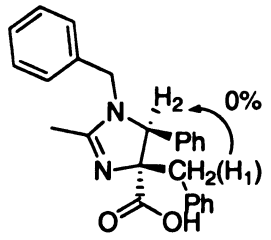

A mixture of 2-methyl-4-methyl-4*H*-oxazolin-5-one (0.69 g, 6.10 mmol) and chlorotrimethylsilane (0.85g, 7.93 mmol) in anhydrous THF was allowed to stir under nitrogen for 0.5 h The imine of benzaldehyde and benzylamine (1.43 g, 7.32 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$: ether (1:3) to give the compound in 1:1 diastereoselectivity; yield: 12%,(0.19 mg, *dr*=1:1); dirty-white solid, 25 mg of the cis product was isolated from this mixture using $CHCl_3$:ether (1:4) for characterization; $^1H$ NMR (300MHz) $CDCl_3$: δ 1.08 (3H, s), δ 2.43 (3H, s), 4.17(1H, d, $J$ = 15.6 Hz), 4.53 (1H, d, $J$ = 15.8 Hz), 5.83(1H, s), 6.99-7.36 (10H, m). $^{13}C$ NMR (125 MHz) $CDCl_3$: 12.35, 21.85, 48.06, 70.22, 71.03, 127.03, 128.61, 128.68, 129.03, 129.35, 133.14, 133.2, 163.71, 174.77. IR (neat): 2924.5 $cm^{-1}$, 1624.3cm $^{-1}$. LRMS (EI) (M-$CO_2H$): 263.1 NOESY1D: ($H_1 \rightarrow H_2$) 0% HRMS (FAB+): *m/z* calcd for $C_{19}H_{21}N_2O_2$ (M+H), 309.1604, found, 309.1603.

**Compound IV-4-1: *dl* -(4R, 5S)-1-benzyl-4, 5-dihydro-2-methyl-4, 5-diphenyl-1H-imidazole-4-carboxylic acid**



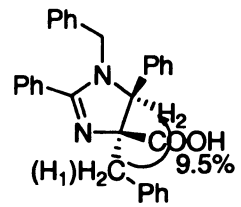

A mixture of 2-methyl-4-phenyl-4*H*-oxazolin-5-one (0.15 g, 0.85 mmol) and chlorotrimethylsilane (0.093 g, 1.11 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and benzylamine(0.17g, 0.85mmol) was then added and the mixture was allowed to reflux for 10 h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using methanol:ether (1:5) to give the titled compound; yield: 73% (0.27g, *dr*=9:1) ; white solid; the cis imidazoline was obtained by re-precipitation from methanol:ether(1:4) of the above mixture. $^1$H NMR (300MHz) $CDCl_3$: δ 2.29 (3H, s), 3.98 (1H, d, *J* = 15.0 Hz), 4.51 (1H, d, *J* = 15.0 Hz), 6.09 (1H, s), 6.87-7.29 (15H, m). $^{13}$C NMR (75MHz) $CDCl_3$: δ 11.68, 48.29, 73.05, 77.91, 123.3, 126.79, 127.22, 127.55, 127.95, 128.56, 128.68, 128.92, 129.52, 133.33, 133.94, 137.26, 164.28, 172.43; IR(neat): 3034.4 $cm^{-1}$, 2924.5 $cm^{-1}$, 1635.8 $cm^{-1}$. LRMS (EI): 324.2(M-$CO_2$H-H); HRMS (FAB+): *m/z* calcd for $C_{24}H_{23}N_2O_2$ (M+H), 371.1760, found, 371.1759.M.P. decomposes at 161 °C.

**Compound IV-5-1: *dl*-(4R, 5S)-1, 4-dibenzyl-4, 5-dihydro-2-methyl-5-phenyl-1H-imidazole-4-carboxylic acid**



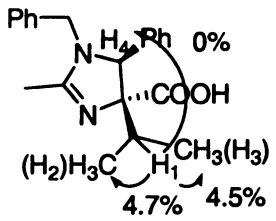

A mixture of 2-methyl-4-benzyl-4*H*-oxazolin-5-one (0.6 g, 2.93 mmol)and chlorotrimethylsilane (0.59 g, 5.49 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and benzylamine (0.99g, 3.80 mmol) was then added and the mixture was allowed to reflux for 9h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was then evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$: ether (1:2). Yield: 55% (0.62g); $^1$H NMR (300MHz) $CDCl_3$: δ 2.24 (3H, s), 2.49(2H, q, *J* = 15.0 Hz), 4.05 (1H, d, *J* = 15.0 Hz), 4.52 (1H, d, *J* = 15.0 Hz), 5.55(1H, s), 6.18-7.46 (15H, m) $^{13}$C NMR (125 MHz) $CDCl_3$: δ 12.89, 41.21, 48.29, 72.11, 73.84, 126.65, 127.19, 127.68, 128.91, 129.27, 129.55, 129.83, 130.82, 131.62, 132.73, 133.22, 135.37, 166.33, 173.41 IR(neat): 2926.5 $cm^{-1}$, 1599.2 $cm^{-1}$. NOESY1D: ($H_1 \rightarrow H_2$) 0% LRMS (EI): 339.2(M-$CO_2H$), 383.2(M-H). HRMS (FAB+): *m/z* calcd for $C_{25}H_{24}N_2O_2$ (M+H), 385.1914, found, 385.1916. M.P. decomposes at 217-218 °C.

**Compound IV-6-1: *dl*-(4S, 5S)-1, 4-dibenzyl-4,5-dihydro-2,5-diphenyl-1H-imidazole-4-carboxylic acid**



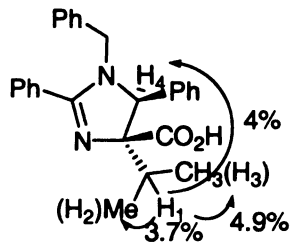

A mixture of 2-phenyl-4-benzyl-4*H*-oxazolin-5-one (0.8 g, 2.97 mmol) and chlorotrimethylsilane (0.42 g, 3.86 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and benzylamine (0.64g, 3.26 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was then evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$: ether (1:3). Yield: 45%, (0.59 g); $^1$H NMR (300MHz) $CD_3OD$: δ 3.42 (2H, q, *J* = 15.0 Hz), 4.035 (1H, d, *J* = 15.0 Hz), 4.81 (1H, d, *J* = 15.0 Hz), 5.17 (1H, s), 6.56-7.85 (20H, m) $^{13}$C NMR (125 MHz) $CD_3OD$: δ 44.4, 50.37, 72.42, 75.65, 123.50, 129.34, 129.99, 130.37, 131.16, 132.06, 133.23, 133.62, 134.39, 135.18, 167.85, 170.45, IR(neat): 2922.5 cm$^{-1}$, 1743.8 cm$^{-1}$. NOESY1D: ($H_1 \rightarrow H_2$) 9.5% LRMS (EI): 401.3(M-$CO_2H$). HRMS (FAB+): *m/z* calcd for $C_{30}H_{27}N_2O_2$ (M+H), 447.2074, found, 447.2073.M.P. decomposes at 232 °C.

**Compound IV-7-1: *dl* –(4R, 5S)1-benzyl-4, 5-dihydro-4-isopropyl-2-methyl-5-phenyl-1H-imidazole-4-carboxylic acid**



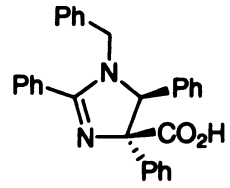

A mixture of 2-methyl-4-iso-propyl-4*H*-oxazolin-5-one (0.4 g, 2.84 mmol) and chlorotrimethylsilane (0.39g, 3.68 mmol) in anhydrous THF was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and benzylamine (0.66 g, 3.4 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$, filtered and evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$: ether (1:5) to give the compound in 1:1 diastereoselectivity; yield: 79% (651mg, *dr*=1:1) as a white solid. ~200 mg of the cis compound was isolated from this mixture using $CHCl_3$:ether(1:4) for characterization; $^1$H NMR (300MHz) $CDCl_3$: δ 0.65 (3H, d, *J* = 6.9 Hz), δ 0.96 (3H, d, *J* = 6.6 Hz), 1.81 (1H, m), 2.37 (3H, s), 3.98(1H, d, *J* = 16.8 Hz), 4.46 (1H, d, *J* = 16.2 Hz), 5.78(1H, s), 6.97-7.36 (10H, m). $^{13}$C NMR (125 MHz) $CDCl_3$: 12.55, 18.4, 18.74, 31.13, 48.07, 72.22, 77.34, 126.92, 128.22, 128.87, 129.33, 132.84, 133.3, 165.20, 172.53. IR (neat): 2926.3 cm$^{-1}$, 1601.1cm $^{-1}$. LRMS (EI) (M-$CO_2$): 355.2 HRMS (FAB+): *m/z* calcd for $C_{21}H_{25}N_2O_2$ (M+H), 337.1916, found, 337.1918. NOESY1D: ($H_1$→$H_2$) 4.67%, ($H_1$→$H_3$) 4.45%, ($H_1$→$H_4$) 0% .M.P. decomposes at 115 °C.

**Compound IV-8-1: *dl*-(4S, 5S)-1-benzyl-4, 5-dihydro-4-isopropyl-2, 5-diphenyl-1H-imidazole-4-carboxylic acid**



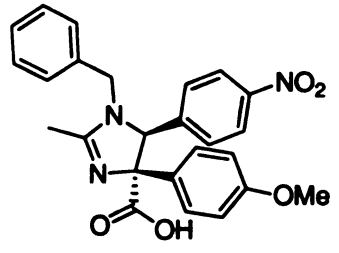

A mixture of 2-phenyl-4-iso-propyl-4*H*-oxazolin-5-one (0.44 g, 2.17 mmol) and chlorotrimethylsilane (0.30g, 2.82 mmol) in anhydrous THF was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and benzylamine (0.51 mg, 2.605 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$: ether (1:5) to give the titled compound; yield, 71% (0.61 g); white solid; $^1$H NMR (300MHz) $CDCl_3$+ 2drops DMSO-*d6*: δ 0.59 (3H, d, $J$ = 6.6 Hz), δ 0.86 (3H, d, $J$ = 6.6 Hz), 2.15 (1H, m), 3.72(1H, d, $J$ = 14.7 Hz), 4.50(1H, s), 4.64 (1H, d, $J$ = 15.0 Hz), 6.66-7.68 (15H, m). $^{13}$C NMR (125 MHz) $CDCl_3$ +2dropsDMSO-*d6*: δ 15.14, 17.42, 36.07, 48.86, 69.71, 77.60, 121.26, 128.48, 128.79, 128.93, 129.26, 129.46, 129.74, 130.82, 132.58, 133.95, 165.50, 168.72. IR (neat): 3345 cm$^{-1}$, 1732cm $^{-1}$. LRMS (EI) (M-$CO_2$): 355.2 HRMS (FAB+): *m/z* calcd for $C_{26}H_{27}N_2O_2$ (M+H), 399.2073, found, 399.2069. NOESY1D: ($H_1$→$H_2$) 3.73%, ($H_1$→$H_3$) 4.98%, ($H_1$→$H_4$) 4.02% .M.P. decomposes at 210 °C.

**Compound IV-9-1: *dl*-(4*S*, 5*S*)-1-benzyl-2, 4, 5 triphenyl-4, 5-dihydro-1*H*-imidazole- 4-carboxylic Acid**



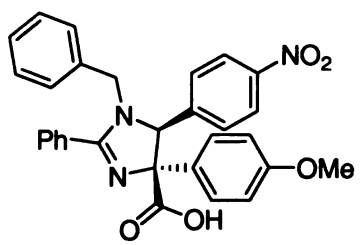

A solution of benzaldehyde (0.6 g, 5.7 mmol), benzylamine (0.61 g, 5.7 mmol) in anhydrous $CH_2Cl_2$ (120 ml) was refluxed under nitrogen for 2 h. 2,4-Diphenyl-4*H*-oxazolin-5-one (1.35 g, 5.7 mmol) and chlorotrimethylsilane (0.8 g, 7.4 mmol) were added and the mixture was refluxed under nitrogen for 6 h and then stirred overnight at r.t. The product was purified by silica-gel column chromatography (EtOH–EtOAc, 1:5); yield: 65% (2.1 g); off-white solid; $^1$H NMR (300MHz) DMSO-$d_6$: δ 3.80 (1 H, d, $J$ = 15.6 Hz), 4.62 (1 H, d, $J$ = 15.6 Hz), 4.98 (1 H, s), 6.58 (2 H, d, $J$ = 8.1 Hz), 7.05–7.65 (16 H, m), 7.90 (2 H, d, $J$ = 7.2 Hz) $^{13}$C NMR (75MHz) DMSO-$d_6$: δ 29.7, 48.3, 75.6, 79.1, 123.1, 125.7, 126.7, 127.3, 127.4, 127.9, 128.1, 128.2, 128.8, 128.9, 129, 129.3, 132.9, 133.8, 136, 143.1, 164.8, 168.1d mp 153– 155 °C. HRMS (EI): *m/z* calcd for $C_{28}H_{23}N_2$ [(M – H) – $CO_2$], 387.1526; found, 387.1539. IR (neat): 3400 cm$^{-1}$, 1738 cm$^{-1}$

**Compound IV-10-2: *dl* - (4R, 5S)-1-benzyl-4, 5-dihydro-4-(4-methoxyphenyl)-2-methyl-5-(4-nitrophenyl)-1H-imidazole-4-carboxylic acid**



A mixture of 2-methyl-4-(p-methoxy)phenyl-4*H*-oxazolin-5-one (0.15 g, 0.628 mmol) and chlorotrimethylsilane (0.088g, 0.82 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of p-nitrobenzaldehyde and benzylamine (0.182 g, 0.76 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred at r. t. for 8h. The reaction mixture was washed with water (3X) and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$/hexane (1:1) to give the cis compound only; yield: 66%, (0.18 g); pale yellow solid, $^1$H NMR (300MHz) $CDCl_3$: δ 2.16 (3H, s), 3.54(3H, s), 3.88(1H, d, *J* = 15 Hz), 4.49(1H, d, J=15 Hz), 6.16(1H, s), 6.41-7.92 (13H, m). $^{13}$C NMR (125 MHz) $CDCl_3$: 12.12, 48.48, 55.02, 71.45, 77.25, 113.35, 123.34, 126.44,127.35, 127.54, 127.75, 129.06, 129.48, 132.17, 140.91, 147.61, 158.94, 164.9, 171.91 IR (neat): 3346.9, 2918.8 $cm^{-1}$, 1608.8cm $^{-1,}$ 1514.3 $cm^{-1}$,1132 $cm^{-1}$. LRMS (EI) (M-$CO_2$H): 399.9 HRMS (FAB+): *m/z* calcd for $C_{25}H_{19}N_3O_5$ (M+H), 446.1716, found, 446.1715. M. P. decomposes at 206-207°C.

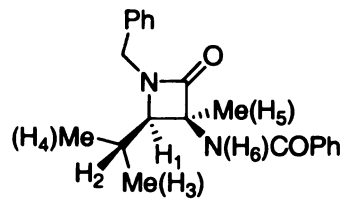
**Compound IV-11-2: *dl*-(4S, 5S)-1-benzyl-4, 5-dihydro-4-(4-methoxyphenyl)-5-(4-nitrophenyl)-2-phenyl-1H-imidazole-4-carboxylic acid**




A mixture of 2-phenyl-4-(p-methoxy) phenyl-4*H*-oxazolin-5-one (0.2 g, 0.791 mmol) and chlorotrimethylsilane (0.11g, 1.03 mmol) in anhydrous $CH_2Cl_2$ was

allowed to stir under nitrogen for 0.5 h. The imine of p-nitrobenzaldehyde and benzylamine (0.23 g, 0.95 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred at r. t. for 8h. The reaction mixture was washed with water (3X) and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$/hexane (1:1) to give the *trans* compound only; yield: 53 %, (0.21 g); bright yellow solid, $^1H$ NMR (500MHz) $CDCl_3$: δ 3.73 (1H, d, J=15 Hz), 3.75(3H, s), 4.64(1H, d, *J*=15 Hz), 4.99(1H, s), 6.16-8.19 (18H, m). $^{13}C$ NMR (125 MHz) $CDCl_3$: 48.98, 55.14, 72.90, 79.29, 113.59, 122.48, 124.02, 126.64, 127.06, 128.69, 128.86, 129.16, 129.28, 129.26, 129.45, 133.00, 134.09, 144.19, 148.15, 158.99, 164.86, 168.78 IR (neat): 2924.8 $cm^{-1}$,2870 $cm^{-1}$,1630 $cm^{-1}$,1608.8 cm $^{-1,}$ 1520.1 $cm^{-1}$,1348.4 $cm^{-1}$. LRMS (EI) (M-$CO_2$H-H): 461.3 HRMS (FAB+): *m/z* calcd for $C_{30}H_{26}N_3O_5$ (M+H), 508.1872, found, 508.1871.M. P. decomposes at 122°C.

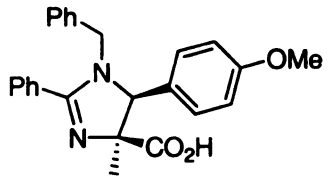
**Compound IV-4-3: N-(1-benzyl-2-isopropyl-3-methyl-4-oxoazetidin-3-yl)benzamide**




A mixture of 2-phenyl-4-methyl-4*H*-oxazolin-5-one (0.7 g, 4.13 mmol) and chlorotrimethylsilane (0.34g, 3.17 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of iso-butyraldehyde and benzylamine (0.88 g, 4.54 mmol) was then added and the mixture was allowed to reflux for 8h

and then stirred at r. t. for 8h. The reaction mixture was washed with water (2X) and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using ether to give the *β-lactam* only; yield: 78.2 %, (1.02 g); white solid, $^1$H NMR (500MHz) $CDCl_3$: δ 0.88(3H, d, *J*=6.6 Hz), 0.98(3H, d, *J*=6.6 Hz), 1.65(3H, s), 2.21(1H,m), 3.05(1H, d, *J*=8.4 Hz), 4.17(1H, d, *J*=15 Hz ), 4.99(1H, d, *J*=15 Hz), 6.20(1H, s, br), 7.23-7.82 (10H, m). $^{13}$C NMR (125 MHz) $CDCl_3$: 20.14, 20.56, 28.65, 46.15, 66.24, 71.75, 127.0, 127.69,128.31, 128.67, 128.81, 131.85, 133.97, 135.89, 167.1, 169.95 IR (neat): 3321.8, 2968.8 $cm^{-1}$,2872.4 $cm^{-1}$,1743.9 $cm^{-1}$,1666.7 $cm^{-1}$, 1537.5 $cm^{-1}$,1315.6 $cm^{-1}$. LRMS (EI): 413.1 (M+); M. P. 141°C. NOESY1D ($H_1 \rightarrow H_2$) 0%;($H_1 \rightarrow H_5$) 2.91% ($H_2 \rightarrow H_3$)2.21% ($H_2 \rightarrow H_4$)3.1 % ($H_6 \rightarrow H_5$) 4.25%

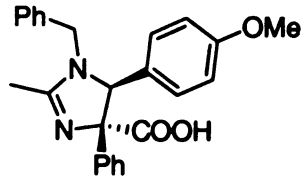
**Compound IV-1-4. *dl*-(4S, 5S)-1-benzyl-5-(4-methoxyphenyl)-4-methyl-2-phenyl- 4, 5-dihydro-1*H*-imidazole-4-carboxylic Acid**




A solution of *p*-anisaldehyde (0.077 g, 0.57 mmol), benzylamine (0.061 g, 0.57 mmol) in anhydrous $CH_2Cl_2$ (15 ml) was refluxed under nitrogen for 2 h. 2-Phenyl-4 methyl-4*H*-oxazolin-5-one (0.1 g, 0.57 mmol) and chlorotrimethylsilane (0.08 g, 0.74 mmol) were added and the mixture was refluxed under nitrogen for 6 h and then stirred overnight at r.t. The reaction mixture was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$–hexanes (1:1);

yield: 78% (0.18 g); $^1$H NMR (300MHz) DMSO-$d_6$: δ 1.8 (3H, s), 3.8 (3H, s), 3.95 (1H, d, $J$ = 15.3Hz), 4.5 (1H, s), 4.9 (1H, d, $J$ = 15Hz), 6.83-6.92 (4H, m), 7.08-7.19 (3H, m), 7.3-7.4 (3H, dd, $J_1$ = 5.1Hz, $J_2$ = 1.8Hz), 7.54-7.62 (2H, t, $J$ = 7.2Hz), 762-7.68 (1H, t, $J$ = 7.2Hz), 7.9 (2H, d, $J$ = 6.9 Hz); $^{13}$C NMR (75MHz) DMSO-$d_6$: □ 25.3, 48.8, 55.6, 70.9, 74.1, 115.2, 122.2, 123, 125.5, 127.9, 128.4, 129.2, 129.3, 129.6, 129.9, 132.8, 134.2, 161.1, 166.3, 168.4; IR (neat): 3388 cm$^{-1}$, 1738 cm$^{-1}$; M.P. 205–208 °C; HRMS (EI): *m/z* calcd. for $C_{25}H_{23}N_2O_3$ [M – H], 399.1709; found, 399.1717.

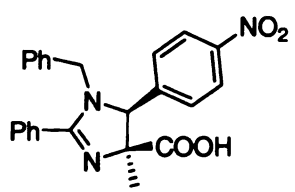
**Compound IV-4-4: *dl*- (4R, 5S)-1-benzyl- 4, 5-dihydro-5-(4-methoxyphenyl)-2-methyl-4-phenyl-1H-imidazole-4-carboxylic acid**




A mixture of 2-methyl-4-phenyl-4*H*-oxazolin-5-one (0.9 g, 5.14 mmol) and chlorotrimethylsilane (0.73 g, 6.7 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of p-anisaldehyde and benzylamine (1.16g, 5.14 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$:ether (1:3) to give the titled compound; yield: 60%, (1.23 g, *dr*=3:2) ; dirty-white solid; the cis imidazoline was obtained by re-precipitation from methanol:ether(1:5) of the above mixture.; white solid; $^1$H

NMR (300MHz) $CDCl_3$: δ 2.19 (3H, s), 3.92 (1H, d, *J* = 15.6 Hz), 4.52 (1H, d, *J* = 15.6 Hz), 6.17 (1H, s), 6.88-7.90 (14H, m) $^{13}C$ NMR + **DEPT** (125 MHz) $CDCl_3$: δ 11.4 ($CH_3$), 47.8 ($CH_2$), 55.04 ($-OCH_3$), 72.3 (-CH), 77.2, 125.6 (-CH), 126.4 (-CH), 127.2 (-CH), 127.7 (-CH), 128.5 (-CH), 129.2 (-CH), 133.1 (-CH), 136.9 (-CH), 159.4, 163.8, 172.5. IR(neat): 2955 $cm^{-1}$, 2924.5 $cm^{-1}$, 2855.0 $cm^{-1}$, 1701 $cm^{-1}$(br), 1606.9 $cm^{-1}$, 1462 $cm^{-1}$. LRMS (EI): 354.2($M-CO_2H$); HRMS (FAB+): *m/z* calcd for $C_{25}H_{25}N_2O_3$ (M+H), 401.1867, found, 401.1865. M.P. decomposes at 138 °C.

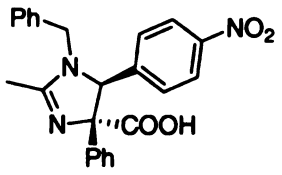
**Compound IV-1-5: *dl*- (4S, 5S)-1-benzyl-4,5-dihydro-4-methyl-5-(4-nitrophenyl)-2-phenyl-1H-imidazole-4-carboxylic acid**




A mixture of 2-phenyl-4-methyl-4*H*-oxazolin-5-one (0.68 g, 3.88 mmol) and chlorotrimethylsilane (0.55 g, 5.04 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of p-nitrobenzaldehyde and benzylamine (0.94g, 3.88 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using methanol:ether (1:5) to give the titled compound; yield: 51% (0.70 g); yellow solid; $^1H$ NMR (500MHz) DMSO-*d6*: δ 2.29 (3H, s), 3.98 (1H, d, *J* = 15.0 Hz), 4.51 (1H, d, *J* = 15.0 Hz), 6.09 (1H, s), 6.87-7.29 (15H, m).

$^{13}C$ NMR (125 MHz) DMSO-*d6*: δ 22.62, 49.61, 70.78, 72.64, 116.51, 122.60, 122.22, 126.88, 128.63, 129.67, 129.93, 130.32, 134.86, 140.64, 148.93, 167.39, 169.78. IR (neat): 2982.5 $cm^{-1}$, 1745.8 $cm^{-1}$, 1606.9 $cm^{-1}$, 1522.5 $cm^{-1}$. LRMS (EI) (M-$CO_2H$): 370.2. HRMS (FAB+): *m/z* calcd for $C_{24}H_{21}N_3O_4$ (M+H), 416.1614, found, 416.1610. M.P. decomposes at 238 °C.

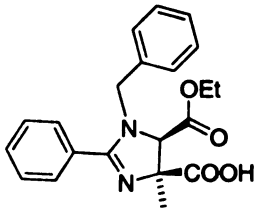
**Compound IV-4-5: *dl*-(4R, 5S)-1-benzyl-4,5-dihydro-2-methyl-5-(4-nitrophenyl)-4-phenyl-1H-imidazole-4-carboxylic acid**




A mixture of 2-methyl-4-Phenyl-4*H*-oxazolin-5-one (0.9 g, 5.14 mmol) and chlorotrimethylsilane (0.73 g, 6.7 mmol) in anhydrous $CH_2Cl_2$ was stirred under nitrogen for 0.5 h. The imine of 4-nitrobenzaldehyde and benzylamine (1.23 g, 5.14 mmol) was then added and the mixture was allowed to gently reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using methanol: ether (1:5) to give the titled compound; yield: 72% (1.54 g); white solid; $^1H$ NMR (300MHz) $CDCl_3$: δ 2.19 (3H, s), 3.92 (1H, d, *J* = 15.6 Hz), 4.52 (1H, d, *J* = 15.6 Hz), 6.17 (1H, s), 6.88-7.90 (14H, m). $^{13}C$ NMR (75MHz) $CDCl_3$: δ 11.8, 48.4, 71.5, 77.5, 123.3, 126.2, 127.4, 127.8, 128.1, 129.1, 129.3, 129.4, 132.2, 135.8, 140.7, 147.6, 165.5, 171.4; IR (neat): 2924.5

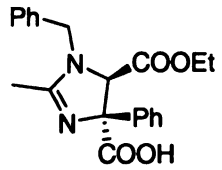
$cm^{-1}$, 1606.9 $cm^{-1}$. LRMS (EI): 369.3(M-$CO_2$H-H); HRMS (FAB+): *m/z* calcd for $C_{24}H_{22}N_3O_4$ (M+H), 416.1610, found, 416.1611. M.P. decomposes at 154 °C.

**Compound IV-1-6: dl-(4S, 5R)-5-(ethoxycarbonyl)-1-benzyl-4, 5-dihydro-4-methyl-2-phenyl-1H-imidazole-4-carboxylic acid**



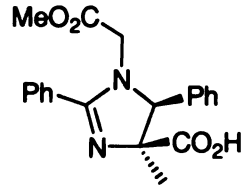

A mixture of 2-phenyl-4-methyll-4*H*-oxazolin-5-one (0.072 g, 0.41 mmol) and chlorotrimethylsilane (0.58 g, 0.53 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of ethyl glyoxylate and benzylamine was then added and the mixture was allowed to reflux for 8h and then stirred for 4h at r. t. The solvent was removed *in vacuo* and 250 ml ether was added. The product was precipitated using ether and then maintaing the mixture at -20°C for 40h. yield: 65%, (0.097 g); white solid; $^1$H NMR (300MHz) $CDCl_3$: δ 1.283 (3H, t, *J* = 6.9 Hz), 1.77(3H, s), 4.24(2H, q, *J* = 7.8Hz), 4.39(1H, d, J=14.7 Hz), 4.88(1H, d, *J* = 14.7 Hz), 4.93 (1H, s), 7.08-7.88 (10H, m). $^{13}$C NMR (75 MHz) $CDCl_3$: δ 13.91, 25.27, 50.82, 62.91, 70.11, 77.21, 121.10, 127.95, 129.47, 129.58, 129.72, 129.80, 131.29, 134.51, 165.62, 166.49, 169.92 IR (neat): 3310 $cm^{-1}$, 2926.4$cm^{-1}$, 2853.1 $cm^{-1}$, 1741.9 $cm^{-1}$,1738 $cm^{-1}$, 1658.9 $cm^{-1}$, 1203.7$cm^{-1}$. LRMS (EI): 321.2(M-$CO_2$H); HRMS (+FAB): *m/z* calcd for $C_{21}H_{23}N_2O_4$ (M+H), 367.1660, found 367.1658; (-FAB): 35, 37, m/z for $C_{21}H_{21}N_2O_4$ (M-H) 365.4; M.P. decomposes at 124°C.

**Compound IV-4-6 : *dl*-(4R, 5R)-5-(ethoxycarbonyl)-1-benzyl-4, 5-dihydro-2-methyl-4-phenyl-1H-imidazole-4-carboxylic acid**



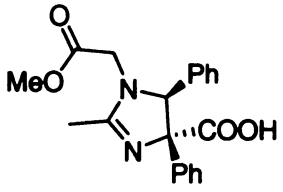

A mixture of 2-methyl-4-phenyl-4*H*-oxazolin-5-one (0.81 g, 4.67 mmol) and chlorotrimethylsilane (0.66 g, 6.07 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of ethyl glyoxylate and benzylamine was then added and the mixture was allowed to reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using methanol: ether (1:5) to give the titled compound; yield: 32% (0.54 g); white solid; $^1$H NMR (300MHz) $CDCl_3$: δ 0.695 (3H, t, *J*=7.2 Hz), 2.66(3H, s), 3.24(1H, m), 3.52(1H, m), 4.36(1H, d, *J*= 15 Hz), 4.79 (1H, d, *J* = 15.0 Hz), 5.33(1H, s), 7.22-7.40 (10H, m). $^{13}$C NMR (125 MHz) $CDCl_3$: δ 13.05, 13.28, 50.16, 62.17, 69.64, 74.75, 126.76, 127.54, 128.62, 128.73, 129.19, 129.34, 129.43, 129.51, 131.41, 131.67, 165.42, 168.45, 168.66 IR (neat): 2924.5 $cm^{-1}$, 1743.9 $cm^{-1}$,1595 $cm^{-1}$, 1211$cm^{-1}$. LRMS (EI): 320.1(M-$CO_2$H-H); HRMS (+FAB): *m/z* calcd for $C_{21}H_{23}N_2O_4$ (M+H), 367.1658, found 367.1657; M.P. decomposes at 86°C.

**Compound IV-1-7. *dl*-(4*S*, 5*S*)-1-methoxycarbonylmethyl-4-methyl-2,5 diphenyl- 4,5-dihydro-1*H*-imidazole-4-carboxylic Acid**




To a well stirred solution of 2-phenyl-4-methyl-4*H*-oxazolin-5-one (0.5 g, 2.85 mmol) and TMSCl (0.37 g, 3.42 mmol) in anhydrous $CH_2Cl_2$ (50 ml) was added a solution of (benzylidene-amino)-acetic acid methyl ester (0.6 g, 3.42 mmol) in anhydrous $CH_2Cl_2$ (20 ml) and the mixture was refluxed under nitrogen for 10 h and then stirred overnight at r.t. The reaction mixture was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$– hexanes (1:1); yield: 70% (0.7 g); white solid; $^1$H NMR (300 MHz) DMSO-$d_6$: δ 1.99 (3H, (1H, d, $J$ = 18.3 Hz), 4.53 (1H, d, $J$ = 18.3 Hz), 5.39 (1H, s), 7.47-7.50 (5H, m), 7.74-7.87 (5H, m); $^{13}$C NMR (75MHz) DMSO-$d_6$: δ 24.23, 52.09, 70.83, 75.38, 121.84, 128.26, 128.69, 129.52, 129.75, 131.78, 134.02, 167.59, 168.62, 169.19; IR(neat): 3468 cm$^{-1}$, 1747 cm$^{-1}$. HRMS (EI): *m/z* calcd. for $C_{20}H_{21}N_2O_4$ [M + H] 352.1501, found, 353.1507.M.P. decomposes at 215–217 °C.

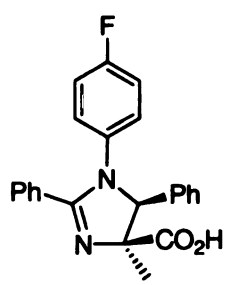
**Compound IV-4-7: *dl*-(4R, 5S)-1-((methoxycarbonyl)methyl)-4,5-dihydro-2-methyl-4,5-diphenyl-1H-imidazole-4-carboxylic acid**




A mixture of 2-methyl-4-phenyl-4*H*-oxazolin-5-one (0.45 g, 2.58 mmol) and chlorotrimethylsilane (0.36 g, 3.35 mmol) in anhydrous $CH_2Cl_2$ was stirred under

nitrogen for 0.5 h. The imine of glycine methyl ester and benzaldehyde (3.099 mmol) was then added and the mixture was allowed to gently reflux for 8h and then stirred overnight at r. t. The reaction mixture was washed with water and the aqueous layer was collected. The aqueous layer was then extracted in n-BuOH. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$ :ether (1:7) to give the titled compound; yield: 44% (0.4 g); dirty-white solid; $^1H$ NMR (300MHz) $CDCl_3$: δ 2.63 (3H, s), 3.61(3H, s),3.84 (1H, d, *J* = 17.4 Hz), 4.36 (1H, d, *J* = 17.4 Hz), 5.94 (1H, s), 7.02-7.74 (10H, m) $^{13}C$ NMR (75MHz) $CDCl_3$: δ 12.83, 45.23, 52.97, 75.96, 77.20, 126.32, 128.19,128.68, 128.92, 129.57, 130.56, 130.95, 138.59, 166.91, 167.77, 170.77; IR(neat): 2924.5 $cm^{-1}$, 1747.73 $cm^{-1}$,1599.19 $cm^{-1}$. LRMS (EI): 308.2(M-$CO_2$H-H); HRMS (FAB+): *m/z* calcd for $C_{20}H_{21}N_2O_4$ (M+H), 353.1501, found, 353.1502. M.P.: decomposed at 78°C

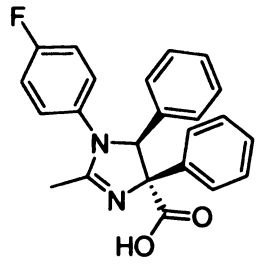
**Compound IV-1-8. *dl*-(4*S*,5*S*)-1-(4-fluorophenyl)-4-methyl-2,5-diphenyl-4,5-dihydro- 1*H*-imidazole-4 carboxylic acid**




A solution of benzaldehyde (0.060 g, 0.57 mmol), 4-fluoroaniline (0.063 g, 0.57 mmol) in anhydrous $CH_2Cl_2$ (15 ml) was refluxed under nitrogen for 2 h. 2 Phenyl-4-methyl-4*H*-oxazolin-5-one (0.1 g, 0.57 mmol) and chlorotrimethylsilane

(0.08 g, 0.74 mmol) were added and the mixture was refluxed under nitrogen for 6 h and then stirred overnight at r.t. The reaction mixture was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$–hexanes (1:1) to give the titled compound; yield: 74% (0.16 g); white solid; $^1H$ NMR (300MHz) DMSO-$d_6$: δ 1.98 (3H, s), 5.98 (1H, s), 7.05-7.65 (14H, m) $^{13}C$ NMR (75MHz) DMSO-$d_6$: δ 25.2, 71.2, 77.9, 116.9, 117, 117.1, 117.3, 123, 125.1, 125.3, 129.3, 129.4, 129.6, 130.1, 130.3, 130.4, 130.5, 132.5, 133.3, 134.5, 160.4, 163.7, 165.3, 170.4; IR(neat): 3450 $cm^{-1}$, 1744 $cm^{-1}$; M.P. 230–232 °C. HRMS (EI): *m/z* calcd for $C_{23}H_{18}FN_2O_2$ [M – H], 373.1352; found, 373.1359.

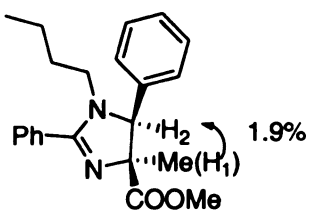
**Compound IV-4-8: *dl*-(4R, 5S)-1-(4-fluorophenyl)-4, 5-dihydro-2-methyl-4, 5-diphenyl-1H-imidazole-4-carboxylic acid**




A mixture of 2-methyl-4-phenyl-4*H*-oxazolin-5-one (0.6 g, 3.43 mmol) and chlorotrimethylsilane (0.48g, 4.45 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and p-fluoroaniline (0.82 g, 4.11 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred at r. t. for 6h. The reaction mixture was washed with water and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using ether to give the cis compound only; yield: 20%, (0. 255 g);

dirty-white solid, $^1$H NMR (300MHz) $CDCl_3$: δ 1.83 (3H, s), 6.64(1H, s), 7.04-7.40 (14H, m). $^{13}$C NMR (125 MHz) $CDCl_3$: 12.68, 76.52, 78.17, 117.32, 126.78, 127.57, 128.13, 128.44, 128.75, 129.05, 129.20, 130.91, 134.13, 136.28, 160.96, 164.82, 172.54. IR (neat): 3346.9, 2918.8 $cm^{-1}$, 1653.2cm $^{-1}$. LRMS (EI) (M-$CO_2H$): 329.3 HRMS (FAB+): *m/z* calcd for $C_{23}H_{19}N_2O_2F$ (M+H), 375.1508, found, 375.1508. M. P. decomposes at 162-163°C.

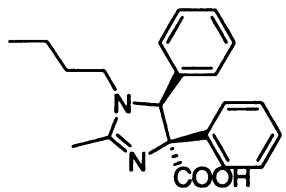
**Compound IV-1-9: *dl*-(4S, 5S)-methyl 1-butyl-4, 5-dihydro-4-methyl-2,5-diphenyl-1H imidazole-4-carboxylate**




A mixture of 2-phenyl-4-methyl-4*H*-oxazolin-5-one (0.35 g, 2.0 mmol) and chlorotrimethylsilane (0.35g, 2.6 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and n-butylamine (0.39 g, 2.4 mmol) was then added and the mixture was allowed to reflux for 10h and then stirred at r. t. for 6h. The reaction mixture was subjected to methylation using $TMSCHN_2$ and further purified over silica using column chromatography (ether:hexane 7:3). yield: 5 %, (0.04 g); $^1$H NMR (300MHz) $CDCl_3$: δ 0.72 (3H, t, J=6.9 Hz), 0.95(2H, m), 1.09(3H, s), 1.29(2H, m), 2.98(1H, m), 3.39(1H, m), 3.89(3H,s), 5.36(1H, s), 7.43-7.85 (10H, m). $^{13}$C NMR (74.9 MHz) $CDCl_3$: 13.49, 19.60, 29.34, 29.51, 45.68, 52.80, 69.38, 77.20, 127.02, 128.1, 128.54, 128.61, 128.69, 129.91, 130.58, 131.79, 166.33, 172.06 NOESY1D ($H_1 \rightarrow H_2$) 1.9%; IR

(neat): 2926.4 $cm^{-1}$, 2840.0 $cm^{-1}$,1741.9 $cm^{-1}$, 1653 $cm^{-1}$. LRMS (EI) (M-$CO_2H$): 291.1 HRMS (FAB+): *m/z* calcd for $C_{22}H_{27}N_2O_2$ (M+H), 351.2073, found, 351.2072.

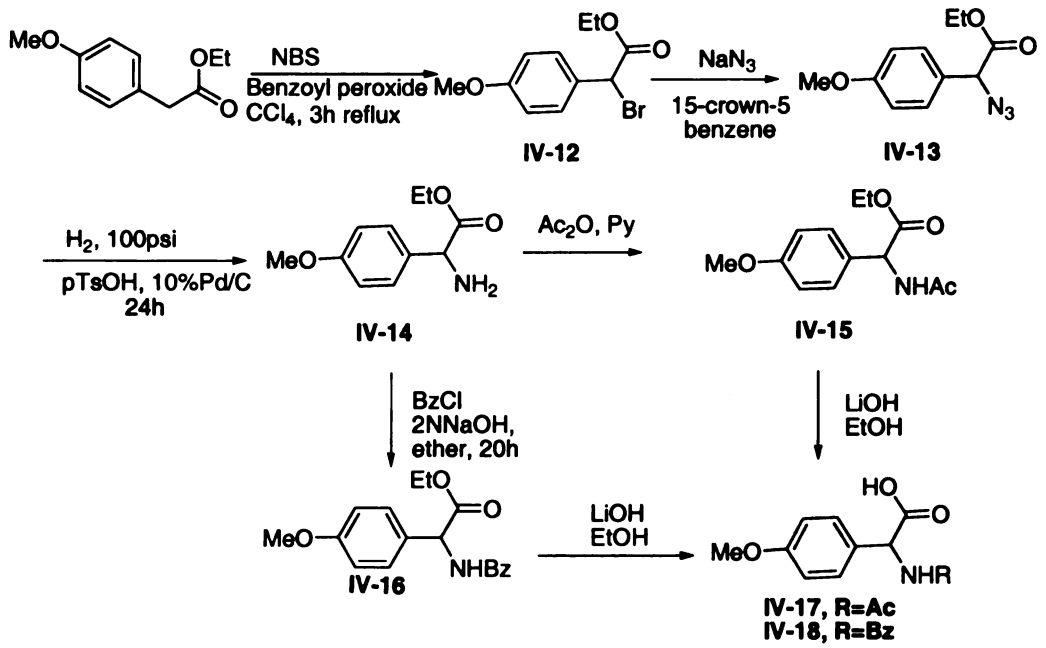
**Compound IV-4-9: *dl* – (4R, 5S)-1-butyl-4, 5-dihydro-2-methyl-4, 5-diphenyl-1H-imidazole-4-carboxylic acid**



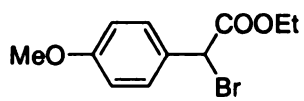

A mixture of 2-methyl-4-phenyl-4*H*-oxazolin-5-one (0.39 g, 2.23 mmol) and chlorotrimethylsilane (0.31g, 2.89 mmol) in anhydrous $CH_2Cl_2$ was allowed to stir under nitrogen for 0.5 h. The imine of benzaldehyde and n-butylamine (0.43 g, 2.67 mmol) was then added and the mixture was allowed to reflux for 8h and then stirred at r. t. for 10h. The reaction mixture was washed with water (2X) and the organic layer was collected. The organic layer was dried with $Na_2SO_4$ and filtered. The organic layer was evaporated to dryness under vacuum. The product was precipitated using $CH_2Cl_2$/hexane/ether (1:1.5:0.3) to give the *cis* compound only; yield: 43 %, (0.318 g); white solid, $^1H$ NMR (300MHz) $CDCl_3$: δ 0.891(3H, t, J=7.2 Hz), 1.26(2H, m), 1.49(2H, m), 2.20(3H, s), 2.89(1H, m), 3.28(1H, m) 6.25(1H, s), 6.97-7.43 (10H, m). $^{13}C$ NMR (74.9 MHz) $CDCl_3$: 11.70, 13.52, 19.69, 29.52, 44. 31, 71.98, 77.19, 125.90, 126.60, 127.16, 127.75, 128.29, 128.52, 129.55, 133.33, 164.34, 172.36 IR (neat): 2930.2 $cm^{-1}$, 2870.0 $cm^{-1}$,1639.7 $cm^{-1}$, 1595.3 $cm^{-1}$, 1344.6 $cm^{-1}$. LRMS (EI) (M-$CO_2H$): 291.1 HRMS

(FAB+): *m/z* calcd for $C_{21}H_{24}N_2O_2$ (M+H), 337.1915, found, 337.1917. M. P. decomposes at 124°C.

**Modification of Wasserman's synthesis for acyl p-methoxyphenylglycine (Compound 30 and 31)**



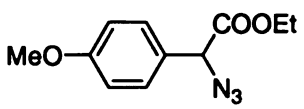

**Compound IV-12: ethyl 2-bromo-2-(4-methoxyphenyl) acetate**




A mixture of ethyl (4-methoxyphenyl) acetate (5g, 0.025 mmol), N-bromosuccinide (4.894g, 0.0275 mmol) and benzoyl peroxide (25 mg) in $CCl_4$ was refluxed for 3h and then allowed to stand overnight to precipitate any dissolved succinimide. The mixture was then filtered and concentrated to give dark yellow oil in quantitative yield. $^1$H NMR(500MHz) $CDCl_3$: δ 1.29 (3H, t, $J$ = 16.5 Hz), 3.81 (3H, s), 4.24 (2H, q, $J$ = 14.0 Hz), 5.36 (1H, s), 6.89 (2H, d, $J$ = 15 Hz), 7.51 (2H, d, $J$ = 15 Hz). $^{13}$C NMR (125 MHz) $CDCl_3$: δ 13.83, 46.73, 55.23,

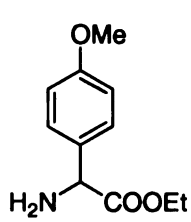
62.29, 114.11, 127.72, 130.00, 160.17, 168.32. IR (neat): 2982.5 $cm^{-1}$, 1745.8 $cm^{-1}$, 1608.8 $cm^{-1}$, 1178.6 $cm^{-1}$. LRMS (EI): 271.0, 273.0

**Compound IV-13: ethyl 2-azido-2-(4-methoxyphenyl) acetate**




A solution of 15-crown-5 (150 $\mu$L, 0.7 mmol) in 60 ml benzene was refluxed for 2h with azeotropic removal of water. On cooling, 3g (10 mmol) of the bromo ethyl ester and 0.71 g (10 mmol) of sodium azide were added. The mixture was stirred at room temperature for 8h and then passed through a silica column eluting with benzene. The filtrate was concentrated and dried to give 82% (2.63 g) of light oil. $^1$H NMR (300MHz) $CDCl_3$: $\delta$ 1.22 (3H, t, $J$ = 6.9 Hz), 3.77 (3H, s), 4.20 (2H, q, $J$ = 6.9 Hz), 4.86 (1H, s), 6.90 (2H, d, $J$ = 8.7 Hz), 7.51 (2H, d, $J$ = 8.7 Hz). $^{13}$C NMR (125 MHz) $CDCl_3$: $\delta$ 13.46, 54.66, 61.45, 64.24, 113.9, 125.58, 128.57, 129.66, 159.80, 168.88. IR (neat): 2982.5 $cm^{-1}$, 2104.6 $cm^{-1}$,1741.9 $cm^{-1}$, 1610.8 $cm^{-1}$, 1514.3 $cm^{-1}$,1178.6 $cm^{-1}$. LRMS (EI): 235.0

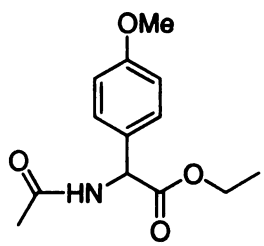
**Compound IV-14: ethyl 2-amino-2-(4-methoxyphenyl) acetate**




A mixture of 1.9 g (8.08 mmol) of azide ester, 1.89 g p-toluenesulphonic acid monohydrate (9.94 mmol) and 0.3 g of 10% Pd/C in 100 ml 95% ethanol was hydrogenated at room temperature at 100 psi for 24h. The mixture was filtered through a pad of celite and concentrated to give yellow crystals. These were then

washed with saturated $NaHCO_3$ and extracted twice in ether to give the aminoacid ester in 71% yield (1.2 g). $^1H$ NMR (300MHz) $CDCl_3$: δ 1.00 (3H, t, $J$ = 8.7 Hz), 2.08(1H, s, br), 3.56 (3H, s), 3.94 (2H, q, $J$ = 7.2 Hz), 4.34 (1H, s), 6.75 (2H, d, $J$ = 8.7 Hz), 7.11 (2H, d, $J$ = 8.7 Hz). $^{13}C$ NMR (125 MHz) $CDCl_3$: δ 13.46, 54.51, 57.48, 60.47, 113.2, 127.42, 129.65, 132.08, 158.65, 173.6. IR (neat): 3400 $cm^{-1}$, 2800 $cm^{-1}$,1734.2 $cm^{-1}$, 1610.8 $cm^{-1}$, 1512.3 $cm^{-1}$, 1138.2 $cm^{-1}$. LRMS (EI): 209.9 (M+H)

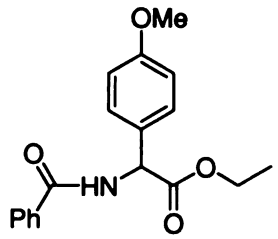
**Compound IV-15: ethyl 2-acetamido-2-(4-methoxyphenyl) acetate**




A mixture of amino acid ester (230 mg, 1.1 mmol) and pyridine (1.65 mmol) in $CH_2Cl_2$ was maintained at 0°C for five minutes. $Ac_2O$ (1.21 mmol) was added drop-wise and the reaction mixture was brought to room temperature and stirred for 8h. The reaction mixture was then washed with saturated solution of $NH_4Cl$ (aq.) and the organic layer collected. The organic layer was further washed with $NaHCO_3$ (aq.) and finally with brine. The organic layer was dried over $Na_2SO_4$ and the solvent removed *in vacuo* to give the product in 80% (0.22 g) yield. $^1H$ NMR (300MHz) $CDCl_3$: δ 1.19 (3H, t, $J$ = 6.0 Hz), 2.00(1H, s), 3.78 (3H, s), 4.16 (2H, q, $J$= 7Hz), 5.47 (2H, d, $J$ = 3.0 Hz), 6.35 (2H, d, $J$ = 6.0 Hz), 6.84 (2H, d, $J$ = 6.0 Hz), 7.25 (2H, d, $J$ = 6.0 Hz). $^{13}C$ NMR (125 MHz) $CDCl_3$: δ 13.68, 22.45, 54.38, 55.87, 61.21, 113.86, 127.52, 128.25, 159.20, 169.43, 170.89. IR (neat):

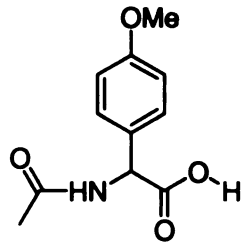
3287.1 $cm^{-1}$, 2980.4 $cm^{-1}$, 2839.6 $cm^{-1}$,1741.9 $cm^{-1}$, 1655.1 $cm^{-1}$, 1514.3 $cm^{-1}$, 1178.7 $cm^{-1}$. LRMS (EI): 250.7 (M+)

**Compound IV-16 : ethyl 2-(benzamido)-2-(4-methoxyphenyl) acetate**



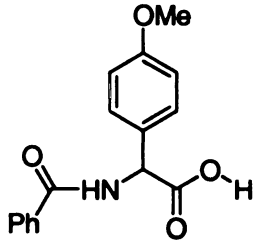

A mixture of amino acid ester (1.5g, 7.17 mmol) in ether (100 ml) and 2N NaOH (150 ml) was maintained at 0°C for 15 minutes. A diluted solution of benzoyl chloride(0.915 ml, 7.89 mmol) in ether (50 ml) was very slowly added using a dropping funnel. The reaction mixture was brought to room temperature and stirred for 20h. The organic layer was collected and dried over $Na_2SO_4$. The solvent was removed *in vacuo* to give the product in 72% yield (1.62 g). $^1H$ NMR (300MHz) $CDCl_3$: δ 1.11(3H, t, *J* = 6.9 Hz), 3.63 (3H, s), 4.17 (2H, q, *J* = 7.2 Hz), 5.67 (1H, d, *J* = 5.7 Hz), 6.85 (2H, d, *J* = 8.1 Hz), 7.07-7.51 (6H, m), 7.78 (2H, d, *J* = 8.1 Hz). $^{13}C$ NMR (125 MHz) $CDCl_3$: δ 14.01, 55.30, 56.27, 61.96, 114.34, 127.11, 128.53, 128.57, 128.81, 131.79, 133.70, 159.68, 166.58, 170.23. IR (neat): 3321.8 $cm^{-1}$, 2936.0 $cm^{-1}$,2837.6 $cm^{-1}$, 1740.0 $cm^{-1}$, 1653.2 $cm^{-1}$, 1512.3 $cm^{-1}$,1250.0 $cm^{-1}$. LRMS (EI): 313.1 (M+)

**Compound IV-17: 2-acetamido-2-(4-methoxyphenyl) acetic acid**




A mixture of Ethyl 2-acetamido-2-(4-methoxyphenyl) acetate (1g, 4.22 mmol) and $LiOH.H_2O$ (8.44 mmol) in EtOH was maintained at r. t. for 20 h. Removed the solvent *in vacuo* and added 2.5 ml water. Acidified using conc. HCl drop-wise to pH=2 while maintaining the reaction mixture at 0°C on ice. Filtered and dried under vacuum to obtain dirty white solid in 81% yield (0.72 mg). $^1H$ NMR (300MHz) DMSO-*d6*: δ 1.86 (3H, s), 3.73 (3H, s), 5.20 (2H, d, $J$ = 7.5 Hz), 6.90 (2H, d, $J$ = 8.7 Hz), 7.27 (2H, d, $J$ = 8.7 Hz), 8.51 (2H, d, $J$ = 6.0 Hz). $^{13}C$ NMR (125 MHz) DMSO-*d6*: δ 22.24, 55.15, 56.63, 113.89, 128.89, 129.12, 158.95, 169.03, 172.30. IR (neat): 3320.0 $cm^{-1}$, 2924.4 $cm^{-1}$, 2839.6 $cm^{-1}$,1716.9 $cm^{-1}$, 1514.3 $cm^{-1}$, 1248.1 $cm^{-1}$. LRMS (EI): 223.1 (M+)

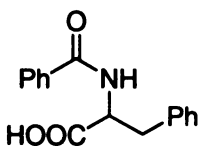
**Compound IV-18: 2-(benzamido)-2-(4-methoxyphenyl) acetic acid**




A mixture of Ethyl 2-acetamido-2-(4-methoxyphenyl) acetate (1.61g, 5.16 mmol) and $LiOH.H_2O$ (10.33 mmol) in EtOH was maintained at r. t. for 20 h. Removed the solvent *in vacuo* and added 2.5 ml water. Acidified using conc. HCl drop-wise to pH=2 while maintaining the reaction mixture at 0°C on ice. Filtered and dried

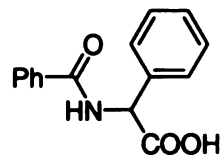
under vacuum to obtain dirty white solid in 73.5% yield (1.08 g). $^{1}$H NMR (300MHz) DMSO-*d6*: δ 3.77 (3H, s), 5.49 (1H, d, *J* = 9 Hz), 6.93 (2H, d, *J* = 8.4 Hz), 7.41-7.502 (5H, m), 7.90 (2H, d, *J* = 8.4Hz) 8.93(1H, d, *J*= 6.9 Hz). $^{13}$C NMR (125 MHz) DMSO-*d6*: δ 55.11, 56.40, 113.67, 127.58, 128.16, 129.24, 129.49, 131.36, 133.88, 158.83, 166.02, 172.04. IR (neat): 3320.0 $cm^{-1}$, 2928.3 $cm^{-1}$, 1724.5 $cm^{-1}$, 1603.1 $cm^{-1}$, 1514.3 $cm^{-1}$,1176.7 $cm^{-1}$; LRMS (EI): 285.1 (M+)

**2-(benzamido)-3-phenylpropanoic acid, IV-19**




A mixture of amino acid ester (10 g, 60.56 mmol) in ether (350 mL) and 2N NaOH (400 mL) was maintained at 0°C for 15 minutes. A diluted solution of benzoyl chloride(7.72 mL, 66.62 mmol) in ether (50 mL) was very slowly added using a dropping funnel. The reaction mixture was brought to room temperature and stirred for 20h. The organic layer was collected and dried over $Na_2SO_4$. The solvent was removed *in vacuo* to give the product in 95.2 % yield, (15.52 g). $^{1}$H NMR (300MHz) DMSO *d6*: δ 3.11 (2H, m), 4.65 (1H, s), 7.15-7.97 (10H, m), 8.76 (1H, d, *J* = 7.5 Hz). $^{13}$C NMR (125 MHz) $CDCl_3$: δ 36.28, 54.35, 126.42, 127.45, 128.24, 128.30, 128.63, 129.15, 129.35, 131.44, 132.93, 133.96, 138.32, 166.50, 173.28. IR (neat): 3400.1$cm^{-1}$, 3278.8 $cm^{-1}$, 2936.0 $cm^{-1}$, 1686.80(br) $cm^{-1}$. LRMS (EI): 269.1 (M+).

**2-(benzamido)-2-phenylacetic acid, IV-20**




$^{1}$H NMR (300MHz), $CDCl_3$: δ: 5.53(d, 1H, *J*=7.5 Hz),7.26-7.88(m, 10H), 8.97(d, 1H, *J*=7.5 Hz, exchangeable), 12.86(s, br, 1H) $^{13}$C NMR (74.4 MHz) DMSO-*d6*: 56.81, 127.67, 127.84, 128.12, 128.16,128.36, 128.53, 129.23, 131.43, 132.83, 133.74,137.14, 166.26, 171.88; IR($CHCl_3$): 2924.5, 1718.8, 1630.1, 1529.8 $cm^{-1}$. EIMS: m/z = 255.0(M+). Anal. Calcd. ($C_{15}H_{13}NO_3$) C, H, N.

## M. References


1. Janvier, P.; Sun, X.; Bienayme, H.; Zhu, J. *J. Am. Chem. Soc.* **2002**, *124*, 11, 2560 – 2567.

2. Breinbauer,R.; Vetter, I. R.; Waldmann, H. *Angew. Chem. Int. Ed. Engl.* **2001**, *41*(16), 2878-2890.

3. Feher, M.; Schmidt, J. M. *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 218-227.

4. (a) Henkel, T.; Brunne, R. M.; Müller, H.; Reichel, F. *Angew. Chem. Int. Ed. Engl.* **1999**, *38*, 643-647. (b) Lee, M. –L.; Schneider, G. *J. Comb. Chem.* **2001**, *3*, 284-289.

5. Abel, U.; Koch, C.; Speitling, M.; Hansske, F. G. *Curr. Opin. Chem. Biol.* **2002**, *6*, 453-458.

6. Bajorath, J. *J. Comput. Aided. Mol. Des.* **2002**, *16*, 431-439.

7. Newman, D. J.; Cragg, G. M.; Snader, K. M. *J. Nat. Prod.* **2003**, *66*, 1022-1037.

8. Boldi, A. M. *Curr. Opin. Chem. Biol.* **2004**, *8*, 281-286.

9. Grieder, A.; Thomas, A. W. *Synthesis* **2003**, 1707-1711.

10. Zou, N.; Liu, J. –F.; Jiang, B. *J. Comb. Chem.* **2003**, *5*, 754-755.

11. Kissau, L.; Stahl, P.; Mazitschek, R.; Giannis, A.; Waldmann,H. *J. Med. Chem.* **2003**, *46*, 2917-2931.

12. (a) Schreiber, S. L.; Nicolaou, K. C.; Davies, K. *Chem. Biol.* **2002**, *9*, 1-2; (b) Webb, T. R. *Curr. Opin. Drug. Discov. Devel.* **2005**, *8*, 303-308. (2)(a) Arya, P.; Joseph, R.; Gan, Z.; Rakic, B. *Chem. Biol.* **2005**, *12*, 163-180; (b) Burke, M. D.; Berger, E. M.; Schreiber, S. L. *Science* **2003**, *302*, 613-618; (c) Burke, M. D.; Schreiber, S. L. *Angew. Chem. Int. Ed. Engl.* **2004**, *43*, 46-58; (d) Kuruvilla, F. G.; Shamji, A. F.; Sternson, S. M.; Hergenrother, P. J.; Schreiber, S. L. *Nature* **2002**, *416*, 653-657.

13. (a) Thompson, L. A.; Ellman, J. A. *Chem. Rev.* **1996**, *96*, 555-560.(b) Balkenhohl, F.; Von dem Bussche-Hünnefeld, C.; Lansky, A.; Zechel, C. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2289-2337.

14. Schreiber, S. L. *Science*, **2000**, *287*, 1964.

15. Brummond, K. M.; Mitasev, B. *Org. Lett.* **2004**, *6*, 2245-2248.

16. Carter, H. E. *Organic Reactions 3*, Jon Wiley and Sons, INC., New York, **1947**, 198-239.

17. Cornforth, J. W. In Clarke, H. T., Johnson, J., Robinson, R., Eds., *Chemistry of Penicillin*; Princeton University Press, Princeton, N. J., **1948**, 688-848.

18. Mukerjee, A. K. *Heterocycles* **1987**, *26*, 1077-1097.

19. George, G. I.; Boge, T. C.; Cheruvallath, Z. S.; Harriman, G. C. B.; Hepperleand, M.; Park, H. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 335-338.

20. Boyd, G. V., In Potts, K. T., Ed., *Comprehensive Heterocyclic Chemistry*, *6*, Pergamon Press, Oxford, UK, **1984**, 177-234.

21. (a) Bergman, J.; Lidgreen, G. *Tetrahedron Lett.* **1989**, *30*, 4597-4600; (b) Benoiton, N. L. *Int. J. Protein. Res.* **1991**, *38*, 285-286.

22. Chen, F. M. F.; Kuroda, K.; Benoiton, N. L. *Synthesis* **1970**, 230.

23. (a) Gothardt, H.; Huisgen, R.; Bayer, H. O. *J. Am. Chem. Soc.* **1970**, *103*, 4340. (b) Padwa, A., In Taylor, E. C.; Weissberger, A., Eds., 1, 3-Dipolar Cycloaddition Chemistry, *General Heterocyclic Chemistry Series*, Jon Wiley and Sons, INC., New Jersey, **1984** .(c) Gribble, G. W., In Palmer, D. C., Ed., Oxazoles: Synthesis, Reactions and Spectroscopy, Volume 60 Part A, *The Chemistry of Heterocyclic Compounds*; Jon Wiley and Sons, INC., New Jersey, **2002**; 473-576.

24. Cativiela, C.; Diaz-de-Villegas, M. D., In Palmer, D. C., Ed., Oxazoles: Synthesis, Reactions and Spectroscopy, Volume 60: Part B, *The Chemistry of Heterocyclic Compounds*; Jon Wiley and Sons, INC., New Jersey, **2002**, 130-299.

25. Bayer, H. O.; Huisgen, R.; Knorr, R.; Schaefer, F. C. *Chem. Ber.* **1970**, *103*, 2581.

26. (a) Funke, E.; Huisgen, R. *Chem. Ber.* **1971**, *104*, 3222. (b) Prajapati, D.; Mahajan, A. R.; Sandhu, J. S. *J. Chem. Soc. Perkin Trans. 1.* **1992**, 734.

27. (a) Gingrich, H. L.; Baum, J. S. In *Oxazoles*; Turchi, I. J., Ed.; Wiley: New York, 1986; Vol. 45, p 731. (b) Potts, K. T. In *1,3-Dipolar Cycloaddition Chemistry*; Padwa, A., Ed.; Wiley: New York, 1984; Vol. 2, p 1. (c) Keating, T. A.; Armstrong, R. W. *J. Am. Chem. Soc.* **1996**, *118*, 2574. (d) Santiago, B.; Dalton, C. R.; Huber, E. W.; Kane, J. M. *J. Org. Chem.* **1995**, *60*, 4947.

28. (a) Bilodeau, M. T.; Cunningham, A. M. *J. Org. Chem.* **1998**, *63*, 2800. (b) Consonni, R.; Dalla Croce, P.; Ferraccioli, R.; La Rosa, C. *J. Chem. Res. Synop.* **1991**, *7*, 188.

29. Huisgen, R.; Gotthardt, H.; Bayer, H. O. *Chem. Ber.* **1970**, *103*, 2368.

30. Nayyar, N. K.; Hutchison, D. R.; Martinelli, M. J. *J. Org. Chem.* **1997**, *62*, 982.

31. (a) Avalos, M.; Babiano, R.; Cintas, P.; Hursthouse, M. B.; Jimenez, J. L.; Light, M. E.; Lopez, I.; Palacios, J. C.; Silvero, G. *Chemistry* **2001**, *7*, 3033. (b) Prasad, K. K.; Petrzilka, T. *Helv. Chim. Acta* **1975**, *58*, 2504.

32. (a) Peddibhotla, S.; Jayakumar, S.; Tepe, J. J. *Org. Lett.* **2002**, *4*(20), 3533-3535. (b) Peddibhotla, S.; Tepe, J. J. *Synthesis* **2003**, *9*, 1433-1440.

33. Peddibhotla, S.; Tepe, J. J. *J. Am. Chem. Soc.* **2004**, *126*(40), 12776-12777.

34. Peddibhotla, S. Lewis acid mediated *in situ* generation of münchnones aimed at diversity oriented synthesis of nitrogen containing dihydroheterocycles, Ph.D. Thesis, Michigan State University, East Lansing, MI, **2004**.

35. (a) Dai, L.; Lin, Y.; Hou, X.; Zhou, Y. *Pure. Appl. Chem.* **1999**, *71*(6), 1033. (b) Lucet, D.; Le Gall, T.; Mioskowski, C. *Angew. Chem. Int. Ed.* **1998**, *37*, 2580. (c) Zhou, X.; Lin, Y.; Dai, L.; Sun, J.; Xia, L.; Tang, M. *J. Org. Chem.* **1999**, *64*, 1331.

36. (a) Jones, R. C. F.; Howard, K. J.; Snaith, J. S. *Tetrahedron Lett.* **1996**, *37*, 1707-1710. (b) Jones, R. C. F.; Howard, K. J.; Snaith, J. S. *Tetrahedron Lett.* **1996**, *37*, 1711-1714. (c) Dalko, P. I.; Langlois, Y. *Chem. Commun.* **1998**, 331-332.

37. (a) Menges, F.; Neuburger, M.; Pfaltz, A. *Org. Lett.* **2002**, *4*, 4713-4716. (b) Herrmann, W. A. *Angew. Chem. Int. Ed.* **2002**, *41*, 1290-1309. (c)Hermann, W. A.; Goossen, L. J.; Speigler, M. Organometallics. **1998**, *17*, 1172. (d) Betschart, C.; Hegedus, L. S. *J. Am. Chem. Soc.* **1992**, *114*, 5010-5017.

38. (a) Bon, R. S.; Hong, C.; Bouma, M. J.; Schmitz, R. F.; de Kanter, F. J.; Lutz, M.; Spek, A. L.; Orru, R. V. *Org. Lett.* **2003**, *5*, 3759-3762. (b) Bon, R. S.; van Vliet, B.; Sprenkels, N. E.; Schmitz, R. F.; de Kanter, F. J.; Stevens, C. V.; Swart, M.; Bickelhaupt, F. M.; Groen, M. B.; Orru, R. V. *J. Org. Chem.* **2005**, *70*, 3542-3553.(c) Dhawan, R.; Dghaym, R. D.; Arndtsen, B. A. *J. Am. Chem. Soc.* **2003**, *125*, 1474-1475. (d) Boland, N. A.; Casey, M.; Hynes, S. J.; Matthews, J. W.; Smyth, M. P. *J. Org. Chem.* **2002**, *67*, 3919-3922.

39. (a) Dardonville, C.; Rozas, I. *Med Res Rev* **2004**, *24*, 639-661; (b) Ueno, M.; Imaizumi, K.; Sugita, T.; Takata, I.; Takeshita, M. *Int. J. Immunopharmacol.* **1995**, *17*, 597-603; (c) Furukawa, O.; Murakami, T.; Sugita, T.; Ueno, M.; Takata, I.; Tosa, T. *Int. J. Immunopharmacol.* **1993**, *15*, 515-519; (d) Schafer, S. G.; Kaan, E. C.; Christen, M. O.; Low-Kroger, A.; Mest, H. J.; Molderings, G. J. *Ann. N. Y. Acad. Sci.* **1995**, *763*, 659-672; (e) Bousquet, P.; Bruban, V.; Schann, S.; Feldman, J. *Pharm. Acta. Helv.* **2000**, *74*, 205-209; (f) Bousquet, P.; Feldman, J. *Drugs* **1999**, *58*, 799-812.

40. (a) Acharya, A. N.; Ostresh, J. M; Houghten, R. A. *J. Comb. Chem.* **2001**, *3*, 612.(b) Concellón, J. M.; Riego, E.; Suárez, J. R.; García-Granda, S.; Rosario Díaz, M. *Org. Lett.* **2004**, *6*, 4499. (c) Acharya, A. N.; Ostresh, J. M; Houghten, R. A. *J. Org. Chem.* **2001**, *66*, 8673. (d) Chern, J. -W.; Liaw, Y. -C.; Chen, C. -S.; Rong, J. -G.; Huang, C. -L.; Chan, C. -H.; Wang, A. H. -J. *Heterocycles* **1993**, *36*, 1091-1103. (e) De Lucca, G. V. *J. Org. Chem.* 1998, 63, 4755-4766.

41. Munk, S. A.; Harcourt, D.; Ambrus, G.; Denys, L.; Gluchowski, C.; Burke, J. A.; Kharlamb, A. B.; Manplapaz, C. A.; Padillo, E. U.; Runde, E.; Williams, L.; Wheeler, L. A.; Garst, M. E. *J. Med. Chem.* **1996**, *39*, 3533.

42. Regunathan, S., Feinstein, D. L., Reis, D. J. *Ann. N. Y. Acad. Sci.* **1999** *881*, 410-419.

43. (a) Gilbert, I. H.; Rees, D. C.; Crockett, A. K.; Jones, R. C. F. *Tetrahedron* **1995**, *51*, 6315. (b) Jones, R. C. F.; Dickson, J. *J. Peptide Sci.* **2000**, *6*, 621-624.

44. Arkin, M. R.; Wells, J. A. *Nature Rev. Drug Discov.* **2004**, *3*(4), 301-317.

45. Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. *Science*, **1996**, *274*, 948-953.

46. Vassilev, L. T.; Vu, B. T.; Graves, B.; Carvajal, D.; Podlaski, F.; Filipovic, Z.; Kong, N.; Kammlott, U.; Lukacs, C.; Klein, C.; Fotouhi, N.; Liu, E. A. *Science* **2004**, *303*, 844-848.

47. Sharma, V.; Lansdell, T.; Peddibhotla, S.; Tepe, J .J. *Chem. Biol.* **2004**, *11*, 1689-1699.

48. Wasserman, H. H.; Hlasta, D. J.; Tremper, A. W.; Wu, J. S. *J. Org. Chem.***1981**, *46*, 2999.

49. (a) Blondeau, P.; van der Lee, Arie.; Barboiu, M. *Inorg. Chem.* **2005**, *44*, 5649-5653. (b) Shan, Shang; Hu, Wei Xiao; Xu, Duan Jun. *Acta Crystallographica, Section E: Structure Reports Online* **2004**, E60 (11), o2059-o2061. (c) Haneline, Mason R.; Tsunoda, Mitsukimi; Gabbaie, Francois P. *J. Am. Chem.Soc.* **2002**, 124(14), 3737-3742.(d) Coates,G. W.; Dunn,A. R.; Henling, L. M.; Ziller, J. W.; Lobkovsky, E. B.; Grubbs, R. H. *J. Am. Chem. Soc.* **1998**, 120 (15), 3641 -3649. (e) Corey, E. J.; Noe, M. C. *J. Am. Chem. Soc.* **1996**, *118*, 319-329.

50. Grigg, R.; Lansdell, M. I.; Thornton-Pett, M. *Tetrahedron* **1999**, *55*, 2025-2044.

# CHAPTER V

## CHECKPOINT KINASE INHIBITORS: AT CROSSROADS TO LIFE AND CELL DEATH

*To be or not to be, that is the question.....William Shakespeare*

### A. Introduction

Despite an improved understanding of the underlying causes of cancer, the disease is on the rise in most developing countries. Most clinical cases are either treated with radiation or some form of chemotherapy. This involves a bi-faceted challenge, to identify new "less-toxic" anti-cancer drugs from the conventional treatments and to improve the existing therapies, so that they become more effective in the battle against cancer. Both radiation as well as chemotherapeutic treatments tend to be more toxic to rapidly dividing cancer cells.[1] However, their specificity towards cancerous cells is far from sufficient. This leads to debilitating side effects that often compromise the efficacy of chemotherapeutic agents in clinic. [2] Resistance, which is a focal point to the failure to cancer therapy often seems to stem from the ability of the tumor to overcome and repair the DNA damage.

DNA replication is a process that requires accuracy and precision and relies on surveillance mechanisms, which monitor DNA damage and initiate DNA repair.[3] DNA repair is a 'double edged sword' in tumor biology. While, the entire repair machinery is vital to the maintenance of genomic integrity, viability and proliferation of normal cells, the introduction of mutations may lead to the development of neoplastic cells and

cancer. Very often, the inability to carry out 'normal' repair functioning on chromosomal insults leads to the transformation of normal cells into malignancies.[4] It is these repair pathways that confer resistance to the genotoxic burden of most established therapies. Thus, in order to utilize the potential of repair mechanisms and develop novel anti-cancer therapies to modulate them, we need a better understanding of the mechanism of these DNA repair pathways. By targeting some of the important proteins in the DNA repair processes it is hoped that, in combination with the existing therapies, we can improve the effectiveness of treatments.

Tumorigenesis is a multi-step process that reveals genetic modifications, which drive the progressive transformation of normal cells into malignant cells. DNA damage fuels this "genomic instability". Due to a plethora of factors (endogenous and exogenous) causing a variety of damages, no single repair process can cope. This has led to numerous sophisticated and interactive repair systems.[5] Multi-cellular organisms respond to chromosomal insults by activating a complex damage response pathway. Upon DNA damage, cell cycle checkpoints get activated, which delay cell cycle progression and allow DNA repair. A multi-faceted involvement of these checkpoint pathways regulates DNA repair, [3,4,6] telomere length[6] and the induction of apoptotic cell death.[3, 8] Protein kinases regulate a host of cellular processes such as growth and differentiation, cell proliferation and apoptosis.[9] DNA damage caused by radiation or chemotherapy triggers the DNA damage-responsive protein kinases ATM and ATR, which activate Chk1 and Chk2. Chk1 and Chk2 in turn phosphorylate Cdc25 and prevent Cdc2 activation, resulting in cell cycle arrest.[10] Hence, small molecules that can

inhibit the checkpoints may enhance the efficacy of DNA damaging chemotherapeutics or radiation therapy.[11]

**B. Synthetic efforts towards natural ends.**

Combinatorial chemistry allows for the synthesis of vast libraries with numerous compounds- millions of compounds are realizable by one chemist on their own in a few weeks using split-pool combinatorial techniques.[12] However, the limitation is that the libraries produced are a focused class of compounds that have limited structural diversity. This technique probably got inspiration from Nature's way of producing primary and secondary metabolites in presence of enzymes. Nature's technique to produce unique bioactive libraries can be considered to be analogous and in harmony to the combinatorial generation of synthetic libraries, each approach providing access to very different types of lead compounds.[13] However, there is a significant difference between these processes. The generation of combinatorial libraries has been constrained primarily by the availability of reagents and suitable reactions. In contrast, the generation of diverse natural product requires subtle chemistry involving variation in restricted number of precursors and the available biosynthetic reactions in the context of a biological utility.

One of the intriguing family of secondary metabolites displayed by marine sponges is the pyrrole-imidazole alkaloid family with members like palau'amine[14] (known as a pharmaceutical from a sponge), oroidin, [15] phakellin,[16] hymenin[17] etc. which provide a wide variety of biological activities (Figure V-1).

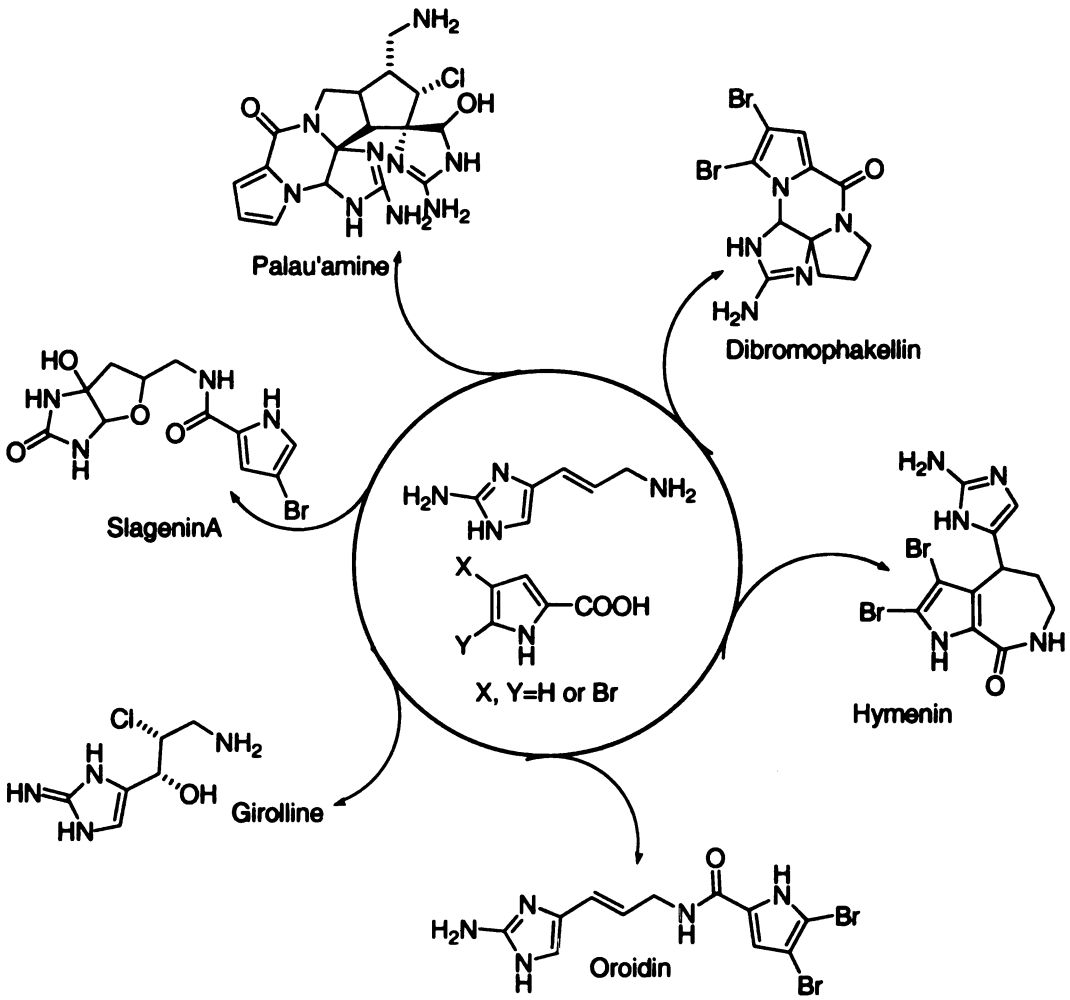



**Figure V-1**. Representative structural groups synthesized by phylum Porifera.

The marine sponge metabolite hymenialdisine (Figure V-2) was originally isolated from the sponges *Hymenialdison aldis*, *Axinella verrucosa* and *Acantella aurantiaca* in early 1980s and its structure was established on the basis of X-ray crystallography.[18a] Hymenialdisine and debromohymenialdisine were shown to possess potent activity against P388 leukemia cell line,[18b] L5178y mouse lymphoma cells[18c] and even some insecticidal activity.[18c] The hymenialdisines form a unique class of natural products including hymenin, stevensine and axinohydantoin (Figure V-2) with a common pyrrolo[2, 3-c]azepine ring attached to a cyclic guanidine or hydantoin ring.

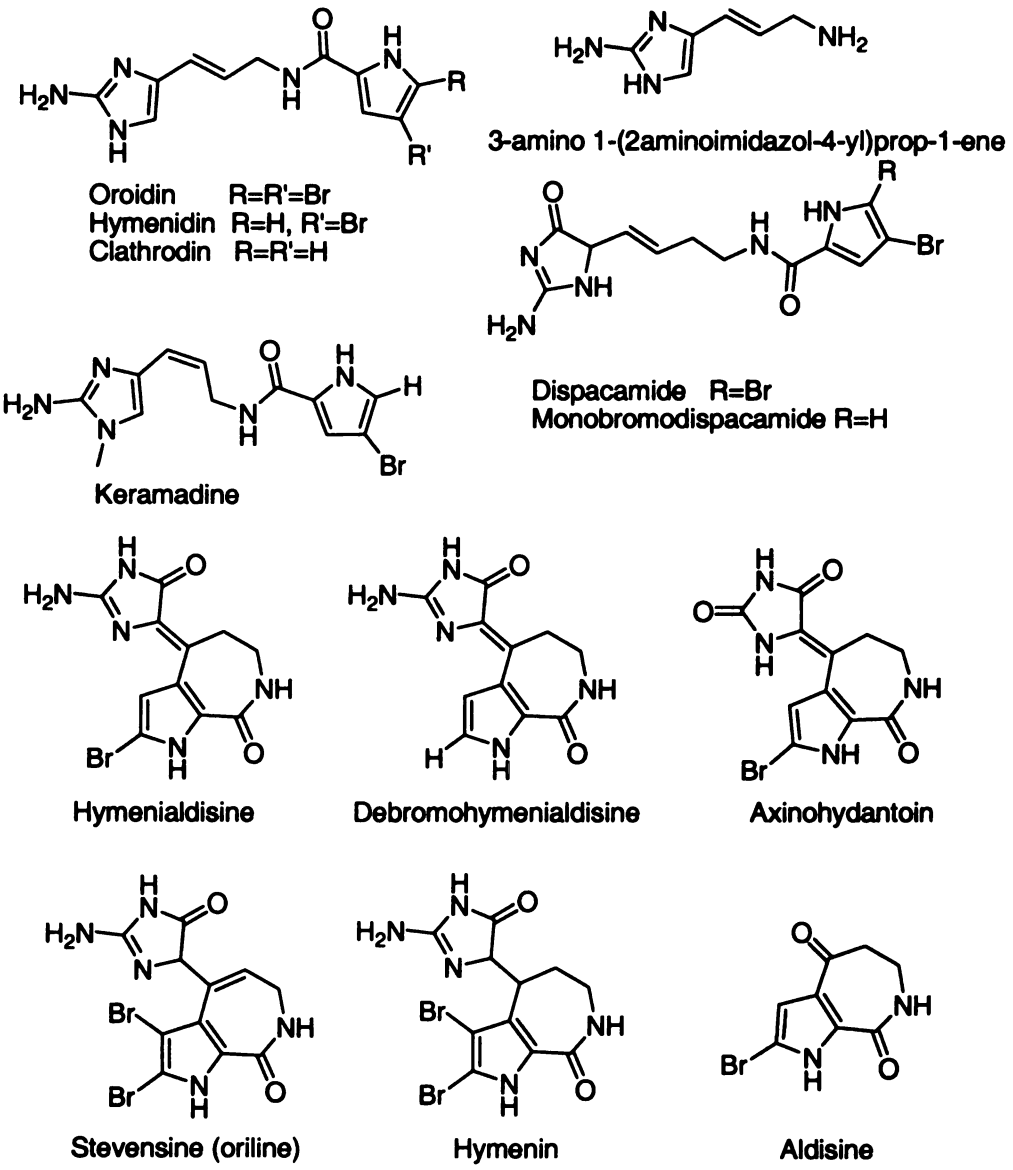



**Figure V-2.** The Oroidin and Hymenialdisine families.

Oroidins however, are chemically distinct due to the presence of the 2-aminoimidazole ring, a brominated or non-brominated pyrrolacarboxamide, and a three C long alkyl chain (functionalized or non- functionalized) connecting these heterocyclic moieties. These are potent antagonists for serotinergic and histaminergic receptors.[19] Hymenialdisine and other oroidin compounds (Figure V-2) have been established to be potent anti-

proliferative, anti- neurodegenerative and anti-inflammatory agents in various cell lines and animal models.[20-23, 26]

**C. Hymenialdisine and kinase inhibition.**

Protein kinases play an essential role in the regulation of a variety of cellular processes such as transcription involving cellular proliferation, growth and metabolism, and apoptosis.[9] Abberant roles in the activity of kinases can lead to diseases such as cancer, alzheimer's disease and diabetes.[27] Several small molecules have been synthesized for kinase inhibition and while most target the ATP binding pocket of the kinase, there are some molecuales which inhibit kinases in a non-ATP binding manner.[28] Most molecules that belong to the former class are heterocycles such as pyrimidines, purines, indolinones, imidazoles etc.[29] Approximately 46 kinase inhibitors are now in clinical trials for targeting cancer, angiogenesis, leukemia, inflammation etc.[20]

The marine sponge metabolites hymenialdisine is a potent inhibitor of a variety of kinases including MEK-1, GSK-3β and CK1.[22] In addition, hymenialdisine and debromohymenialdisine exhibit inhibition of the $G_2$ cell cycle checkpoint at micromolar concentrations.[10] Hymenialdisine was found to be a potent kinase inhibitor of several closely related CDK's.[21] In addition, hymenialdisine was found to be a potent NF-κB inhibitor[20] and acts as a competitive nanomolar inhibitor of the cyclin-dependent kinases, GSK-3β and CK1 with $IC_{50}$'s of 10 nM and 35 nM, respectively.[21] Crystallographic data portrayed the binding of the kinase inhibitor in the ATP binding pocket of the kinases.[22] In addition, Ireland and co-workers have identified hymenialdisine as a very potent

mitogen-activated protein kinase kinase-1 (MEK-1) inhibitor with low nanomolar $IC_{50}$ values ($IC_{50}$ = 6 nM).[23]

Hymenialdisine was also isolated from the sponge *Styllisa massa* as a compound for the inhibition of Raf/Mek-1/MAPK cascade.[23] Raf/MEK/MAPK proteins play a very important role in cellular signaling processes. All eukaryotic organisms conserve this Ras-MAPK signaling cascade (MAPK module, Figure V-3) where it is mainly involved in relaying signals from the extracellular matrix into the cytosol and nucleus.[24]

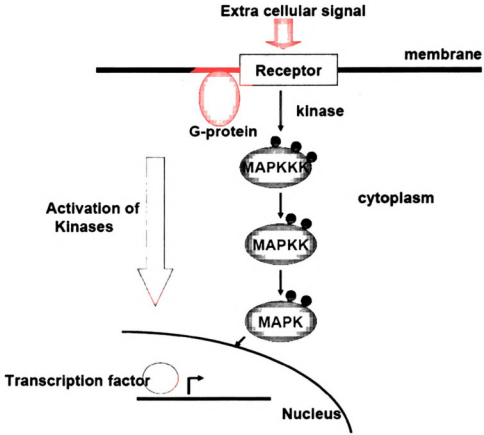



**Figure V-3**. Schematic representation MAPK pathway.

The cascade is activated by 'active' Ras (guanosine 5'-triphosphate-loaded), which recruits Raf-1 to the inner cell membrane. Here, Raf-1 gets phosphorylated and activated.

This activated Raf-1 phosphorylates and activates the kinase, MEK (MAP kinase kinase), on two different serine residues.[24] This triggers a series of phosphorylation events which culminate in activation of transcription factors important in cell survival and differentiation.[25]

**D. Biological relevance of the amino imidazolone ring and aminoimidazoles.**

Heterocyclic compounds have long been known to exhibit remarkable biological and pharmacological properties. While traditional approaches in understanding the mediators in chronic inflammation and cancer include direct target intervention such as use of an enzyme inhibitor, recent advances in elucidation of regulatory mechanisms involved in transcription have shifted the focus at the level of gene transcription. NF-κB is one such important transcription factor that may be therapeutically modulated and find applications in clinical use.

The 2-aminoimidazolone ring is a structural component of compounds that inhibit NF-κB activation and protein kinase C.[21] Meijer *et al*[20] showed a structure based activity relationship with Cdks, CK1 and GSK-3β and propose that the binding pattern of these molecules was similar for all three kinases. Figure V-4 highlights the important regions for the activity of hymenialdisine-like molecules. The activity of glycoamidine ring reduced dramatically when alkylated or acylated. Similarly, the presence of free NH in the indole or pyrrole moiety was important for activity. Further, while halogen substitutions ($R_1$ and $R_2$) did not affect the activity on the pyrroles or indoles, $NO_2$ or $MeSO_2$ reduced the activity.

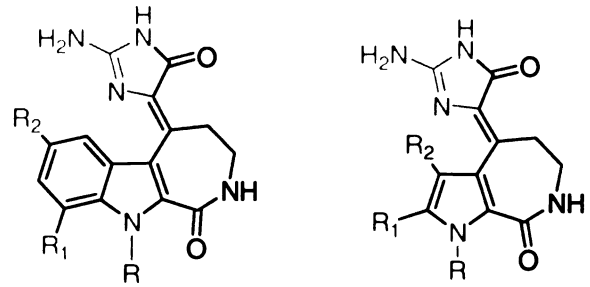
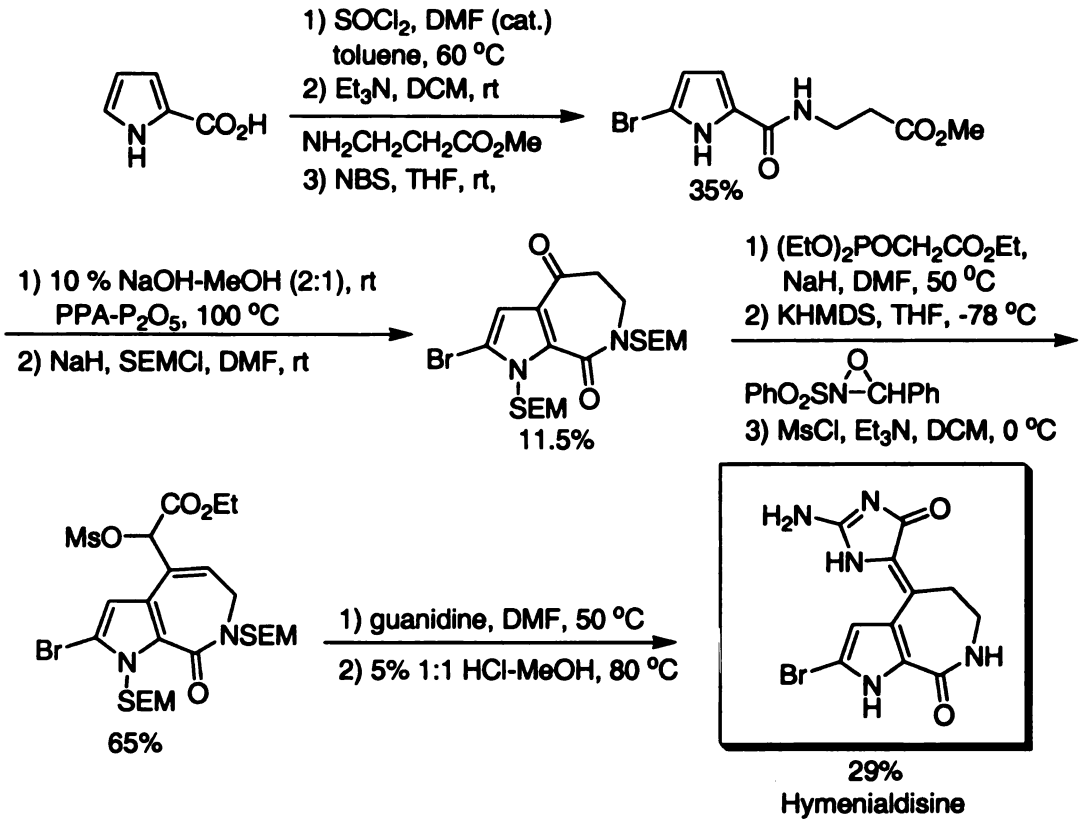



**Figure V-4.** Structure based activity for indoleazepine and hymenialdisine analogs. Functionalities in red are essential and those in blue are non-essential.

## E. Total syntheses of hymenialdisine family.

Due to excellent biological and medicinal activities along with unique structural features, the oroidin and hymenialdisine families have attracted the attention of various research groups. Lindel and Hoffmann showed the synthesis of dispacamide, (a member of the oroidin family assumed to be a direct precursor of pyrroloazepinone such as hymenialdisine) from thiohydantoin.[30] Only three total syntheses have been reported in literature for the synthesis of hymenialdisine. The first synthesis was accomplished by Annoura and coworkers in 1995 [31] and is illustrated in Scheme V-1.

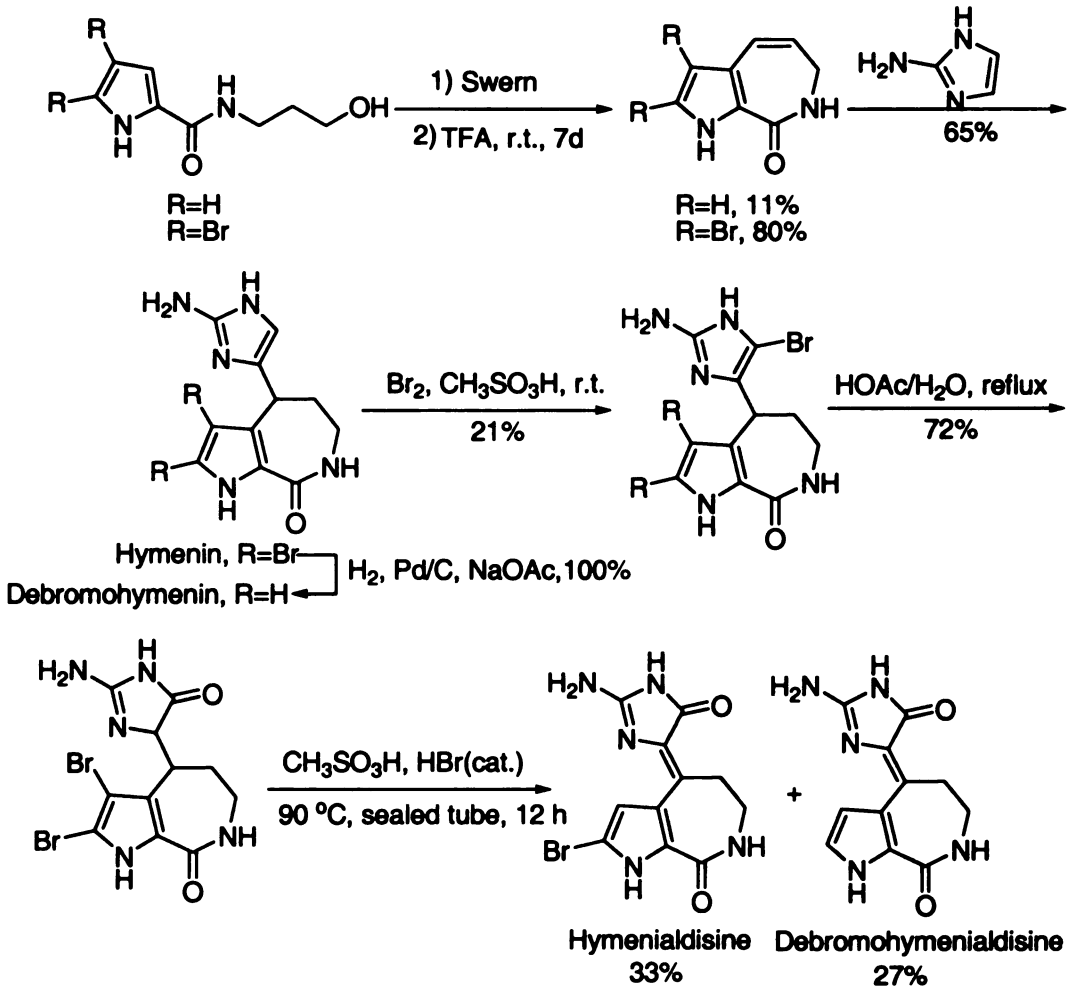



**Scheme V-1.** Total synthesis of hymenialdisine by Annoura *et al.*

Commercially available pyrrole -2- carboxylic acid was treated with $SOCl_2$ /cat.DMF in toluene followed by condensation with β-alanine methyl ester to give the corresponding ester which was then brominated with NBS to afford the 2-bromopyrrole derivative in modest yields. Hydrolysis of the ester and subsequent cyclization in presence of PPA afforded a mixture of 2-bromo and 3-bromo product which could only be separated after conversion into N-SEM protected derivatives. Horner –Emmons reaction with ethyl diethyl phosphonoacetate/NaH gave the α,β- and β,γ-unsaturated esters which when deprotonated with KHMDS in THF generated the ester anion, which was then quenched with 2-benzenesulfonyl-3-phenyloxaziridine (Davies's salt) to give the α-hydroxy-β,γ-

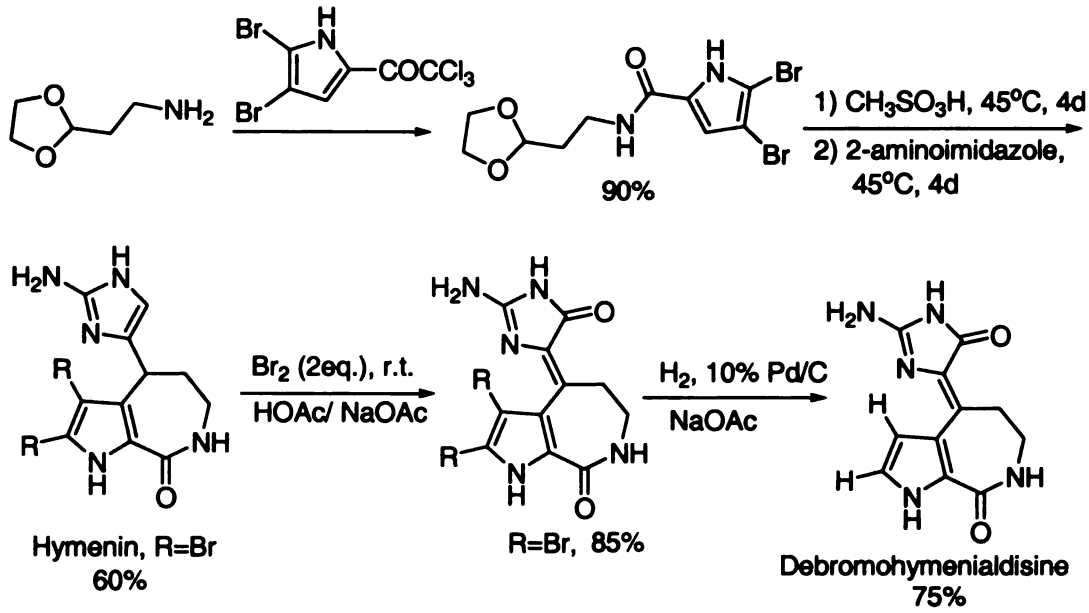
unsaturated esters. These were then cyclised when treated with guanidine in DMF. Finally, SEM groups were deprotected to yield hymenialdisine and debromohymenialdisine in good yields. This synthesis yielded hymenialdisine in overall 0.7% yield in ten steps.

Horne and co-workers synthesized hymenialdisine and debromohymenialdisine in 1997 [32] as shown in Scheme V-2. Most importantly, the key step in the synthesis was the preferential heterodimerization of two azafulvene and 2-aminoimidazole under acidic conditions. This coupling strategy allowed a successful creation of the carbon-carbon bond that connects the two heterocyclic moieties of the natural product. A competing reaction critical to the success of this approach was monomer self-dimerization, which is commonly observed for pyrroles and indoles. However, for 2, 3-dibrominated pyrrole moieties, the self-dimerization was not a problem since the nucleophilic position was blocked (Scheme V-2). A successful route to hymenialdisine required a regioselective debromination and conversion of aminoimidazole moiety to aminoimidazolone on hymenin. Use of mild conditions such as $H_2O$/HOAc yielded 3-bromo-4, 5' dihydrohymenialdisine which when subjected to $CH_3SO_3H$, HBr (cat.) afforded a mixture of hymenialdisine and debromohymenialdisine via a photodebromination/ oxidation step. This synthesis provided hymenialdisine and debromohymenialdisine in overall 2.0% and 0.3% yield in 6 steps. While the synthesis involves some time consuming reaction conditions requiring upto 7 days, the effort is concise and accesses the desired molecules in 6 steps.



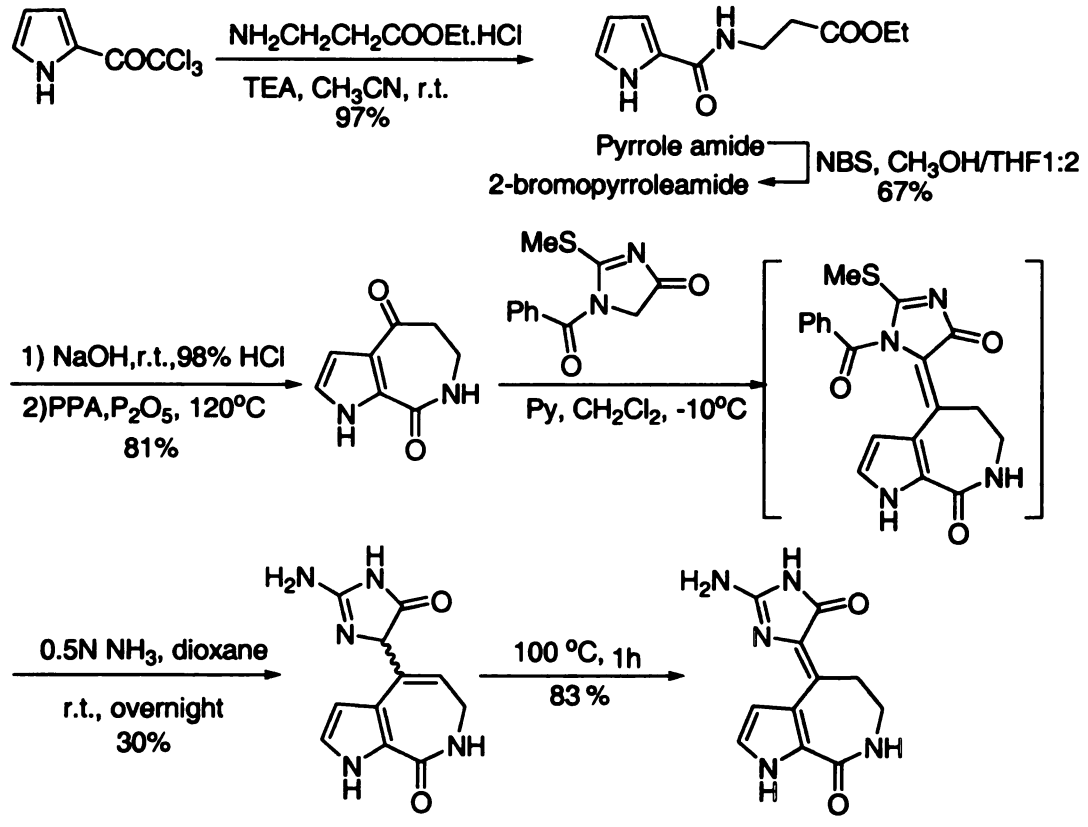

**Scheme V-2.** Total synthesis of hymenialdisine and debromohymenialdsine by Horne *et al.*

Further, Horne and co-workers synthesized debromohymenialdisine in 4 steps in 38% yield starting with 2-(1,3-dioxolan-2-yl) ethanamine and the commercially available 4,5-dibromopyrrol-2-yl trichloromethyl ketone as shown in Scheme V-3.[33]




**Scheme V-3**. Horne's practical synthesis of debromohymenialdisine.

Very recently, in an effort to synthesize the $C_{11}H_5$ marine alkaloids, Papeo *et al* synthesised (*Z*)- hymenialdisine and (*Z*)- debromohymenialdisine with a key step being the condensation between aldisine and the imidazolinone based glycociamidine presursor (Figure V-7).[34] This synthesis afforded hymenialdisine and debromohymenialdisine in 8% and 15% respectively. This synthesis provided a facile methodology to prevent the scrambling of Br under acidic conditions when subjected to cyclization under PPA.[31,32]




**Scheme V-4.** Synthesis of debromohymeniladisine and hymenialdisine by Papeo *et al.*

## F. Synthesis of Indoleazepines

Concurrent to our studies, Meijer *et al* reported the use of affinity chromatography and *in vitro* enzymatic profiling against 60 kinases to identify additional targets for hymenialdisine and its analogs.[20] They adopted a synthetic methodology similar to Horne *et al* to access the indole azepines. As a part of a program to develop analogs of the oroidin family of natural products, and study their important biological targets, we were interested in similar scaffolds (Scheme V-1).

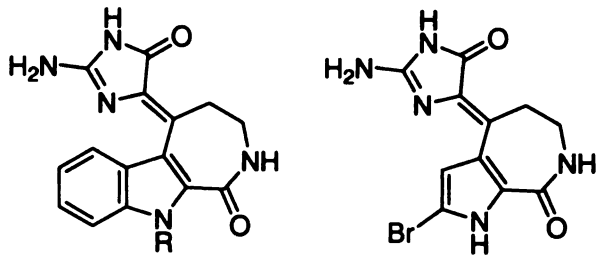



**Figure V-5.** Structure of **V-1**, **V-2** compared to natural product hymenialdisine

In order to keep the synthesis concise, we wanted to conserve the global strategy of condensing the two fragments together as for the natural product. Retrosynthetically, we envisioned the indoleazepine to be derived from the azepine ring (Figure V-6).

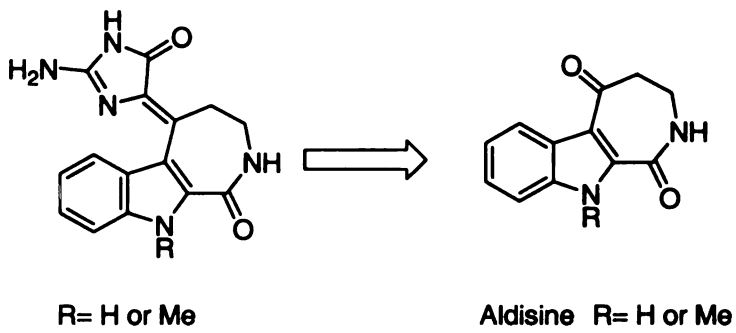
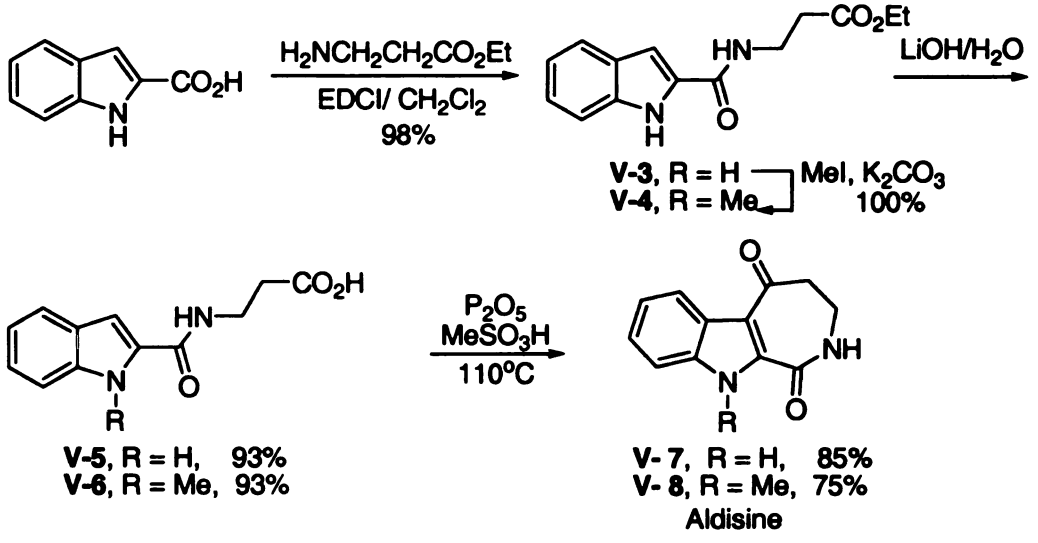



**Figure V-6.** Retrosynthetic analysis for indoleazepine.

The synthesis of the indol-aldisine or indoloazepine sketeton was achieved *via* a slightly modified route as reported in the synthesis of aldisine.[31-34] The aldisine could be synthesized in a similar manner a described by others starting from the indolecarboxylic acid. Starting with the commercially available 2-indolecarboxylic acid, condensation with

the ethyl ester of β-alanine in the presence of EDCI.HCl and DMAP, provided the indole **V-3**. Methylation of the indole nitrogen with MeI and $K_2CO_3$ proceeded in near quantitative yields, rendering the *N*-methyl indole, **V-4**, which was used for the synthesis of **V-2**. Hydrolysis of esters **V-3** and **V-4** followed by the $P_2O_5/MeSO_3H$ mediated cyclization provided the key intermediate aldisine derivatives **V-7** and **V-8** (Scheme V-2).




**Scheme V-5**. Synthesis of N-H (**V-7**) and N-Me aldisine (**V-8**).

## G. Condensation with hydantoin and thiohydantoin to the aldisine.

Lindel and Hoffmann showed the synthesis of dispacamide (Figure V-7), (a member of the oroidin family originally isolated from *Agelas dispar* and assumed to be a direct precursor of pyrroloazepinone such as hymenialdisine) from thiohydantoin.[30] This could then be subjected to methylation at S and nucleophillic displacement by

$NH_3/NH_4Cl$.[35]

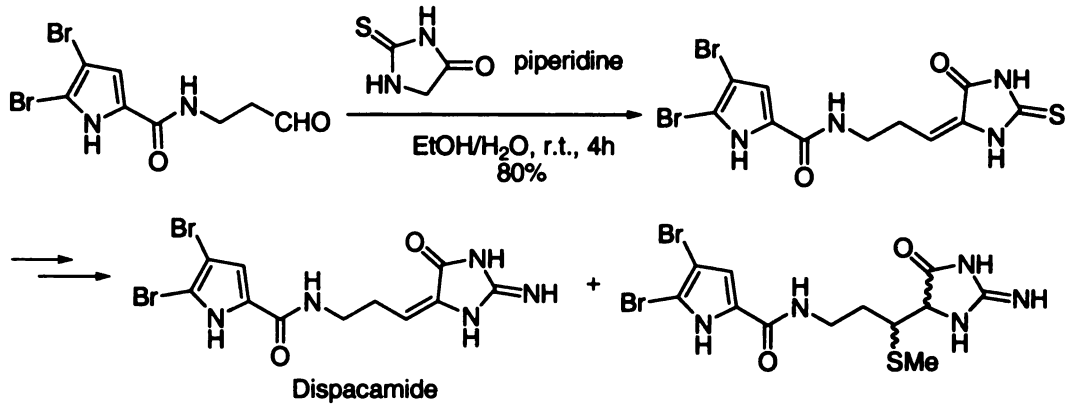



**Figure V-7**. Condensation with thiohydantoin.

This strategy had a two fold advantage, first, it served as a precursor for indoleazepines and secondly, itself was an analog to the natural product axinohydantoin which is an inhibitor of protein kinase C. Horne *et al* have accoplished a linear synthesis of bromoaxinohydantoins and debromoaxinohydantoins from ornithine methyl ester (Figure V-8).[36]

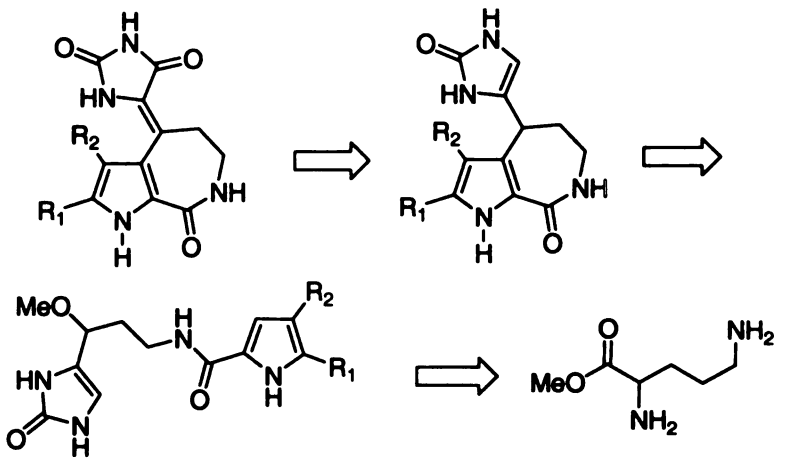
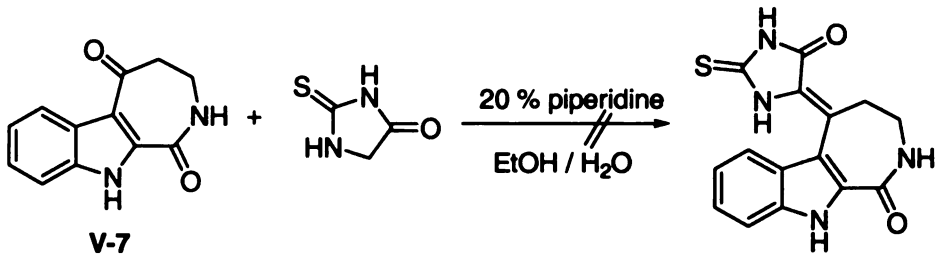


**Figure V-8**. Horne's strategy for the synthesis of axinohydantoin.

However, our efforts to couple the aldisine to thiohydantoin did not afford the desired product at room temperature (Scheme V-6).




**Scheme V-6.** Condensation between aldisine **V-7** and thiohydantoin.

Upon heating, a new product was seen which was partially characterized to be a self-condensation product of the thiohydantoin. The aldisine remained unreacted even after 24 h of reflux. Hence, at this time it was speculated that the aldisine is unreactive to these conditions due to conjugation of the C=O with the aromatic indole system and probably requires more rigorous conditions.

Meanwell *et al* have shown Horner-Wadsworth-Emmons reaction at C-5 of the hydantoin and aldehydes and ketones with conjugated systems such as isatin under basic conditions. The reaction proceeds via the formation of the phosphonate of the hydantoin.[37]

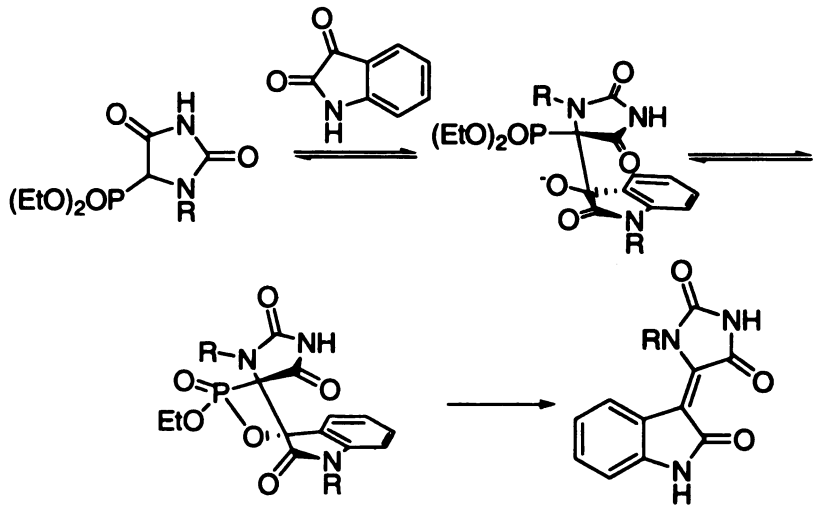
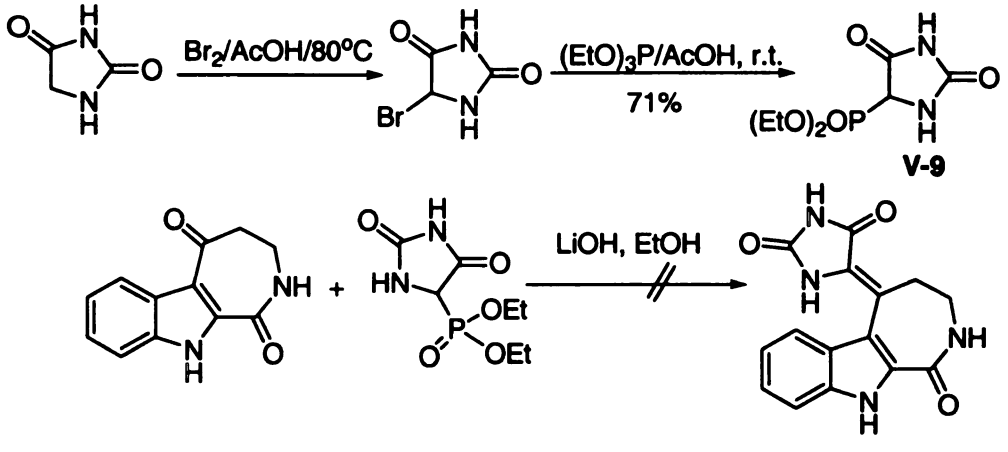


**Figure V-9.** Condensation of isatin with hydantoin phosphonate.

However, our attempts to use this strategy proved to be futile presumably due to a sterically encumbered betaine ring in the transition state and a poor electrophilic carbonyl group in the aldisine. These results correlated to their results with no or poor yields for cyclopentanone and acetophenone (Scheme V-7).



**Scheme V-7.** Preparation of C-5 unsaturated hydantoin derivatives under Horner-Wadsworth-Emmons conditions.

We revised our synthetic strategy and envisioned that the analogs **V-2** and **V-3** could be synthesized by condensation of aldisine and the oxazalone moeities to give a scaffold

similar to the natural product. The key step in this strategy (Figure V-10) involved self condensation products making the purification challenging.

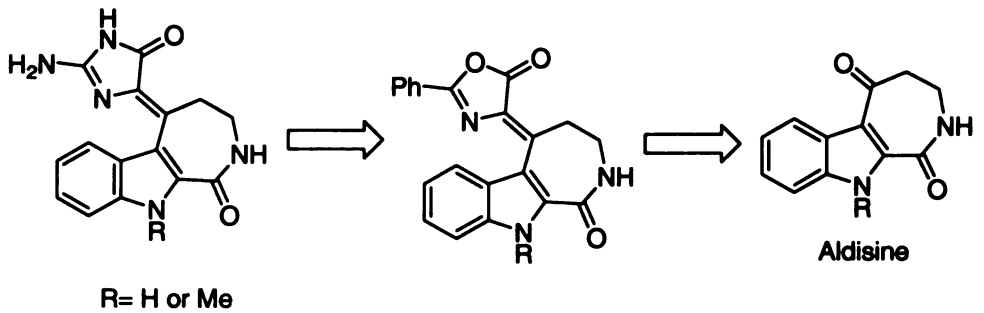



**Figure V-10.** Revised retrosynthesis for **V-2** and **V-3**.

Prager and Tsopelas [38a] have shown a knoevenagel reaction with diethylmalonate which was then used to undergo nucleophilic substitution and which could further cyclize to yield hymenialdisine.

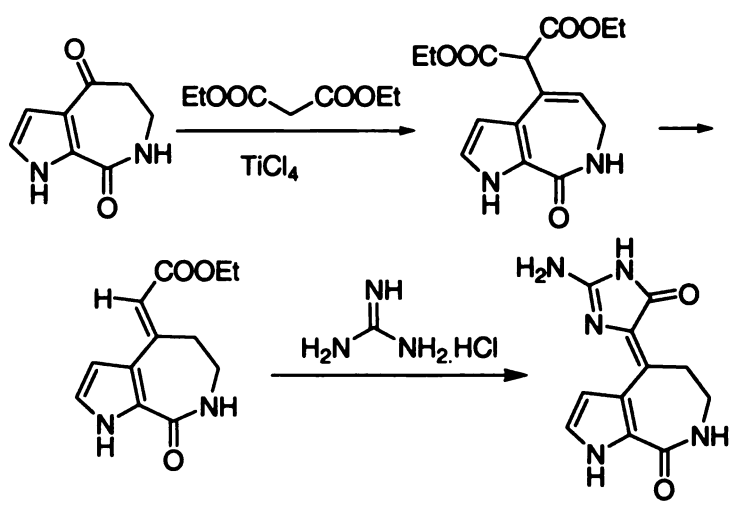
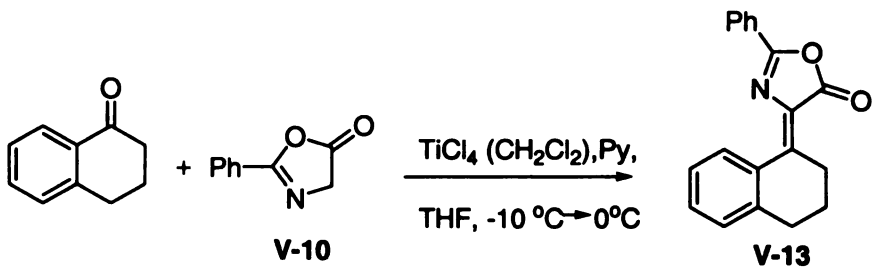


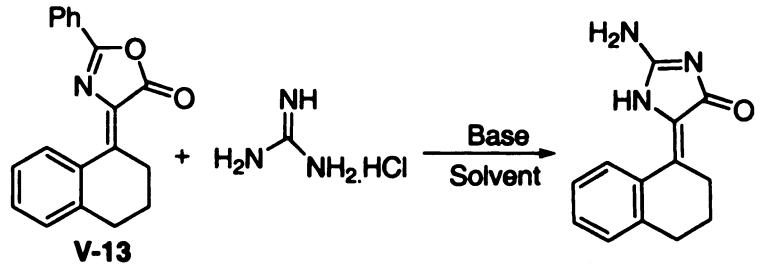

**Figure V-11.** Prager's Knovenegel's approach to synthesize debromohymenialdisine

We devised a model system for quickly test various conditions the conversion of azlactone into the imidazolone (as in **V-2**). α-Tetralone was used as a model for aldisine and was coupled with azlactone (previously synthesized from hippuric acid using EDCI) under slightly modified lewis acid mediated knoevenagel conditions described by Prager and Tsopelas (Scheme V-8).




**Scheme V-8.** α-Tetralone condensed to oxazolone under knoevenagel conditions.

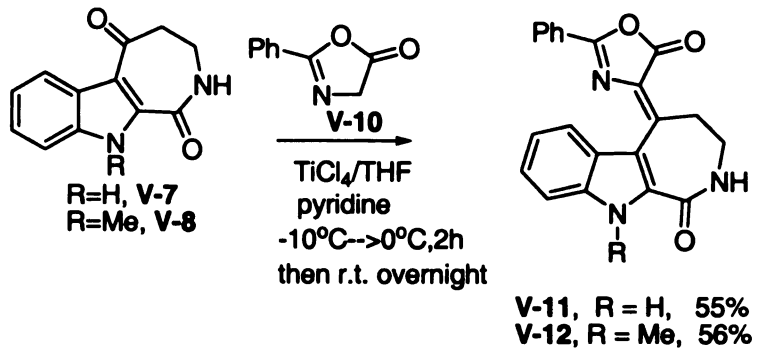
This azlactone coupled tetralone was then subjected to nucleophillic ring opening using guanidine under various alkaline conditions. Table V-1 illustrates the efforts towards this ring opening reaction.




| Base | Solvent | Result |
|---|---|---|
| None | Toluene, reflux | starting material decomposed |
| TEA | Toluene, reflux | starting material decomposed |
| $K_2CO_3$ | Dioxane, reflux | starting material decomposed |
| glacial AcOH, KOH | water, reflux | traces of product |

**Table V-1.** Nucleophilic substitution with guanidine on model compound **V-13.**

Several efforts involving 2-methylazlactone condensed to tetralone **V-14** as well as aldisine **V-15** were abandoned due to instability of the compounds. The 2-methyl azlactone was particularly susceptible to hydrolysis on contact with moisture. Similar to scheme V-8, $TiCl_4$ mediated aldol condensation with the phenyl oxazolone **V-10** provided the oxazolone successfully in 55% and 56% yield for compounds **V-11** and **V-12.**




**Scheme V-9**. Knoevenagel condensation between aldisine (**V-7**, **V-8**) and azlactone (**V-10**).

Previously, other researchers in our group (Dr. Guang Yi Jin and Kevin W. Anderson)[38b] had used modified procedures of those described in Scheme V-9 in attempted syntheses of hymenialdisine, debromohymenialdisine and the N-methyl indoleazepine.

Mukerjee *et al* have shown a TEA mediated reaction of S-benzylisothiouronium chloride with unsaturated 2-oxalidinon-5-one to synthesize the glycoamidine ring.[39]

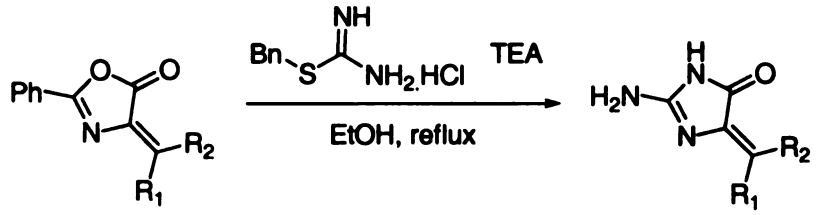



**Figure V-12**. Mukerjee's TEA mediated conversion of oxazolone into imidazolone.

Fu *et al* have shown a solid phase synthesis of 2 aminoimidazolones.[40]

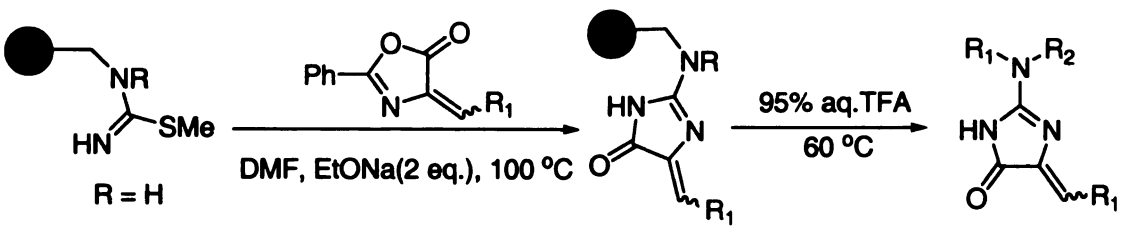
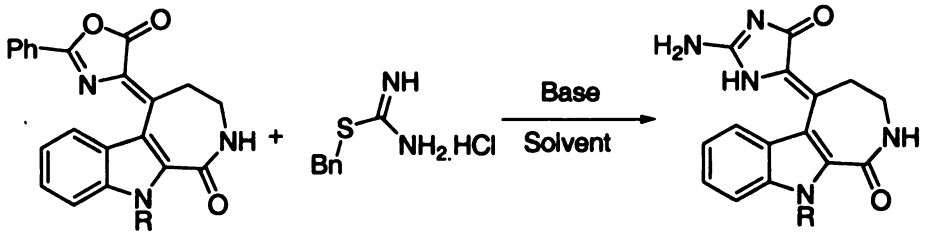



**Figure V-13.** Solid support synthesis of 2-aminoimidazolones.

Treatment of the oxazolone derivatives **V-9** and **V-10** with the S-benzyl thiourea under basic conditions provided the final products **V-1** and **V-2** in modest yields. Table V-2 illustrates the various reaction conditions used. We modified our reaction conditions based on studies done earlier by Dr. GuangYi Jin in our laboratory.




R=H, **V-11**
R=Me, **V-12**

R=H, **V-1**
R=Me, **V-2**

| Entry | Base | Solvent | Result |
|---|---|---|---|
| 1 | 1.5 eq. TEA | EtOH, reflux | Ring opening, ethyl ester formed |
| 2 | 3 eq. TEA | EtOH, reflux | Ring opening, ethyl ester formed |
| 3 | 3 eq. TEA | EtOH, sealed tube | Ring opening, ethyl ester formed |
| 4 | 10 eq. NaOEt | EtOH, reflux | traces |
| 5 | 2eq. NaOMe | DMF | traces |
| 6 | LiH, 5eq. | EtOH | 28% |
| 7 | LiH, 5eq. | dioxane | SM decomposed* |
| 8 | LiH, 5eq. | t-BuOH | SM decomposed* |

* generation of ketene caused rapid decomposition of the starting material

**Table V-2.** Nucleophilic ring opening of the azlactone under alkaline conditions.

Use of DMF as a solvent instead of EtOH only afforded the product in trace amount. Generation of LiOEt using LiH and ethanol (entry 6 in table V-2), afforded the product and the synthesis for **V-1** and **V-2** was accomplished in five steps and overall yield of 12 % and 31% respectively.[26, 41] All our attempts in using high boiling, polar but aprotic solvents led to decomposition of the starting material presumably due to the formation of the ketene from the azlactones **V-11** and **V-12**. Compound **V-2** was successfully crystallized from methanol and its crystal structure is illustrated in Figure V-8. Clearly indicated in this crystal structure is the *Z*- double bond at the C(8)-C(9) position of **V-2** as found in the natural product hymenialdisine (Figure V-14).

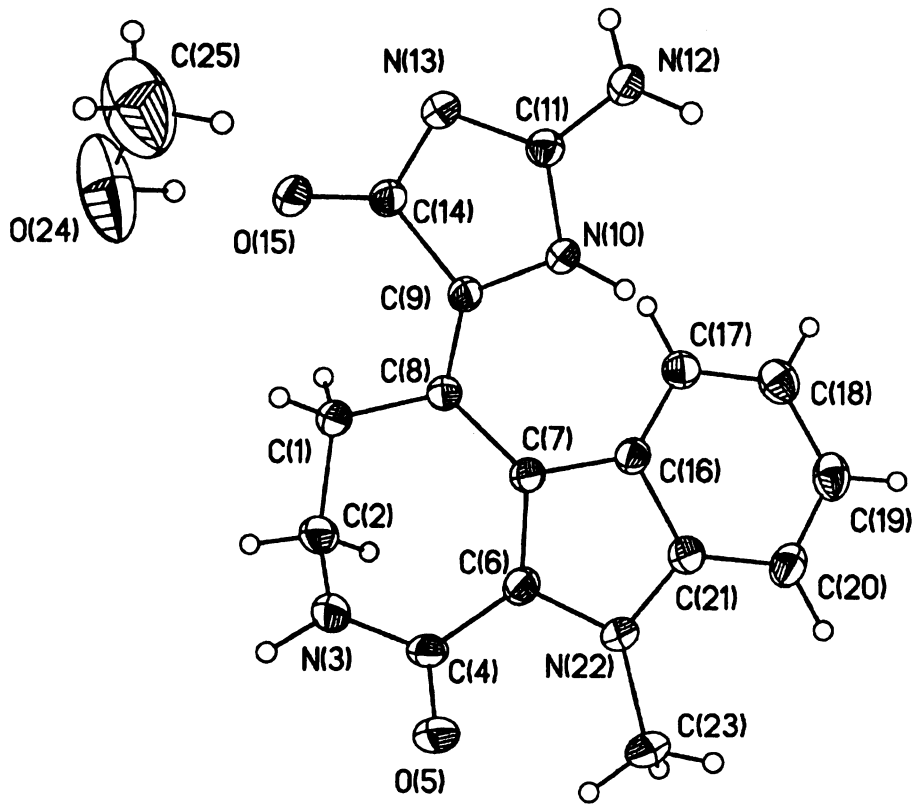
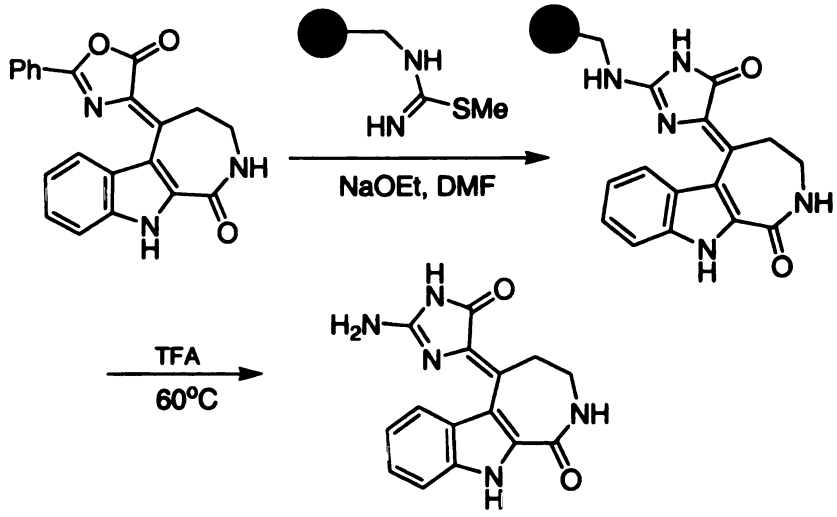



**Figure V-14.** X-ray crystal structure of **V-2**.MeOH.

The purification steps for **V-1** and **V-2** were very tedious. A more advantageous method for incorporating the aminoimidazolone ring could be through a solid-phase method which might make the purification easier and might increase the yield. We tried to use the solid phase synthesis used by Fu *et al* earlier.[40]

Accordingly, Rink Amide MBHA resin was treated with FMoc-NCS in $CH_2Cl_2$ to afford the FMoc protected isocyanate addition product. This was then deprotected with 20% piperidine in DMF and methylated in DMF to yield S-methyl isothiouronium salt on solid support. Treatment of the α, β unsaturated oxazolone derivative **V-11** was treated with the S-methyl isothiouronium salt on solid support. Unlike our expected result, this strategy yielded the product only in very small traces probably due to the sterically demanding ketone functionality (Scheme V-10) and has now been abandoned.




**Scheme V-10.** Solid support synthesis of **V-1.**

## H. Studies towards imidazole based hymenialdisine analogs

We have shown the synthesis of the indole based derivatives of hymenialdisine. We turned out focus now to imidazole based analogs **V-16** and **V-17** (Figure V-15).

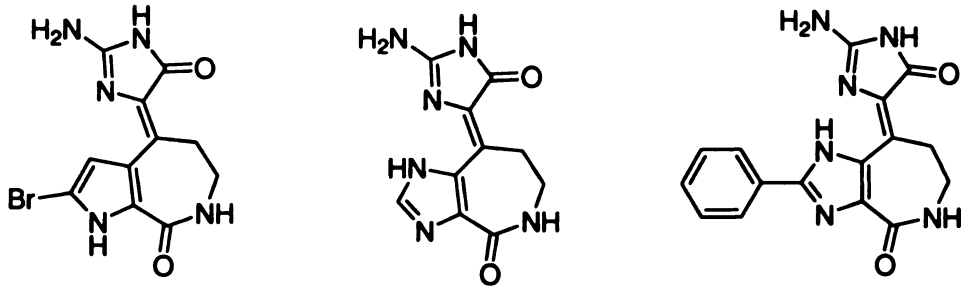



**Figure V-15.** Structures of imidazole based **V-16** and **V-17** compared to hymenialdisine.

Previously, as part of our program to develop small molecular weight scaffolds containing a high degree of diversity, our laboratory focused on developing a stereoselective synthesis of substituted imidazolines.[42] In 1970, Steglich reported that 4-(dimethylamino) pyridine (DMAP) and 4-(pyrrolidino) pyridine (PPY) catalyze the rearrangement of *O*-acylated azlactones to their *C*-acylated isomers, thereby generating both a new carbon-carbon bond and a new quaternary stereocenter (Figure V- 16).[43]

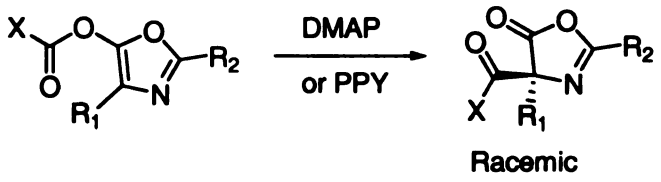
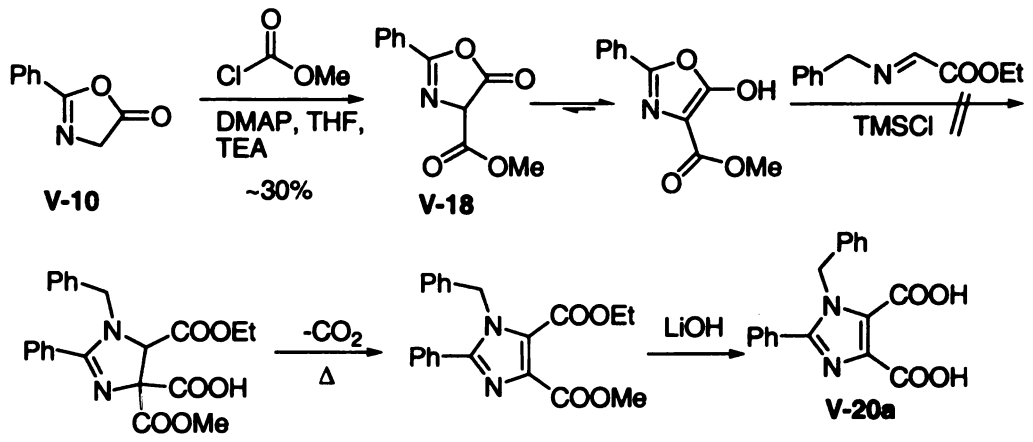



**Figure V-16**. Rearrangement of *O*-acylated product to *C*-acylated isomer.

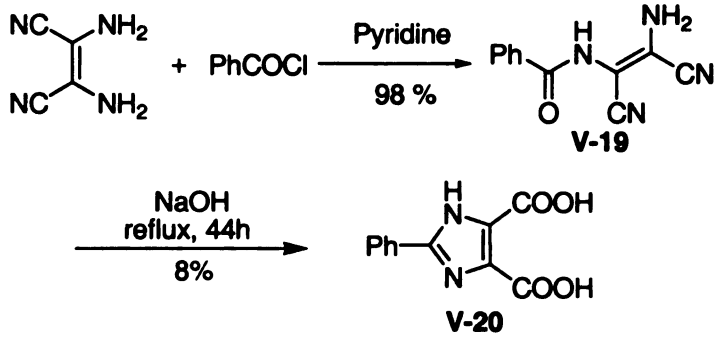
Fu *et al* have used planar chiral derivatives of DMAP as enantioselective catalysts for this rearrangement. [44] We examined our in-house 1, 3 dipolar cycloaddition to furnish for the

synthesis of **V-20**. Hippuric acid was first converted into the oxazalone in presence of EDCI.HCl to give **V-10** in 80% yield. **V-10** was then subjected to C-4 acylation using methyl orthochloroformate in presence of TEA and DMAP in 30% yield (Scheme V-11). However, **V-18** was found to occur primarily in the enol form which did not afford the imidazoline when subjected to the TMSCl mediated 1, 3 dipolar cycloaddition reaction conditions due to the inability for the setting up of the dipole.




**Scheme V-11**. Attempted conversion of hippuric acid to 2-phenyl imidazole dioic acid

Our next strategy was a modified route described by Daly *et al*[46] for synthesis of 8-phenyl xanthines. DAMN was condensed with PhCOCl in presence of pyridine to yield mono-acylated DAMN (**V-19**) in nearly quantitative yield. **V-19** was then subjected to alkaline hydrolysis in presence of NaOH, which yielded only 8% of the desired product (Scheme V-12).




**Scheme V-12.** Conversion of DAMN into 2-phenyl imidazole-diacid.

Based on the unsuccessful attempts for synthesis of the starting material (commercially available at $250 per 10mg) we revised the retrosynthetic plan to start with tartaric acid (commercially available $ 7 per 25g) (Figure V-17).

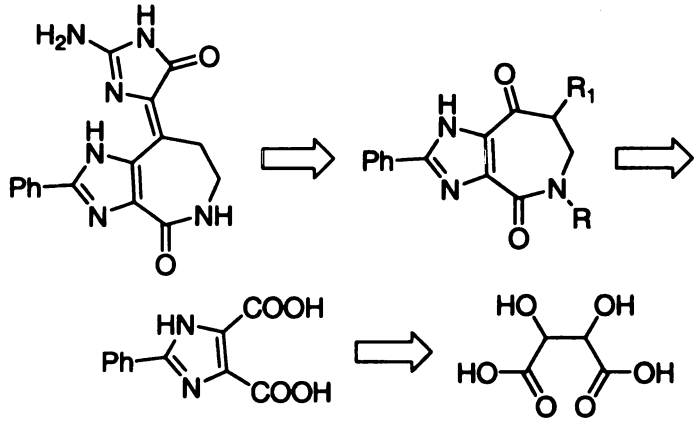
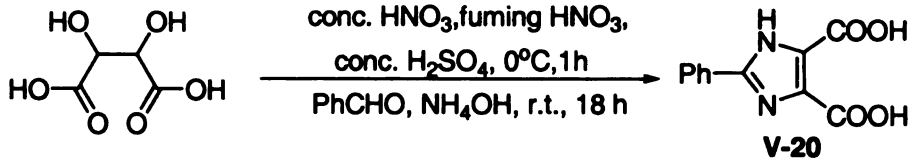


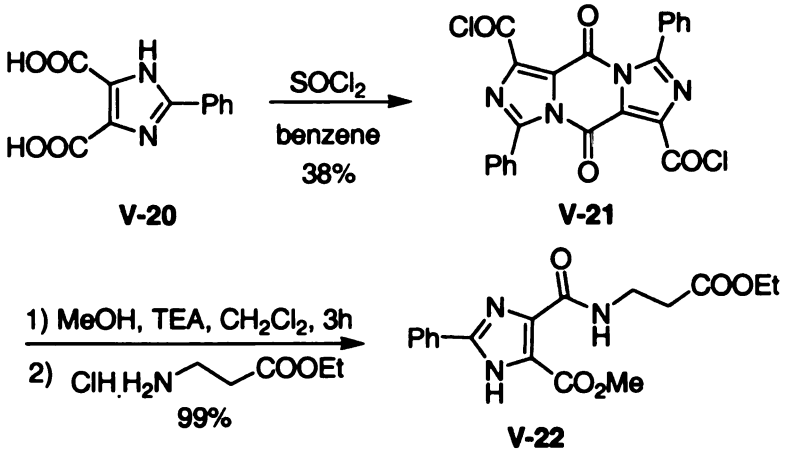

**Figure V-17.** Retrosynthetic analysis for **V-20**.

Tartaric acid was treated with conc. $HNO_3$, fuming $HNO_3$, and conc. $H_2SO_4$ at 38 °C which was cooled for 1h in an ice bath to form the dinitrate (Scheme V-13) which was neutralized and condensed with PhCHO to form **V-20**.




**Scheme V-13.** Synthesis of **V-20** from tartaric acid.

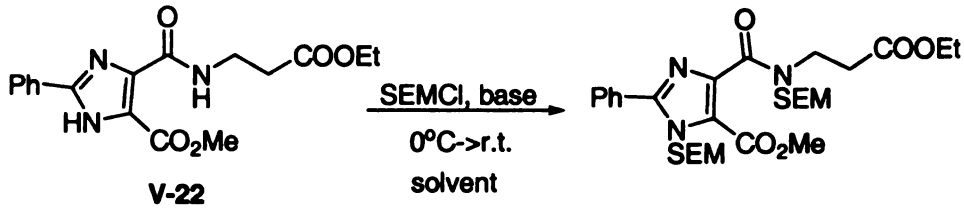
**V-20** was treated with $SOCl_2$ to afford the acid chloride which underwent self condensation to give a dimer, **V-21** (Scheme V-14).[48] The unstable acid chloride dimer was first converted to the methyl diesters and the dimer was finally cleaved with β-alanine ethyl ester to afford **V-22** in 99% yield. Some initial steps of this synthesis were based on efforts by Dr. Guang Yi Jin who used a N-Bn protected amine to open the dimer ester. We optimized these reaction conditions (Scheme V-14) and used SEM and PMB group on the N.




**Scheme V-14.** Conversion of **V-20** to **V-22** via a dimerized acid chloride.

Our next step was to protect the imidazolic and amide nitrogen and cyclize the molecule via a favored 7 *exo*-trig Dieckmann's cyclization reaction (Scheme V-15). Our first choice for this reaction was SEM protection as used by Annoura *et al.* [31] SEM protections

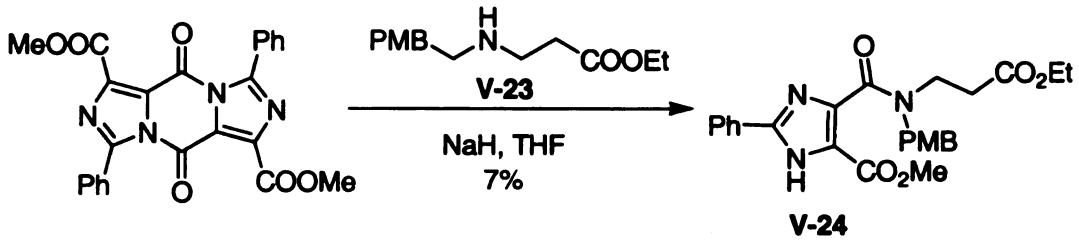
on imidazoles has also been shown by several other groups.[49] However, all attempts to protect both the imidazole and the amide were futile and the reaction decomposed during isolation on silica column.




| Entry | Solvent | Base | Yield isolated |
|---|---|---|---|
| 1 | DMF | NaH, 2.1eq | 0% |
| 2 | THF | KH, 2.1eq | 0% |

**Scheme V-15.** SEM protection on the imidazole ring.

An excess upto 10 eq. base to carry out the protections and the cyclization as a one pot procedure was also unsuccessful. At this point we revised our earlier approach for the ring opening of the dimer with a primary amine to a secondary amine (Scheme V-16). We targeted two secondary amine, N-benzyl –β alanine ethyl ester and N-p-methoxybenzyl-β-alanine ethyl ester **V-23**. Previous studies by Dr. Guang Yi Jin, showed that deprotection of N-benzyl group from the azepine ring was tedious and had to be abandoned. Use of PMB group however was tedious at the ring opening step since the amine was more active and light sensitive. Purification on silica gel led to decomposition of a lot of product material.




**Scheme V-16.** Ring opening with secondary amines.

We tried reversing the sequence of addition for conversion from acid chloride dimer to the 4, 5 substituted imidazole ring. However, none of these efforts improved the results any further. **V-24** was then converted to the N-benzylated product **V-25** under Finkelstein conditions and was subjected to KHMDS for cyclization. Preliminary results showed a successful reaction, though further optimization is required towards the total synthesis of **V-17.**

**I. Kinase inhibition with indoleazepines**

Roberge and co-workers have shown the inhibition of the $G_2$ DNA damage checkpoint by debromohymenialdisine as well as hymenialdisine in cells.[10] Inhibition of Chk1 and Chk2 by debromohymenialdisine was reported to be at $IC_{50}$ values of 3 μM and 3.5 μM, respectively. Both debromohymenialdisine and hymenialdisine showed comparable $IC_{50}$ values for inhibition of $G_2$ checkpoint at 8 $\mu$M and 6 $\mu$M, respectively. This indicates that the bromine at the C2 position of hymenialdisine did not significantly influence the inhibition of the $G_2$ checkpoint.[10]

The indole-based hymenialdisine derivative **V-1** was found to contain potent anti-inflammatory activity as well as anti-proliferative activity.[26] Concurrently with our

studies, the inhibition of purified GSK-3β, CDK5/p25 and CDK1/cyclin B by several hymenialdisine derivatives was investigated; the indole analogues exhibited kinase inhibition profiles similar to those of the pyrrole-based compound.[20]

We discovered potent inhibitory activity of indoloazepine **V-1** against checkpoint kinases Chk1 and Chk2. **V-1** and **V-2** were sent to Upstate N. Y. for kinase inhibition studies. Table V-3 illustrates the comparison between the *in vitro* activity of indoloazepine **V-1, V-2** hymenialdisine and debromohymenialdisine. Compound **V-1** exhibits very potent inhibition of Chk2 activity in the low nanomolar range. Interestingly, unlike the natural product hymenialdisine and debromohymenialdisine, compound **V-1** exhibits also very potent selectivity for the checkpoint kinase Chk2.

| **Kinase** | **V-1** | **V-2** | **Hymenialdisine $IC_{50}$(nM)** | **Debromohymenialdisine $IC_{50}$(nM)** |
|---|---|---|---|---|
| CK1(h) | 1352 | >10000 | 35 | NA |
| CK2(h) | >10000 | >10000 | 7000 | NA |
| MEK1(h) | 89 | >10000 | 6 | 824 |
| PKC(h) | 2539 | >10000 | 700 | NA |
| PKCII(h) | 3381 | >10000 | 1200 | NA |
| Chk1 | 237 | >10000 | 1950 | 725 |
| Chk2 | 8 | 12500 | 42 | 183 |

**Table V-3.** $IC_{50}$ values for kinase inhibition by compound **V-1, V-2,** hymenialdisine and debromohymenialdisine. NA: value not available.

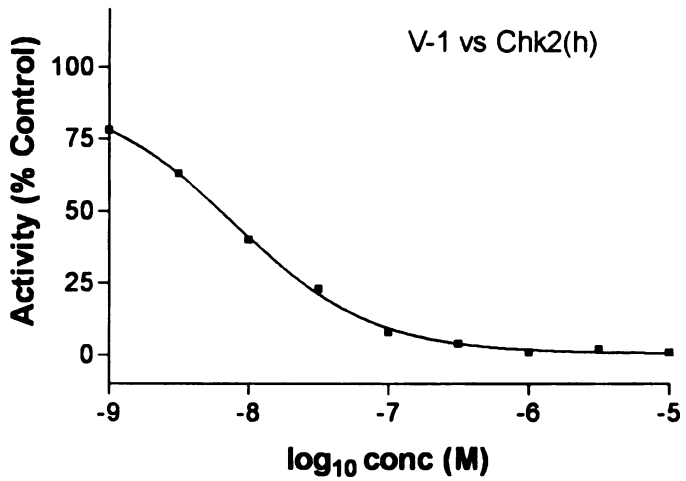



**Figure V-18**. Dose dependence activity of Chk2 with varying **V-1.**

These kinase inhibition studies suggest that analogs of hymenialdisine may improve its overall kinase profile and significantly affect its kinase selectivity. Further *in vivo* studies would help in validating the drug therapeutic potential of hymenialdisine analogs for cancer therapy and neurodegenerative diseases.

**J. Inhibition of cytokine production by hymenialdisine derivatives**

Elevated levels of cytokines, such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin–6 (IL-6), interleukin–8 (IL-8) and tumor necrosis factor-α (TNF-α) have been linked to many disorders including Crohn's disease and psoriasis. In addition, these proinflammatory cytokines play an essential role in the pathogenesis of rheumatoid arthritis and osteoarthritis.[50-52] The inhibition of cytokines, in particular TNF-α, has been successful in several clinical trials for the treatment of rheumatoid arthritis.[53] However, variability in responses to these anti-inflammatory drugs is found due to the complex network of alternative cytokine mediated pathways.[54] Inhibition of transcription factors

that control the expression of several pro-inflammatory mediators, such as the nuclear transcription factor NF-κB, may overcome these problems and provides an alternative strategy in the treatment of these inflammatory disorders.[55] Due to its critical role in the regulation of inflammatory responses, NF-κB has become an increasingly significant therapeutic target in diseases such as asthma, rheumatoid arthritis, multiple sclerosis and Alzheimer's disease.[56]

Hymenialdisine was found to inhibit various proinflammatory cytokines, such as IL-1, IL-6, IL-8 and nitric oxide in a variety of cell lines.[21] Investigation of the promising anti-inflammatory properties of hymenialdisine revealed that the novel bromopyrrole alkaloid inhibits cytokine production through inhibition of the NF-κB signaling pathway. Gel shift analysis indicated that hymenialdisine selectively reduced NF-κB nuclear binding and not the DNA binding of other transcription factors such as C/EBP, AP-1 or SP1.[21] Recently, Meijer *et al* reported that the potent NF-κB inhibitor hymenialdisine acts as a competitive nanomolar inhibitor of the cyclin-dependent kinases, GSK-3β and CK1.[22] Crystallographic data portrayed the binding of the kinase inhibitor in the ATP binding pocket of the kinases. Considering the potential relationship between NF-κB activation and GSK-3, this might suggest a potential pathway for this kinase inhibitor.[27] In addition, Ireland and co-workers have recently also identified hymenialdisine as a very potent mitogen-activated protein kinase kinase-1 (MEK-1) inhibitor with low nanomolar $IC_{50}$ values.[23]

**K. Indoleazepines inhibit PMA/PHA induced cytokine production in jurkat cells and LPS induced TNF-α production in macrophages.**

Hymenialdisine and indoloazepines **V-1** and **V-2** were evaluated for their potential anti-inflammatory activity by examining the transcriptional activity of NF-κB in human Jurkat leukemia T-cells and THP-1 cells (human monocytic leukemia cell line) by other researchers in our laboratory. Compound **V-1** was shown to inhibit IL-2 and TNF-α production, whereas the methylated product **V-2** was depleted of any significant activity. The natural product hymenialdisine was found to inhibit IL-2 and TNF-α production as well, with similar $IC_{50}$ values. Exposure of human Jurkat leukemia T-cells to phorbol myristate acetate (PMA/PHA) and THP-1 cells to lipopolysaccharide (LPS), activates the NF-κB mediated gene transcription of several pro-inflammatory cytokines, including IL-2, IL-6, IL-8 and TNF-α. The anti-oxidant and non-selective NF-κB inhibitor, pyrrolidinedithiocarbamate (PDTC) has been reported to repress activation of NF-κB and was used as a control in all experiments. The effect of hymenialdisine and the indoloazepines **V-1** and **V-2** on NF-κB's transcriptional activity was evaluated by measuring the level of IL-2 and TNF-α production using a competitive enzyme immunoassay (EIA). The inhibition of IL-2 was evaluated in Jurkat cells after PMA/PHA activation.[57] The inhibition of TNF-α was evaluated in THP-1 cells after LPS activation. NF-κB mediated transcription of IL-2 production was examined by exposing the cells to PDTC or compounds **V-1, V-2,** or hymeniladisine, 30 minutes prior to PMA activation. After 24 hours, the cell free supernatants were collected and subjected to EIA for the quantification of total IL-2 production.

| Compound | IL-2 production $IC_{50}(\mu M)$ | TNF-α production $IC_{50}(\mu M)$ |
|---|---|---|
| PDTC | 5.12 (+/- 0.41) | 6.11 (+/- 0.53) |
| Hymenialdisine | 2.41 (+/- 0.71) | 1.36 (+/- 0.25) |
| V-1 | 3.55 (+/- 0.09) | 8.16 (+/- 0.31) |
| V-2 | >10 | >10 |

**Table V-4.** Inhibition of Cytokine (IL-2 and TNF-α) production measured by EIA for PDTC, hymenialdisine and compounds **V-1** and **V-2.** [a]Values are means of two experiments; standard deviation is given in parentheses.

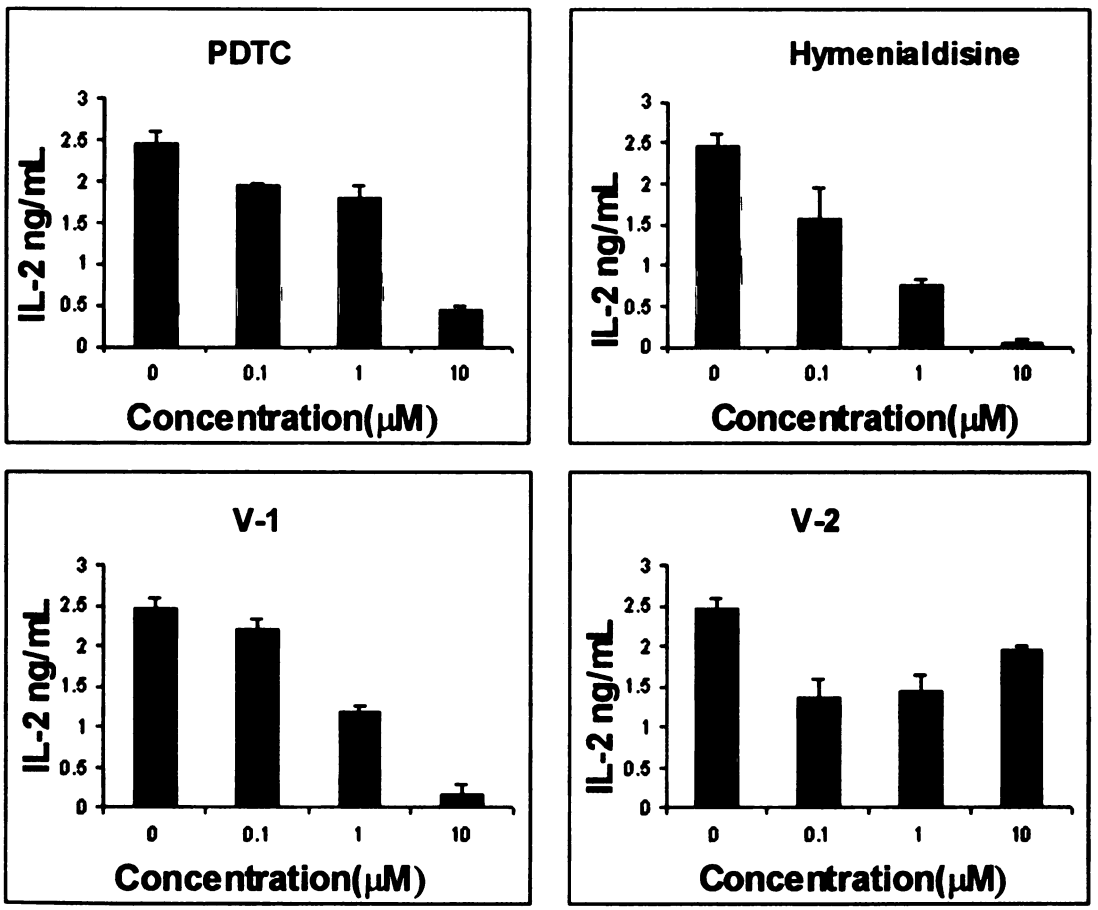



**Figure V-19.** IL-2 production measured by EIA with PDTC, hymenialdisine and compounds **V-1** and **V-2.**

hymenialdisine exhibited significant inhibition of IL-2 production with an $IC_{50}$ value of 2.4 μM (Table V-4). Treatment of the Jurkat cells with compounds **V-1** at concentrations

ranging from 0.1 μM to 10 μM exhibited also a significant dose response inhibition of IL-2 production (Figure V-19). Compound **V-1** exhibited an inhibition of IL-2 production with an $IC_{50}$ value of 3.5 μM. Interestingly, blockage of the *N*-indole position with a methyl group, compound **V-2**, indicated no significant inhibition of IL-2 production (Figure V-3, measured up to 10 μM). This further supports the significance of the pyrrolic *N-H*-moiety in potential hydrogen bonding interactions.

We also examined hymenialdisine and compounds **V-1** and **V-2** for their ability to inhibit TNF-α production in LPS stimulated THP-1 cells. Treatment of THP-1 cells with PDTC and compounds **V-1**, **V-2** or hymenialdisine, 30 minutes prior to LPS activation, resulted in a significant inhibition of TNF–α production after a 3 hour incubation period (Table V-4). Hymenialdisine was found to be a potent inhibitor of TNF-α production with an $IC_{50}$ value of 1.4 μM. Compound **V-1** was also found to inhibit TNF-α production, albeit less potent than hymenialdisine ($IC_{50}$ = 8.2 μM). The methylated indoloazepine **V-2** was again depleted of any significant activity (tested up to 10 μM, Table V-4).

**L. Inhibition of NF-κB-DNA binding by hymenialdisine analogs.**

Inhibition of the DNA-binding of NF-κB by indoleazepines or hymenialdisine was evaluated by examining the nuclear extracts of PMA/PHA activated Jurkat cells using gel electrophoresis (EMSA). Nuclear extracts were prepared (by Teri Lansdell) from Jurkat cells that were treated with various concentrations of PDTC, hymenialdisine, compound **V-1** and compound **V-2** for 30 minutes before PMA/PHA stimulation for an additional 30 minutes. Figure V-16 shows the NF--κB DNA binding with supershift. PDTC ( Lane 4) shows a reduction in the NF-κB-DNA binding induced by PMA/ PHA. Compound **V-1**

(5.0 μM) indicated approximately 49% inhibition of NF-κB-DNA binding, as compared to approximately 46% for hymenialdisine (5.0 μM).

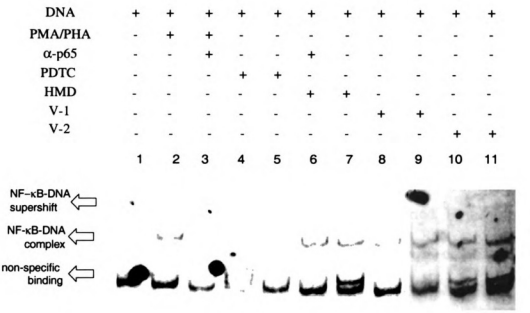



**Figure V-20.** EMSA assay for NF-κB-DNA binding inhibition by compound **V-1, V-2,** and hymenialdisine. Lane 1: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (+PMA/ PHA) + Antibody p65; Lane 2: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA); Lane 3: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (-PMA/- PHA); Lane 4: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 5 μM PDTC; Lane 5: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 1 μM PDTC; Lane 6: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 5 μM hymenialdisine; Lane 7: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 1 μM hymenialdisine; Lane 8: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 5 μM **V-1**; Lane 9: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 1 μM **V-1**; Lane 10: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 5 μM compound **V-2**; Lane 11: NF-κB consensus oligo (0.16 pmol/λ) + nuclear extract (PMA/ PHA) + 1 μM **V-2**. HMD:Hymenialdisine; PDTC: Pyrrollidine dithiocarbamate

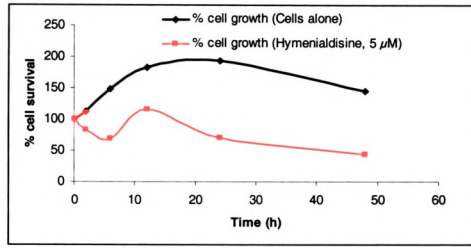
Compound **V-2**, however, failed to show any significant inhibition. Similar to the studies reported on hymenialdisine, no significant inhibition of DNA binding was observed when indoleazepines **V-1** or **V-2** were tested for inhibition of DNA binding with the transcription factor AP-1.[15]

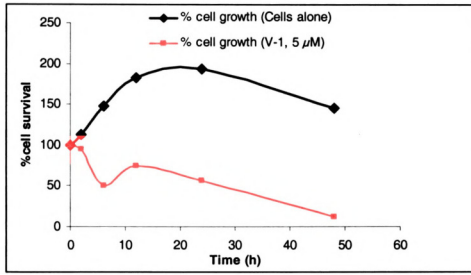
**M. Cytotoxicity profile of hymenialdisine and its indoleazepine analogs.**

We observed inhibition of PMA/PHA induced cytokine (IL-2) production in jurkat cells and LPS induced TNF-α production in macrophages. We also observed noticeable inhibition of NF-κB –DNA binding by EMSA studies. However, these effects would be seen if any of the compounds tested were highly cytotoxic, especially in case of IL-2 production, since incubation was carried out for a period of 24h. Hence, hymenialdisine and compounds **V-1** and **V-2** were tested for their cytotoxicity and were found to exhibit significant inhibition of cell growth. Cells were treated at different concentrations ranging from 10 nM to 5 μM concentrations over a 48 hour time period. Hymenialdisine and compound **V-1** indicated similar inhibition of cell growth with $GI_{50}$ of 1.61 μM and 1.73 μM respectively, whereas compound **V-2** indicated weaker inhibition of cell growth ($GI_{50}$ = 14.3 μM).

| Compound | Inhibition of cell growth $GI_{50}$(μM)[a] |
|---|---|
| PDTC | N/T |
| Hymenialdisine | 1.61 (+/- 0.62) |
| V-1 | 1.73 (+/- 0.088) |
| V-2 | 14.3 (+/- 2.41) |

**Table V-5.** Inhibition of cell growth with hymenialdisine and compounds **V-1** and **V-2** in CEM leukemia T-cells. [a]Values shown are average of two experiments. Standard deviation is given in parentheses.



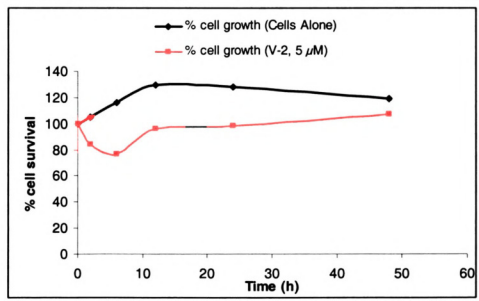




**Figure V-21.** Survival curves showing cell growth in presence of 5 $\mu$M hymenialdisine, **V-1**, **V-2**.

The inhibition of cell growth with indoleazepines or hymenialdisine could therefore account for some of the inhibition of IL-2 production seen in the Jurkat cells, where IL-2 production was measured 24 hours after PMA stimulation. However, inhibition of TNF-α production was measured within 3 hours after LPS stimulation and no significant inhibition of cell growth was apparent at this time interval.

**N. Conclusion**

We successfully synthesized **V-1** and **V-2**, analogs of the natural product hymenialdisine. The kinase inhibition studies suggest that analogs of hymenialdisine may improve its overall kinase profile and significantly affect its kinase **selectivity**. While further *in vivo* studies are required in validating the drug therapeutic potential of hymenialdisine analogs for cancer therapy and neurodegenerative diseases, these small molecules show remarkable promise in targeting Chk2 inhibition in a 'tailor-made' fashion.

These were a new class of indoloazepine inhibitor of the pro-inflammatory protein NF-κB. Inhibition of the transcriptional activity of NF-κB by the indoloazepine compounds **V-1** and **V-2** was evaluated by measuring IL-2 and TNF-α production in Jurkat and THP-1 cells. The free indoloazepine **V-1** and hymenialdisine exhibited potent inhibition of IL-2 and TNF-α production, whereas the *N*-methyl indoloazepine **V-2** was depleted of any significant activity. Hymenialdisine and indoloazepine **V-1** were found to inhibit DNA binding by NF-κB. Gel electrophoresis assays indicated that none of the compounds had any notable effect on the DNA binding of the transcription factor AP-1. The indoloazepine **V-1** provides therefore a new synthetically readily available scaffold for further optimization for cytokine inhibition. The further clinical potential and details on

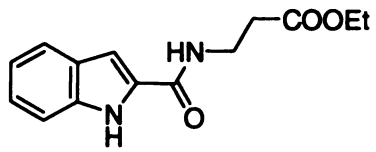
the mode of action of this new class of indoloazepines is currently under investigation in our laboratory.

## O. Experimental Section

Reactions were carried out in flame-dried glassware under nitrogen or argon atmosphere, unless otherwise noted. Commercial solvents and reagents were used as received. Anhydrous solvents were dispensed from a delivery system which passes the solvents through packed columns (tetrahydrofuran, methylene chloride: dry neutral alumina). All reactions were magnetically stirred and monitored by TLC with 0.25 μM pre-coated silica gel plates and column chromatography was carried out on Silica Gel 60 (230–400mesh) supplied by EM Science. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Melting points were determined on a Mel-Temp (Laboratory devices) apparatus with a microscope attachment. Infrared spectra were recorded on a Nicolet IR/42 spectrometer. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Varian Gemini-300 spectrometer, Varian VXR-500 spectrometer and Unity+-500 spectrometer. Chemical shifts are reported relative to the residue peaks of the solvent $CDCl_3$ (7.24 ppm for $^{1}H$ and 77.0 ppm for $^{13}C$). HRMS were obtained at the Mass Spectrometry Facility of the Michigan State University with a JEOL JMS HX-110 mass spectrometer. Gas chromatography/ low resolution mass spectra were recorded on a Hewlet–Packard 5890 Series II gas chromatograph connected to a TRIO-1 EI mass spectrometer. Combustion analysis was carried out on Perkin Elmer Series II 2400 CHNS/O Analyzer.

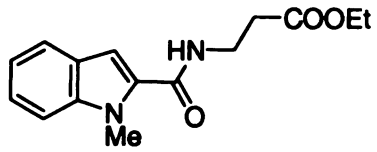
**General Procedure.**

**3-[(1H-Indole-2-carbonyl)-amino]-propionic acid ethyl ester, V-3**



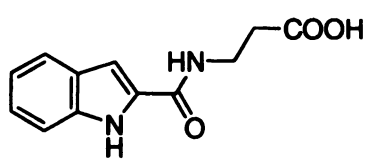

To a mixture of 2-indolecarboxylic acid (5.01 g, 31.11 mmol), 4-(dimethylamino)pyridine(6.44 g, 52.72 mmol) in 150 mL anhydrous $CH_2Cl_2$ was added N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI.HCl) (7.13 g, 37.21 mmol) and β-alanine ethyl ester hydrochloride (5.71 g, 37.21 mmol) at 0°C. The mixture then was stirred at 0°C for 4 hours and allowed to warm to room temperature for 20 hours. The solution was washed with water (50 mL) and 10% HCl (50 mL). The reaction mixture was then dried over anhydrous $Na_2SO_4$, filtered and concentrated to yield product **V-3** as a white solid (7.95 g, 98%). The solid was recrystallized in methanol. M.P. 158-160°C. $^1H$ NMR: (500 MHz, $CDCl_3$ – 2 drops acetone) δ 1.17 (t, 3H, J = 7.5Hz), 2.58 (t, 2H, J = 6.5Hz), 3.67 (q, 2H, J = 6.0 Hz), 4.08 (q, 2H, J = 7 Hz), 6.81 (s, 1H), 7.01(t, 1H, J = 7.5 Hz), 7.09 (s, 1H), 7.14 (t, 1H, J = 7 Hz), 7.35 ( d, 1H, J = 8.5 Hz), 7.52(d, 1H, J = 8.5 Hz), 9.86 (s, 1H). $^{13}C$ NMR: (74.47 MHz, $CDCl_3$) δ 13.9, 33.9, 35.0, 60.6, 102.3, 111.9, 120.3, 121.7, 124.2, 127.5, 130.6, 136.4, 161.7, 172.3. IR: (NaCl) 3377, 3352, 1716, 1624, 1552, 1325, 1207, 748 $cm^{-1}$. HRMS m/e ($C_{14}H_{16}N_2O_3$) (M) 260.1161, found 260.1159. Anal. ($C_{14}H_{16}N_2O_3$) Calcd: C, 64.6; H, 6.20; N, 10.76; found: C, 64.22; H, 6.34; N, 10.67.

**3-[(1-Methyl-1H-indole-2-carbonyl)-amino]-propionic acid ethyl ester, V-4**



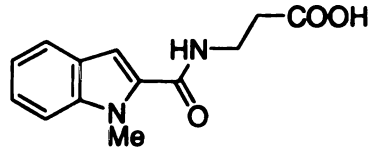

A solution of compound **V-3** (2.5 g, 9.57 mmol), $K_2CO_3$ (5.94 g, 43.06 mmol) and iodomethane (1.49 mL, 23.92 mmol) in acetonitrile (75 mL) was stirred at reflux for 24 hours. The mixture was cooled, filtered and the filtrate was evaporated *in vacuo*. The solid was then washed with 1N HCl and extracted in $CH_2Cl_2$. The crude material was further purified by column chromatography on silica gel ($CH_2Cl_2$-EtOAc 3:1) to yield compound **V-4** (2.64 g) as white solid (100%). The product was recrystallized with dichloromethane: ether (1:1) M.P. 75-77°C. $^1H$ NMR: (300 MHz, $CDCl_3$) δ 1.28 (t, 3H, J = 7.2Hz), 2.67 (m, 2H), 3.72 (m, 2H), 4.07 (s, 3H), 4.22 (q, 2H, J = 6.9Hz), 6.86 (s, 1H), 7.05-7.65 (m, 4H). $^{13}C$ NMR: (74.47 MHz, $CDCl_3$) δ 14.1, 31.4, 33.9, 34.8, 60.7, 103.8, 110.0, 120.4, 121.7, 123.9, 125.9, 131.8, 138.9, 162.4, 172.7. IR (NaCl): 3265, 1730, 1630, 1554, 1460, 1383, 1319, 1184 $cm^{-1}$. HRMS m/e ($C_{15}H_{18}N_2O_3$)(M) 274.1317, found 274.1315. Anal. ($C_{15}H_{18}N_2O_3$) Calcd: C, 65.68; H, 6.61; N, 10.21; found: C, 64.55; H, 6.44; N, 10.10.

**3-[(1H-indole-2-carbonyl)-amino]-propionic acid, V-5**



A mixture of compound **V-3** (6.1 g, 23.4 mmol ) and $LiOH.H_2O$ (1.96 g, 46.7 mmol) was stirred at room temperature in ethanol (300mL) for 20 hours, the ethanol was evaporated to dryness *in vacuo*. The residue was dissolved in water (50 mL) and acidified the solution to pH = 3 with concentrated HCl to form a white solid. The mixture was allowed to stand at 0°C for 30 minutes and the product was isolated by filtration. The solid was washed with water and recrystallized with methanol to afford compound **V-5** (5.03 g 93%). M. P. 232°C. $^1H$ NMR: (300 MHz, $d^6$-DMSO) δ 2.55 (t, 2H, J = 7.2 Hz), 3.47 (q, 2H, J = 6.9 Hz), 7.01(t, 1H, J = 7.2 Hz), 7.04 (s, 1H), 7.13 (t, 1H, J = 7.2 Hz), 7.41 (1H, J = 8.1 Hz), 7.57 (1H, J = 8.1 Hz), 8.52 (s, 1H), 11.55 (s, 1H), 12.25 (s, 1H); $^{13}C$ NMR: (74.47 MHz) ($d^6$-DMSO) δ 34.6, 35.9, 103.2, 112..9, 120.3, 122.1, 123.9, 127.8, 132.4, 137.1, 161.8, 173.4; IR (NaCl): 3422, 3273, 1745, 1707, 1643, 1549, 1417, 1341, 1259, 746$cm^{-1}$; HRMS m/e ($C_{12}H_{12}N_2O_3$)(M) 232.0848, found 232.0844. Anal. ($C_{12}H_{12}N_2O_3$ ) Calcd: C; H, 5.21; N, 12.06; found: H, 5.27; N, 11.79.

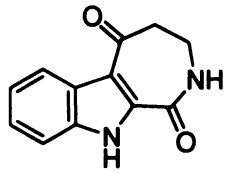
**3-[(1-Methyl-1H-indole-2-carbonyl)-amino]-propionic acid, V-6**




Procedure similar as for compound **V-5** to provide compound **V-6** from **V-4** (2.16 g, 93%). The product was recrystallized in methanol. M. P. 160-162°C. $^1H$ NMR: (300MHz, $d^6$-DMSO) δ 2.49 (t, 2H, J = 7.2 Hz), 3.46 (q, 2H, J = 6.6 Hz), 3.95 (s, 3H), 7.07-7.64 (m, 5H), 8.53 (t, 1H, J = 5.7 Hz). $^{13}C$ NMR: (74.47 MHz,

d$^6$-DMSO) δ 31.1, 33.7, 35.0, 104.0, 110.2, 119.9, 121.3, 123.3, 125.5, 132.1, 138.2, 161.8, 172.6. IR (NaCl): 3377, 2922, 1714, 1601, 1550, 1464, 1423, 1280, 1226, 1190, 908 cm$^{-1}$. HRMS m/e ($C_{13}H_{14}N_2O_3$)(M) 246.1004, found 246.1016. Anal. ($C_{13}H_{14}N_2O_3$) Calcd: C, 63.40; H, 5.73; N, 11.38; found: C, 62.39; H, 5.94; N, 11.12.

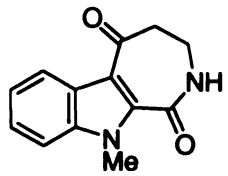
**3, 4-Dihydro-2H, 10H-azepino [3, 4-b]indole-1,5-dione, V-7**




Compound **V-5** (2 g, 8.6 mmol) was added to a clear solution of $P_2O_5$ (1.33 g, 9.44 mmol ) in $MeSO_3H$ (70 mL) at 60°C. The mixture was heated to 110°C for 1.5 hour, and then cooled to room temperature. The reaction mixture was poured into ice-water and stirred for 30 minutes. The buff-colored solid was then filtered and dissolved in acetone (250 mL), filtered again, and the filtrate was concentrated to give a light brown solid which was further purified by column chromatography (20 % acetone in $CH_2Cl_2$ ) on silica gel to yield compound **V-7** (1.56 g, 85%) Mp: 257-260°C. $^1$H NMR (300 MHz, d$^6$-DMSO) δ 2.80-2.85 (m, 2H), 3.40-3.46 (m, 2H), 7.22-7.38 (m, 2H), 7.51 (d, 1H, *J* = 9.0 Hz), 8.28 (d, 1H, *J* = 9.0 Hz), 8.72 (m, 1H), 12.41 (s, 1H); $^{13}$C NMR (74.47 MHz, d$^6$-DMSO) δ 36.5, 44.0, 112.6, 113.7, 122.6, 122.7, 124.7, 126.0, 134.5, 135.6, 162.3, 195.0; IR: (NaCl) 3163, 1662, 1628, 1523, 1437, 1408 cm$^{-1}$; LRMS (EI): $M^+$ = 214.3;

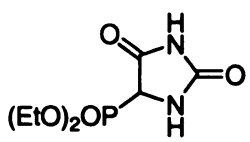
HRMS m/e ($C_{12}H_{10}N_2O_2$)(M) 214.0742, found 214.0740. Anal. ($C_{14}H_{16}N_2O_3$ ) Calcd: C, 67.28; H, 4.71; N, 13.08; found: C, 65.36; H, 4.66; N, 12.53.

**10-Methyl-3,4-dihydro-2H,10H-azepino[3,4-b]indole-1,5-dione, V-8**




Procedure similar as for compound **V-7** to provide compound **V-8** from **V-6** (1.3 g, 75%). The product was recrystallized in methanol. Mp 200-205° C. $^1$H NMR: (500MHz, d$^6$-DMSO) δ 2.77 (t, 2H, J = 5.5 Hz), 3.39 (t, 2H, J = 5.5, 4.5 Hz), 3.99 (s, 3H), 7.27 (t, 1H, J = 7.0, 8.0 Hz), 7.37 (t, 1H, J = 7.5 Hz), 7.62 (d, 1H, J = 8.0 Hz), 8.27 (d, 1H, J = 8.0 Hz), 8.77 (t, 1H, J = 5.0, 6.0 Hz); $^{13}$C NMR: ( 124.1 MHz, d$^6$-DMSO) δ 32.4, 36.6, 44.9, 110.9, 114.7, 122.6, 123.1, 124.5, 125.0, 134.7, 137.9, 162.3, 195.6. IR (NaCl): 3204, 3000, 2924, 1662, 1641, 1506, 1473, 1371, 724 cm$^{-1}$; HRMS m/e ($C_{13}H_{12}N_2O_2$)(M) 228.0899, found 228.0887. Anal. ($C_{13}H_{12}N_2O_2$) Calcd: C, 68.41; H, 5.30; N, 12.27; found: C, 65.80; H, 5.37; N, 11.42.

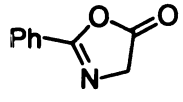
**diethyl 2,5-dioxoimidazolidin-4-yl-4-phosphonate, V-9:**




A mixture of hydantoin (200 mg, 2 mmol),and acetic acid (1.0 mL) was heated to 85 °C in an oil bath. An addition funnel was charged with bromine (352 mg, 0.113

mL, 2.2 mmole) and a small amount of bromine (4 drops) introduced into the reaction mixture with vigorous stirring. Once the color dissipated, the remainder of the bromine was added rapidly dropwise over approximately 5 min to afford a clear solution. After being stirred at 85 °C for 30 min, the reaction mixture was cooled to 30 °C in an ice bath and triethyl phosphite (0.465 g, 0.48 mL, 2.8 mmole) introduced slowly such that the internal temperature was at 40-45 °C. After the addition was completed, the mixture was stirred at ambient temperature for 90 min. The solvent was removed *in vacuo* and the residue diluted with $Et_2O$ (10 mL) with stirring to induce precipitation of a white solid. The mixture was poured onto diethyl ether (50 mL) with vigorous stirring. After 30 min, filtration afforded V-9 (71%), which was used without further purification. M. P. 161-163 °C. $H^1$ NMR (DMSO-*d6*) δ 1.25 (6 H, t, *J* = 7 Hz), 4.10 (4 H, m), 4.76 (1 H, d, *J* = 15 Hz), 8.42 (1 H, bs, NH), 10.92 (1 H, bs, NH); IR (KBr) 1775, 1720, 1250, 1035 cm-'

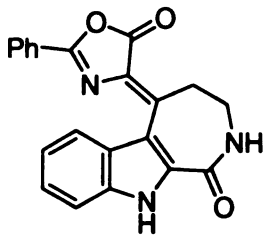
**2-phenyloxazol-5(4H)-one, V-10:**




Pulverized Hippuric acid (1g, 5.5 mmol) was taken in a flame dried flask in 100 ml $CH_2Cl_2$. EDCI.HCl (1.2 g, 6.1mmol) was added and the reaction allowed to stir for 1h at room temperature. The reaction micture was then extracted with 1X $NaHCO_3$(sat.) twice. The organic layer was collected and washed with NaCl(sat.) once. Again the organic layer was collected dried over anhydrous $Na_2SO_4$ and dried *in vacuo*. Yield: 0.7g, 80% $^1H$ NMR (300MHz, $d^6$-DMSO) δ 4.41 (s, 2H),

7.47 (d, 2H, $J$ = 7.5 Hz), 7.56 (t, 1H, $J$ = 7.5 Hz), 7.96 (d, 2H, $J$= 7.5 Hz); $^{13}C$ NMR (74.47 MHz, $d^6$-$CDCl_3$) δ 55.02, 125.81, 127.79, 128.82, 132.83, 163.46, 175.95; IR: (neat) 3107, 3042, 2949, 1816.3, 1658.9, 1452.6 $cm^{-1}$; LRMS m/e ($C_9H_7NO2$)(M) 161.0.

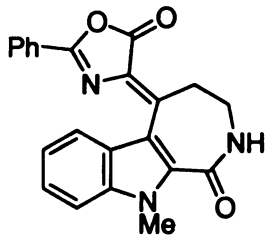
**5-(5-oxo-2-phenyl-oxazol-4-ylidene)-3, 4, 5, 10-tetrahydro-2H-azepino [3,4-b]indol-1-one, V-11**




A solution of $TiCl_4$ (2.04 mL, 18.68 mmol ) in $CH_2Cl_2$ (21 mL) was added into THF (65 mL) at -10°C. Compound **V-7** (1 g, 4.67 mmol) and 2-phenylazlactone (1.50 g, 9.34 mmol) were subsequently added and allowed to stir at 0°C for 20 minutes. Pyridine (2.09 mL, 37.36 mmol) was then added over a 30 minutes period. The reaction mixture was stirred at 0°C for additional 2 hours and then allowed to stir overnight at room temperature. $NH_4Cl$ (80 mL, saturated solution in water) was added and the mixture was stirred for 10 minutes and subsequently extracted with ethyl acetate (3X). The organic extracts were combined dried, filtered, concentrated and the residue was purified using column chromatography on silica gel (EtOAc-hexane 7:3) to yield compound **V-11** (0.91 g, 55%) as reddish-yellow solid. Mp: 247-250°C. $^1H$ NMR (300MHz, $d^6$-DMSO) δ 3.36-3.47 (m, 4H), 7.14 (t, 1H, J = 8.1 Hz), 7.30 (t, 1H, J = 9.0 Hz), 7.49-7.58 (m, 4H),

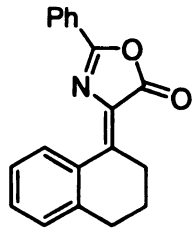
7.81-7.87 (m, 3H), 8.45-8.56 (m, 1H), 12.11(s, 1H); $^{13}C$ NMR (74.47 MHz, $d^6$-DMSO) δ 37.7, 38.6, 112.9, 115.3, 121.0, 125.1, 125.3, 126.5, 127.4, 129.0, 129.8, 133.2, 134.0, 136.9, 146.3, 158.4, 165.0, 166.1; IR: (KBr-pellet) 3358, 3179, 1749, 1653, 1633, 1448 $cm^{-1}$; HRMS m/e ($C_{21}H_{15}N_3O_3$)(M) 357.1113, found 357.1106.

**10-Methyl-5-(5-oxo-2-phenyl-oxazol-4-ylidene)-3, 4, 5, 10-tetrahydro-2H-azepino [3,4-b]indol-1-one, V-12**



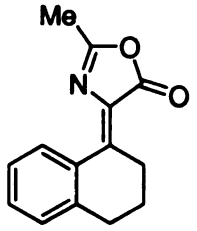

Procedure similar as for compound **V-11** to provide compound **V-12** from **V-8** (0.73 g, 56%) as a yellow solid. Mp: 189-192°C. $^1H$ NMR: (300MHz, $CDCl_3$ ) δ 3.54 (t, 2H, J =3.3, 3.6 Hz), 3.59 (t, 2H, J = 2.7, 3.3 Hz), 4.09 (s, 3H), 6.94 (s, 1H), 7.24-7.27 (m, 1H), 7.38-7.49 (m, 5H), 7.78 (d, 1H, J = 4.8 Hz), 7.92 (d, 2H, J = 6.0 Hz); $^{13}C$ NMR: ( 74.47 MHz, $CDCl_3$) δ 31.9, 38.2, 38.4, 110.1, 115.8, 121.3, 124.3, 125.0, 125.1, 125.8, 127.5, 128.7, 130.0, 131.7, 132.4, 138.5, 144.5, 159.4, 165.3, 165.9. IR: (NaCl) 1784, 1753, 1662, 1633, 1473 $cm^{-1}$; LRMS (EI): 371.1(M); HRMS m/e ($C_{22}H_{17}N_3O_3$)(M) 371.1270, found 371.1268.

**(4Z)-4-(2,3-dihydronaphthalen-4(1H)-ylidene)-2-phenyloxazol-5(4H)-one, V-13:**



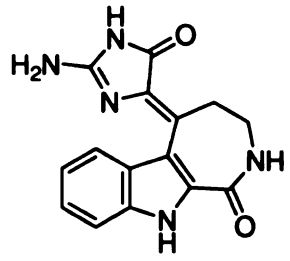

A solution of $TiCl_4$ (1.5 mL, 13.68 mmol ) in $CH_2Cl_2$ (14 mL) was added into THF (45 mL) at -10°C. α-tetralone (0.5 g, 3.42 mmol) and 2-phenylazlactone (1.1 g, 6.84 mmol) were subsequently added and allowed to stir at 0°C for 20 minutes. Pyridine (1.53 mL, 27.35 mmol) was then added over a 30 minutes period. The reaction mixture was stirred at 0°C for additional 2 hours and then allowed to stir overnight at room temperature. $NH_4Cl$ (60 mL, saturated solution in water) was added and the mixture was stirred for 10 minutes and subsequently extracted with ethyl acetate (3X). The organic extracts were combined dried, filtered, concentrated and the residue was purified using column chromatography on silica gel using hexane to yield compound **V-13** (0.52 g, 57%) as yellow solid. $^1H$ NMR (300MHz, $CDCl_3$) δ 1.90-1.99 (m, 2H), 2.85 (t, 2H, $J$ = 6.3 Hz), 3.33 (t, 2H, $J$= 6.3 Hz), 7.18-8.93 (m, 9H); $^{13}C$ NMR (74.47 MHz, $CDCl_3$) δ 22.33, 28.05, 30.41, 125.94, 126.07, 127.75, 128.74, 128.83, 130.71, 132.47, 132.65, 132.96, 142.53, 148.57, 160.11, 166.86; LRMS m/e ($C_{19}H_{15}NO_2$)(M) 289.1.

**(4Z)-4-(2,3-dihydronaphthalen-4(1H)-ylidene)-2-methyloxazol-5(4H)-one, V-14.**




A solution of $TiCl_4$ (1.11 mL, 10.09 mmol ) in $CH_2Cl_2$ (5 mL) was added into THF (25 mL) at -10°C. α-tetralone (0.36 g, 2.52 mmol) and 2-phenylazlactone (0.5 g, 5.04 mmol) were subsequently added and allowed to stir at 0°C for 20 minutes. Pyridine (1.13 mL, 20.18 mmol) was then added over a 30 minutes period. The reaction mixture was stirred at 0°C for additional 2 hours and then allowed to stir overnight at room temperature. $NH_4Cl$ (40 mL, saturated solution in water) was added and the mixture was stirred for 10 minutes and subsequently extracted with ethyl acetate (3X). The organic extracts were combined dried, filtered, concentrated and the residue was purified using column chromatography on silica gel using hexane to yield compound **V-14** (0.35 g, 61%) as yellow solid. Partial characterisation of **V-14**. $^1H$ NMR (300MHz, $CDCl_3$) δ 1.90-1.99 (m, 2H), 2.85 (t, 2H, *J* = 6.3 Hz), 3.33 (t, 2H, *J*= 6.3 Hz), 7.18-8.93 (m, 9H); $^{13}C$ NMR (74.47 MHz, $CDCl_3$) δ 15.31, 22.29, 27.68, 30.26, 125.94, 126.03, 126.69, 127.99, 128.68, 130.59, 132.43, 142.27, 147.96, 162.24, 167.14. IR(neat): 2935.1, 2855, 1756.8, 1648.8, 1270.9 $cm^{-1}$; LRMS ($C_{14}H_{13}NO_2$) m/e (M)227.3

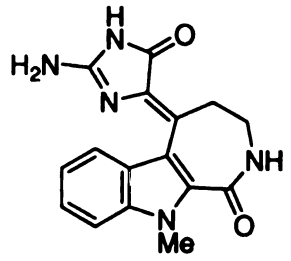
**5-(2-Amino-5-oxo-1,5-dihydro-imidazol-4-ylidene)-3,4,5,10-tetrahydro-2H-azepino[3,4-b]indol-1-one, V-1**



LiH (16 mg, 2 mmol ) was dissolved in ethanol (60 mL) and to this solution S-benzylisothiouronium chloride (405 mg, 2 mmol) was added and allowed to stir until the solution became clear. Compound **V-11** (150 mg, 0.4 mmol) was added and the reaction was heated at reflux for 48 hours. After cooling the solvent was evaporated *in vacuo*. Ethanol (20 mL) added and subsequently evaporated 3 times. Ethanol (5 mL) was added again and the reaction was refluxed for 3 hours. The solvent was evaporated in vacuum, 1N $HCl_{(aq)}$ (15 mL) was added and the mixture was extracted with n-butanol (3 × 15 mL), the extracts were washed with brine (3×10 mL), dried, concentrated and the product was purified twice by column chromatography on silica gel ($CH_2Cl_2$ : MeOH, 4 : 1) to yield compound **V-1** (35 mg, 28 %) as a light yellow solid. Mp:>260°C; **V-1** : $^1$H NMR (300 MHz, d$^6$-DMSO) δ 3.20-3.40 (br, 4H), 7.16 (t, 1H, J = 12.0 Hz), 7.29 (t, 1H, J = 12.0 Hz), 7.52 (m, 2H), 8.30-8.50 (m, 2H), 9.05-9.25 (br., 1H), 10.30-10.40 (m, 1H), 12.48 (s, 1H); $^{13}$C NMR: (124.1 MHz), d$^6$-DMSO ) δ 36.5, 39.2, 112.7, 113.5, 121.9, 122.3, 122.8, 124.5, 125.0, 128.6, 132.8, 137.0, 154.6, 163.4, 165.5; IR: (KBr-pellet) 3294, 1620, 1475, 1251 cm$^1$; LRMS (EI): 295.1 (M); HRMS (FAB) ($C_{15}H_{14}N_5O_2$)(M+H) 296.1148, found 296.1144. **V-1.HCl:** $^1$H NMR (500 MHz,

$d^6$-DMSO) δ 3.27-3.38 (br, 4H), 7.16 (t, 1H, J = 7.5 Hz), 7.28 (t, 1H, J = 7.5Hz), 7.40 (d, 1H, 8 Hz), 7.49 (d, 1H, 8Hz) 8.18 (br, 1H), 8.49 (br, 1H), 12.08 (s, 1H); $^{13}C$ NMR: (148.9MHz), $d^6$-DMSO ) δ 34.4, 40.0, 112.7, 114.2, 116.6, 120.4, 121.5, 123.9, 128.3, 128.6, 130.4, 136.2, 160.1, 166.5, 177.1 LRMS (EI): 295.2 (M); FAB+(LRMS): 296.07 FAB-(LRMS): 34.99, 37.0 Anal. Calcd ($C_{15}H_{15}N_5O_2Cl$): C, 54.3; H, 4.25; N, 21.11; found: C, 55.85; H, 4.27; N, 20.61.

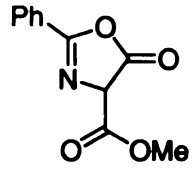
**5-(2-Amino-5-oxo-1,5-dihydro-imidazol-4-ylidene)-10-methyl-3,4,5,10-tetrahydro-2H-azepino[3,4-b]indol-1-one, V-2**




Procedure similar as for compound **V-1** to provide compound **V-2** from **V-12**. The product **V-2** was purified by column chromatography on silica gel eluting with $CH_2Cl_2$: MeOH, (85:15) followed by $CH_2Cl_2$: MeOH, (88:12) to yield compound **V-3** as light yellow solid (96 mg, 31%) . Mp: >260°C. $^1H$ NMR: (300MHz, $d^6$-DMSO) δ 2.99-3.48 (m, 4H) 3.92 (s, 3H), 7.19 (t, 1H, J = 7.2Hz), 7.36 (t, 1H, J = 6.6Hz), 7.55 (d, 1H, J = 8.1Hz), 7.65 (d, 1H, J = 8.4Hz), 8.57 (t, 1H, J = 5.1Hz), 9.15-9.25 (br-s,1H), 10.48 (s, 1H); $^{13}C$ NMR: (74.47 MHz, $d^6$-DMSO) δ 32.1, 37.1, 38.4, 111.7, 112.6, 122.1, 122.2, 123.5, 123.7, 125.1, 128.1, 132.8, 138.5, 154.6, 163.0, 165.1; IR: ( KBr-pellet) 3211, 1699, 1635, 1508, 1477

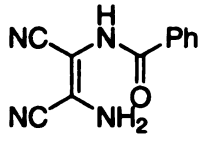
$cm^{-1}$; LRMS (EI): 308.7 (M); HRMS m/e ($C_{16}H_{15}N_5O_2$)(M) 309.1304, found 309.1291.

**Methyl 4,5-dihydro-5-oxo-2-phenyloxazole-4-carboxylate, V-18.**




A solution of **V-10** (1.86 mmol) in THF (20 mL) at 0 °C was mixed with methylchloroformate (2.046 mmol), DMAP(0.093 mmol) and TEA(2.046 mmol), and allowed to stir for 3h. A dark brown precipitate was formed which was filtered, dried and characterized. Yield: 100 mg, 30%. $^1$H NMR (300 MHz) (DMSO-d6) δ TMS: 3.658 (3 H, s), 7.504 (3 H, s), 7.914 (2 H, s). $^{13}$C NMR (75 MHz) (DMSO-d6) δ: 50.1, 125.8, 127.6, 128.3, 129.0, 130.9, 146.2, 162.0, 166.3, 167.7 IR (NaCl, $CDCl_3$): 3200, 2924.4, 1778.6, 1635.8 $cm^{-1}$. MS (EI): M-(COOMe), 161.0.

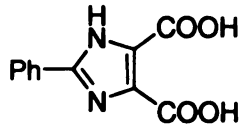
**N-((Z)-2-amino-1, 2-dicyanovinyl)benzamide, V-19.**




A suspension of DAMN (9.25 mmol) in pyridine (40mL, dry) at 0 °C was mixed with benzoyl chloride (10.18 mmol) with continuous stirring. The mixture was stirred for 12h and the solvent was evaporated. Added 10mL $H_2O$ and filtered. Dried to obtain V-19 in 2.1 g, 98% yield. $^1$H NMR (300 MHz) (DMSO-d6) δ TMS: 7.424 (2H, s, exchangeable), 7.494-7.519 (3 H, m), 7.928 (2 H, d, J = 7.8 Hz), 9.6(1H, s, exchangeable). $^{13}$C NMR (75 MHz) (DMSO-d6) δ: 50.1, 125.8,

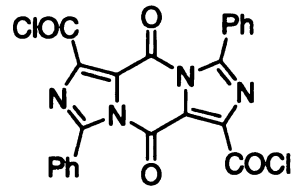
127.6, 128.3, 129.0, 130.9, 146.2, 162.0, 166.3, 167.7 IR (NaCl, $CDCl_3$): 3321.8, 3204.1, 2924.4, 2208.7, 1776.6, 1639.7 $cm^{-1}$. MS (EI): $M^+$: 212.4 (expected=212 g/mol).Anal. Calcd. For. $C_{11}H_8N_4O$: C; H, 3.8; N, 26.4. Found H, 4.1; N, 22.87.

**2-phenyl-1H-imidazole-4,5-dicarboxylic acid, V-20:**



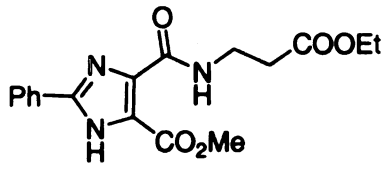

Tartaric acid (1g, 6.67 mmol) was suspended in a mechanically stirred mixture of conc. $HNO_3$ and fuming $HNO_3$. Conc. $H_2SO_4$ was added until temperature rose to 38 °C. The temperature at this stage was maintained between 38-43 °C.The reaction mixture was then chilled in ice for 1h and then filtered through glass wool or a sintered funnel. Dried and added crushed icewith vigorous stirring. Cooled to -10 °C. Neutralized with conc. $NH_4OH$ (~45 ml). Added PhCHO (0.7 g, 6.66 mmol) and $NH_4OH$ (20 ml) and stirred at 0 °C for 20h. Neutralized with conc. HCl. Filtered, dried and characterized **V-20**. Yield (50%) $^1H$ NMR (300 MHz) (DMSO-d6) δ: 7.04 (1H, s, exchangeable), 7.216 (1H, s, exchangeable), 7.386 (1H, s, exchangeable), 7.54-7.56 (3 H, m), 7.164 (2 H, d, $J$ = 8.1 Hz). $^{13}C$ NMR (124.4 MHz) (DMSO-d6) δ: 124.6, 127.8, 128.8, 130.5, 131.4, 145.2, 160.5 IR (NaCl, $CDCl_3$): 2924.4(broad), 1595.3, 1487.3 $cm^{-1}$. MS (EI): $M^+$: 232.3 (expected=232.2 g/mol). Anal. Calcd. For. $C_{11}H_8N_2O_4$: C, 56.9; H, 3.47; N, 12.06. Found C, 55.05; H, 3.3; N, 12.23.

**Dimerized acid chloride of the 2-phenylimidazole dicarboxylic acid, V-21.**



A suspension of **V-20** (1g, 4.31 mmol) in dry benzene (10 mL) containing DMF (0.5 mL) and $SOCl_2$ (7.5 mL) was stirred at 85 °C for 6h. Evaporated the solvent and concentrated *in vacuo* to give the dimer as a yellow solid .Yield: 0.75 g, 38 % yield. M. P. >260 °C $^1$H NMR (300 MHz) (DMSO-d6) δ: 7.34-7.76 (10 H, m) $^{13}$C NMR (124.4 MHz) (DMSO-d6) δ: 121.9, 127.8, 128.9, 129.6, 130.7, 141.7, 149.113, 150.1, 162.6 IR (NaCl, $CDCl_3$): 2926.4, 1751.9, 1610.7 cm$^{-1}$. MS (EI): $M^+$: 464.0 (expected=464.0 g/mol). Anal. Calcd. For. $C_{22}H_{10}N_4Cl_2O_4$: C, 56.79; H, 2.17; N, 12.04. Found C, 50.12; H, 2.02; N, 10.25(corresponds to the monomer, the molecule probably fragmented on heating).

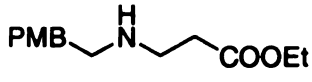
**Methyl 4-(2-(ethoxycarbonyl) ethylcarbamoyl)-2-phenyl-1H-imidazole-5-carboxylate, V-22.**



A suspension of **V-21** (0.43 mmol) was stirred in MeOH for 3h at room temperature. The yellow solid (diester) was filtered and dried *in vacuo*. This solid was then taken in $CH_2Cl_2$ and mixed with β-alanine.HCl (0.86 mmol) and TEA ( 2.58 mmol and stirred at room temperature overnight. The solid dissolved. Evaporated the solvent and extracted in EtOAc/2N HCl. Washed the organic layer and dried to obtain 99.3% product. $^1$H NMR (300 MHz) (CDCl3) δ TMS: 1.25

(3H, t, J = 7.2 Hz), 2.61 (2H, t, J = 6 Hz), 3.71 (2H, t, J = 6.3 Hz), 4.004(3, s), 4.170(2H, q, J = 6.9 Hz), 4.737(1H, s, broad), 7.42-7.44 (3 H, m), 7.96- 8.002 (2 H, m). $^{13}C$ NMR (124.4 MHz) (CDCl3) δ: 13.8, 34.0, 34.9, 52.5, 60.8, 125.9, 126.2, 127.9, 128.5, 129.4, 130.6, 133.0, 149.2, 159.7, 165.4, 171.9 IR (NaCl, $CDCl_3$): 3231.1, 3076.8, 2928.3, 1734.2, 1687.9, 1643.5, 1188.3 $cm^{-1}$. MS (EI): $M^+$: 345.1 (expected=345.1 g/mol). Anal. Calcd. For. $C_{17}H_{19}N_3O_5$: C, 59.1; H, 5.55; N, 12.17. Found C, 58.32; H, 5.6; N, 11.38.

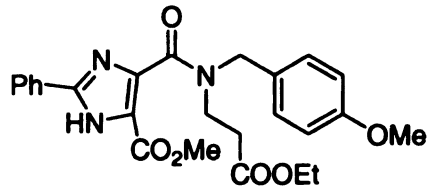
**Ethyl 3-(4-methoxyphenethylamino)propanoate, V-23.**




A suspension of β-alanine ethyl ester (6.16g, 40.1 mmol) in 150 ml absolute ethanol was maintained at room temperature. p-anisaldehyde (5g, 36.4 mmol) and TEA( 4.05 ml, 40.1 mmol) were added and the mixture was stirred for about 6h. The reaction mixture was concentrated *in vacuo* to yield a yellow white solid. Ethanol (50 ml) was added to dissolve this solid. $NaCNBH_3$ (2.52 g, 40.1 mmol) was added to this reaction mixture. The mixture turned turbid in about 3 minutes. Allowed to stir for 30 minutes for the reaction to go to completion. Evaporated the solvent and added EtOAc. Washed with 1N HCl. Collected the organic layer. Dried the organic layer over $Na_2SO_4$ , filtered and evaporated *in vacuo* to obtain 6.54 g, 76% of the secondary amine. $^1H$ NMR (300 MHz) (CDCl3) δ: 1.06 (3H, t, J = 7.2 Hz), 2.32 (2H, t, J = 6.6 Hz), 2.69 (2H, t, J = 6.6 Hz), 3.54(2H, s), 3.58(3H, s), 3.93(2H, q, *J* = 7.2 Hz), 6.65(2 H, d, *J* =9.0 Hz), 7.03 (2 H, d, *J*= 8.7 Hz). $^{13}C$ NMR (124.4 MHz) (CDCl3) δ: 13.9, 34.43, 44.07, 52.81, 54.80, 59.99,

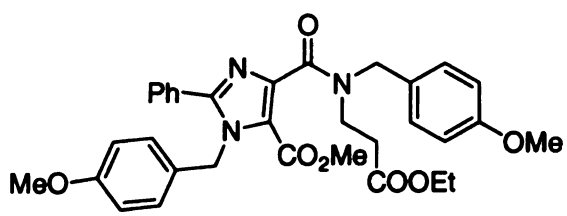
113.40, 128.88, 132.02, 158.27, 172.40 IR (NaCl, $CDCl_3$): 3300.1, 2980.2, 2835.7, 1732.3, 1512.4, 1035.9 $cm^{-1}$.

**Methyl 4-(N-(2-(ethoxycarbonyl)ethyl)-N-(4-methoxybenzyl)carbamoyl)-2-phenyl-1H-imidazole-5-carboxylate, V-24.**




A suspension of **V-21** (0.15 mmol) was stirred in MeOH for 3h at room temperature. The yellow solid (diester) was filtered and dried *in vacuo*. This solid was then taken in $CH_2Cl_2$ and mixed with N-p-methoxy benzyl β-alanine theyl ester.HCl (76.1 mg, 0.32 mmol) and TEA (92.7 mg, 0.91 mmol) and stirred at room temperature for 16h. Evaporated the solvent and extracted in EtOAc/2N HCl. Washed the organic layer and dried. The product was purified by column chromatography in EtOAc to obtain 7% product. Partial characterization: $H^1$ NMR ($CDCl_3$): δ 1.14 (3H, t, $J$ = 6.6Hz), 2.64 (2H, m), 3.46 (2H, m), 3.76 (3H, s) 3.872 (3H, s), 4.12 (2H, m), 4.45(1H, s) ,4.75(1H, s), 6.89-7.92 (9H, m).

**Methyl 4-(N-(2-(ethoxycarbonyl)ethyl)-N-(4-methoxybenzyl)carbamoyl)-1-(4-methoxybenzyl)-2-phenyl-1H-imidazole-5-carboxylate,V-25**




**V-24** (200 mg, 0.42 mmol), p-methoxy benzyl chloride (121.1 mg, 0.85 mmol), KI (10 mg, 0.06 mmol), $K_2CO_3$ (236.8 mg, 1.72 mmol) in acetone were refluxed

for 8h. The solvent was removed *in vacuo* and extracted in EtOAc and $NH_4Cl$. The organic layer was collected and dried over $Na_2SO_4$. The reaction was filtered and dried *in vacuo*. The product was purified on a silica gel column under a gradient column hexane:ether (1:9). The product was dried and characterized. Partial characterization of V-25. $H^1$ (NMR) ($CDCl_3$): 1.18 (m, 3H), 2.548 (2H, m), 2.107 (2H, m), 3.69 (3H, s), 3.70(3H, s), 3.71(3H, s), 4.05(q, 2H, $J$ = 6.6 Hz), 4.40 (s, 3H), 4.50(s, 1H), 6.73-7.25(13H, m)

**Inhibition of cell growth:** CEM cells (CCRF-CEM; Amer. Type Culture Collection, Rockville, MD) were grown in RPMI-1640 Media (Gibco-BRL, Rockville, MD) supplemented with 10% Fetal Bovine Serum, penicillin (614 ng/mL), streptomycin (10 μg/mL) and HEPES buffer, pH 7.2 at 37°C, 5% $CO_2$. DMSO was used as the vector for all drugs and added in the control experiments. Cell cultures were then treated with 10 μM, 1 μM, 0.1 μM or 10 nM of the drug in duplicate and allowed to incubate at 37°C, 5% $CO_2$. Cells were stained with 4% trypan blue solution in PBS, and counted under the microscope (Thomas Scientific, Fisher Scientific) in duplicate. This was repeated for 0h, 2h, 6h, 12h, 24h and 48h. The data points obtained were then plotted and a point -to- point curve was drawn to determine the effect of the drug on the cells.

## P. References


1. Kiberstis, P.A. *Science* **2001**, *292* , 399–401.

2. Ferber , D. *Science* **1999**, *285*, 1651–1653.

3. Zhou, B.-B. S., Elledge, S. J. *Nature* **2000**, *408*, 433-439.

4. Martin, N. M. B. *J. Photochem. Photobiol. B* **2001**, *63*, 162-170.

5. Ponder , B. A. J. *Nature* **2001**, *411*, 336–341.

6. Zhao, S.; Weng, Y.- C.; Yuan, S. -S. F. ; Lin, Y.- T.; Hsu, H. -C.; Lin, S.- C. J.; Gerbino, E.; Song, M.- H.; Zdzienicka, M. Z.; Gatti, R. A.; Shay, J. W., Ziv, Y. S.; Lee, E. Y.-H. P. *Nature* **2000**, *405*, 473.

7. (a) Naito, T. Matsuura, A.; Ishikawa, F. *Nat. genet.* **1998**, *20*, 203-206. (b)Ritchie, K. B.; Mallory, J. C.; Petes, T. D. *Mol. Cell. Biol.* **1999**, *10*, 6065-6075.

8. Lowe, S. W.; Schmitt, E. M.; Smith, S. W.; Osborne, B. A.; Jacks, T. *Nature* **1993**, *362*, 847-849.

9. (a) Sielecki, T. M.; Boylan, J. F.; Benfield, P. A.; Trainor, G. L. *J. Med. Chem.* **2000**, *43*, 1-18.(b) Sridhar, R. Hanson-Painton, O.; Cooper, D. R. *Pharm. res.* **2000**, *17*, 1345-1353. (c) Traxler, P. ; Bold, G.; Buchdunger, E.; Caravatti, G.; Furet, P.; Manley, P.; O'Reilly, T.; Wood, J; Zimmermann, J. *Med. Res. Rev.* **2001**, *21*, 499-512. (d) Scapin, G. *Drug discovery today* 2001, *7*. (e) Toogood, P. l. *Med. Res. Rev.* **2001**, *21*, 487-498. (f) Bridges, A. J. *Chem. Rev.* **2001**, *101*, 2541-2572.

10. Curman, D.; Cinel, B.; Williams, D. E.; Rundle, N.; Block, W. D.; Goodarzi, A. A.; Hutchins, J. R.; Clarke, P. R.; Zhou, B.-B.; Lees-Miller, S. P.; Andersen, R. J.; Roberge, M. *J. Biol. Chem.***2001**, *276*, 17914-17919.

11. (a) Rundle, N. T.; Xu, L.; Anderson, R. J.; Roberge, M. *J. Biol. Chem.* **2001**, *276*, 48231-48236. (b) Jackson, J. R. ; Gilmartin, A.; Imburgia, C.; Winkler, J. D.; Marshall, L. A.; Roshak, A. *Cancer Res.* **2000**, *60*, 566-572. (c) Koniaras, K.; Cuddihy, A. R.; Christopoulos, H.; Hogg, A.; O'Connell, M. J. *Oncogene* **2001**, *20*, 7453-7463. (d) Zhou, B.-B. S., Anderson, H. J.; Elledge, S. J. *Cancer Biology and Therapy* **2003**, *2*, S16-S22. (e)Yu, Q.; La Rose, J.; Zhang, H.; Takemura, H.; Kohn, K. W.; Pommier, Y. *Cancer Res.* **2002**, *62*, 5743-5748.

12. Terrett, N. K. *Combinatorial Chemistry*, Oxford University Press, **1998.**

13. (a) Schreiber, S. L. *Science* **2000**, *287*, 1964-1969. (b) Ortholand, J. – Y.; Ganesan, A. *Curr. Opin. Chem. Biol.* **2004**, *8*, 271-280.

14. (a)Garrido-Hernandez, H.; Nakadai, M.; Vimolratana, M.; Li, Q.; Doundoulakis, T.; Harran, P. G. *Angew. Chem. Int. Ed.* **2005**, *44*, 765-769. (b) Kinnel, R. B.; Gehren, H. -P.; Scheuer, P. J.; Gravalos, D. G.; Faircloth, G. T. *Eur. Pat. Appl.* **1994**, EP 94-302770 , 19940419.

15. Olofson, A.; Yakushijin, K.; Horne, D. A. *J. Org. Chem.* **1998**, *63*, 1248-1253.

16. Poullennec, K. G.; Kelly, A. T.; Romo, D. *Org. Lett.* **2002**, 4, *16*, 2645-2648.

17. Mourabit, A. A.; Potier, P. *Eur. J. Org. Chem.* **2001**, 237-243.

18. (a)Cimino, G.; De Rosa, S.; De Stefano, D.; Mazzarella, L.; Puliti, R.; Sodano, G. *Tetrahedron Lett.* **1982**, *23*, 767-768.(b) Pettit, G. R.; herald, C. L.; Leet, J. E.; Gupta, R.; Schaufelberger, D. E.; Bates, R. B.; Clewlow, P. J.; Doubek, D. L.; Manfredi, K. P. *Can. J. Chem.* **1990**, *68*, 1621. (c) Supriyono, A.; Schwartz, B.; Wray, V.; Witte, L.; Muller, W. E. G.; van Soest, R.; Sumaryono, W.; Proksch, P. *Naturforsch.* **1996**, *50c*, 669.

19. (a) Cafieri, F.; Fattorusso, E.; Mangoni, A.; Taglialatela-Scafati, O. *Tetrahedron Lett.* **1996**, *37*, 3587. (b) Kobayashi, J.; Ohizumi, Y.; Nakamura, H.; Hirata, Y. *Experientia* **1986**, *42*, 1176.

20. Wan, Y.; Hur, W.; Cho, C. Y.; Liu, Y.; Adrian, F. J.; Lozach, O.; Bach, S.; Mayer, T.; Fabbro, D.; Meijer, L.; Gray, N. S. *Chem. Biol.* **2004**, *11*, 247-259.

21. (a) Breton, J. J.; Chabot-Fletcher, M. C. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 459-466; (b) Badger, A. M.; Cook, M. N.; Swift, B. A.; Newman-Tarr, T. M.; Gowen, M.; Lark, M. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 587-593. (c) Roshak, A.; Jackson, J. R.; Chabot-Fletcher, M.; Marshall, L. A. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 955-961.

22. Meijer, L.; Thunnissen., A. -M.W.H.; White, A. W.; Garnier, M.; Nikolic, M.; Tsai, L.-H.; Walter, J.; Cleverley, K. E.; Salinas, P. C.; Wu, Y. Z.; Biernat, J.; Mandelkow, E. M.; Kim, S.- H.; Pettit, G. R. *Chem. Biol.* **2000**, *7*, 51-63.

23. Tasdemir, D.; Mallon, R.; Greenstein, M.; Feldberg, L. R.; Kim, S. C.; Collins, K.; Wojciechowicz, D.; Mangalindan, G. C.; Concepción, G. P.; Harper, M. K.; Ireland, C. M. *J. Med. Chem.* **2002**, *45*, 529-532.

24. Kolch, W. *Biochem. J.* **2000**, *351*(2), 289-305.

25. Mulder, K. M. *Cytokine Growth Factor Rev.* **2000**, *11*(1-2), 23-35.

26. Sharma, V.; Lansdell, T. A.; Jin, G. Y.; Tepe, J. J. *J. Med. Chem.* **2004**, *47*(14), 3700-3703.

27. (a) Garrett, M. D.; Fattaey, A. *Curr. Opin. Gene Dev.***1999**, *9*, 104–111. (b) Malumbres, M.; Barbacid, M. *Nat. Rev. Cancer* **2001**, *1*, 222–231.(c) Ali, A.; Hoeflich, K. P.; Woodgett, J. R. *Chem. Rev.* **2001**, *101*, 2527–2540. (d) Imahori, K.; Uchida, T. *J. Biochem. (Tokyo)* **1997**, *121*, 179–188. (e) Martinez, A.; Castro, A.; Dorronsoro, I.; Alonso, M. *Med. Res. Rev.* **2002**, *22*, 373–384.

28. (a) Profit, A. A.; Lee, T. R.; Lawrence, D. S. *J. Am. Chem. Soc.* **1999**, *121*, 280–283. (b) Martinez, A.; Alonso, M.; Castro, A.; Pérez, C.; Moreno, F. J. *J. Med. Chem.* **2002**, *45*, 1292–1299.

29. (a) Traxler, P.; Bold,G.; Frei, J.; Lang, M.; Lydon, N.; Mett, H.; Buchdunger, E.; Meyer, T.; Mueller, M.; Furet, P. *J. Med. Chem.* **1997**, *40*, 3601–3616. (b) Vesely, J.; Havlicek, L.; Strnad, M.; Blow, J. J.; Donella-Deana, A.; Pinna, L.; Letham, D. S.; Kato, J.; Detivaud, L.; Leclerc, S.; et al., *Eur. J. Biochem.* **1994**, 224, (2), 771-86. (c) Fong, T. A. T.; Shawver, L. K; Sun, L.; Tang, C.; App, H.; Powell, T. J.; Kim, Y. H.; Schreck, R.; Wang, X.; Risau, W. Ullrich A.; Hirth K. P.; McMahon G. *Cancer Res.* **1999**, *59*, 99–106.(d) Lee, J. C.; Badger, A. M.; Griswold, D. E.; Dunnington, D.; Truneh, A.; Votta, B.; White, J. R.; Young, P. R.; Bender, P. E. *Ann. N Y Acad. Sci.* **1993**, *696*, 149–170.

30. Lindel, T.; Hoffmann, H. *Tetrahedron Lett.* **1997**, *38*, 52, 8935-8938.

31. Annoura, H.; Tatsouka, T. *Tetrahedron Lett.* **1995**, *36*, 3, 413-416.

32. Xu, Ying-Xi, Yakushijin, K.; Horne, D. A. *J. Org. Chem.* **1997**, *62*, 456-464.

33. Sosa, A. C. B.; Yakushijin, K.; Horne, D. A. *J. Org. Chem.* **2000**, *65*, 610-611.

34. Chacun-Lefèvre, L.; Joseph, B.; Mérour, J.- Y. *Tetrahedron*, **2000**, *56*, 4491-4499.

35. Ware, E. *Chem. Rev.* **1950**, *46*, 403-470.

36. Sosa, A. C. B.; Yakushijin, K.; Horne, D. A. *J. Org. Chem.* **2002**, *67*, 4498-4500.

37. Meanwell, N. A.; Roth, H. R.; Smith, E. C.; Wedding, D. L.; Wright, J. J. K. *J. Org. Chem.*, **1991**, *56*, 6897-6904.

38. (a)Prager, R. H.; Tsopelas, C. *Aust. J. Chem.* **1992**, *45*, 1771-1777. (b) Anderson, K. W. Trifluoromethanesulfonic acid catalyzed Friendel-Crafts acylation with β-lactams and studies towards total synthesis of hymenialdisine, M.S. Thesis, Michigan State University, East Lansing, MI, **2002**.

39. Mukerjee, A. K.; Joseph, K.; Homami, S.- S.; Tikdari, A. M. *Heterocycles*, **1991**, *32*, 7, 1317-1325.

40. Fu, M.; Fernandez, M.; Smith, M. L.; Flygare, J. A. *Org. Lett.* **1999**, *1*, 1351-1353.

41. Sharma, V.; Tepe, J. J. *Bioorg. Med. Chem. Lett.* **2004**, *43*, 4319-4321.

42. (a) Peddibhotla, S.; Jayakumar, S.; Tepe, J. J. *Org. Lett.* **2002**, *4*, 3533-3535. (b) Peddibhotla, S.; Tepe, J. J. *Synthesis* **2003**, *9*, 1433-1440.

43. (a) Steglich, W.; Höfle, G. *Tetrahedron Lett.* **1970**, 4727-4730. (b) Höfle, G.; Steglich, W.; Vorbrüggen, H. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 569-583. (c) Hassner, A.; Krepski, L. R.; Alexanian, V. *Tetrahedron***1978**, *34*, 2069-2076.

44. Ruble, J. C.; Fu. G. C. *J. Am. Chem. Soc.* **1998**, *120*, 11532-11533.

45. Steglich, W.; Höfle, G. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 981-981.

46. Daly, J. W.; Padgett, W.; Shamim, M. T.; Butts-Lamb, P.; Waters, J. *J. Med. Chem.* **1985**, *28*, 487-492.

47. (a) Anderson, W. K.; Bhattacharjee, D.; Houston, D. M. *J. Med. Chem.* **1989**, *32*, 119-127. (b) Snyder, H. R.; Hendrick, R. G.; Broks, L. A. *Org. Synth.* **1947**, *22*, 65.

48. (a) Yasuda, N.; Iwagami, H.; Nakanishi, E.; Nakamiya, T.; Sasaki, Y.; Murata, T. *J. Antibiot.* **1983**, *36*, 242. (b) Yasuda, N. *J. Heterocyclic Chem.* **1985**, *22*, 413-416.

49. (a) Muchowski, J. M.; Solas, D. R. *J. Org. Chem.* **1984**, *49*, 203-205.(b) Mattews, D. P.; Whitten, J. P.; McCarthy, J. R. *J. Heterocyclic Chem.* **1987**, *24*, 689-692. (c) Whitten, J. P.;Mattews, D. P.; McCarthy, J. R. *J. Org. Chem.* **1986**, *51*, 1891-1894. (d) Lipshutz, B. H.; Vaccaro, W.; Huff, B. *Tetrahedron Lett.* **1986**, *27*, 4095-4098.

50. Gerard, C.; Rollins, B. J. *Nat Immunol* **2001**, *2*, 108-115.

51. Harris, E. D., Jr. *N. Engl. J. Med.* **1990**, *322*, 1277-1289.

52. Baeuerle, P. A.; Henkel, T. *Annu. Rev. Immunol.* **1994**, *12*, 141-179.

53. Moreland, L. W. *J. Rheumatol.* **1999**, *26 Suppl 57*, 7-15.

54. Handel, M. L.; Nguyen, L. Q.; Lehmann, T. P. *Clin Exp Pharmacol Physiol* **2000**, *27*, 139-144.

55. Tak, P. P.; Firestein, G. S. *J Clin Invest* **2001**, *107*, 7-11.

56. Yamamoto, Y.; Gaynor, R. B. *Curr Mol Med* **2001**, *1*, 287-296.

57. Mahon, T. M.; O'Neill, L. A. J. *J. Biol. Chem.* **1995**, *270*, 28557-28564.

# CHAPTER VI

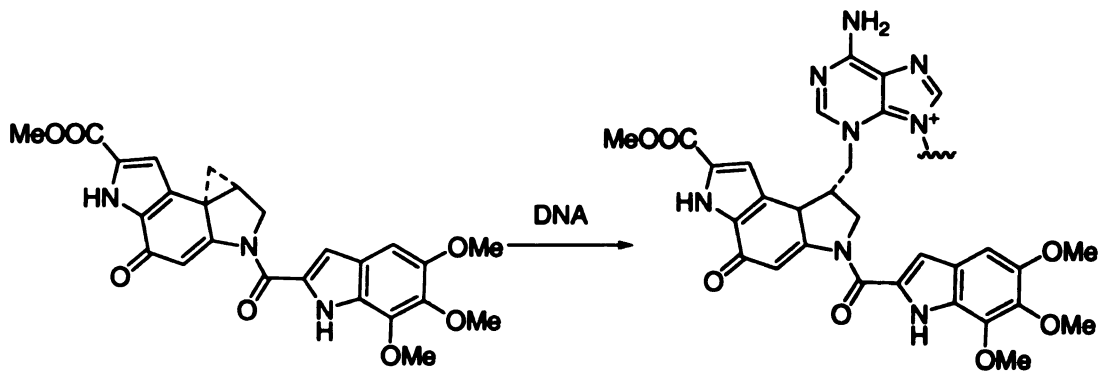
## STRATEGIC BIO-REDUCTIVE ACTIVATION OF THE PRODRUG SELECTIVELY IN TUMOR CELLS

*You can't tell a book by its cover!* ..... .....

### A. Introduction

The double helical DNA is the richest source of information in a living organism. The sequence codes for RNA synthesis via transcription which further gets translated into protein and enzyme synthesis important for metabolism, growth, and survival of living beings. Current chemotherapy relies primarily on drugs such as camptothecin, [1] cis-platin, [2] etoposide, [3] bleomycin[4] etc. which cause genotoxic stress to the cancer cell. However, the clinical efficacy of these drugs is compromised due to activation of survival factors such as NF-κB[5] and $MDM_2$, [6] resistance due to activated P-glycoproteins[7] or poor availability at the site of action. Further, most chemotherapeutic agents are non-selective. They target cells which replicate fast, including those of the gut and bone marrow.[8] Chemotherapeutic agents require efficient penetration into the cytosol of the target cells in order to reach their intracellular targets and exert their therapeutic activity. Nitro-analogs for camptothecin- irinotecan and topotecan are more water soluble, however, they too suffer of the gastrointestinal toxic side effects.[8] Some clinically used chemotherapeutic agents, such as bleomycin and cisplatin, exert a nonpermeant or semipermeant character.[9] The sensitivity of tumor cells to bleomycin is highly variable due to its limited penetration through the plasma membrane. However, a new technology photochemical internalization (PCI) improves the utilization of macromolecules in cancer therapy in a site-specific manner.[9] Some antitumor agents such as CC-1065 and

duocarmycins are naturally occurring antibiotics which selectively alkylate the DNA in a sequence specific manner.[10] These form a unique class of compounds showing *in situ* activation which might be considered specialized subsets of prodrugs. These undergo a conformational change upon DNA-binding and get activated for nucleophilic attack. [11]




**Duocarmycin SA**

**Figure VI-1.** DNA alkylation reaction with Duocarmycin SA.

A prodrug is a pharmacological substance (drug) which is administered in a latent, inactive (or significantly less active) form. Once administered, the prodrug is metabolized in the body under specific physiological conditions (*in vivo*) into the active compound. The rationale behind the use of a prodrug is generally for ADME (Absorption, Diffusion, Metabolism, Excretion) optimization. Prodrugs are usually designed to improve oral bioavailability - poor absorption from the gastrointestinal tract is usually a limiting factor, and is often due to the chemical properties of the drug. In rational drug design, the knowledge of chemical properties likely to improve absorption and the major metabolic pathways in the body allows the modification of the structure of new chemical entities for improved bioavailability. However, sometimes the use of a prodrug is unintentional, especially in the case of serendipitous drug discoveries, and the drug, is only identified as a prodrug after extensive drug metabolism studies. Currently used prodrug formulations include enalapril (angiotensin converting enzyme, ACE inhibitor), for the treatment of

heart failure which has been developed as its prodrug by Merck Co. The active form enilaprilat is activated *in situ* in presence of esterase.

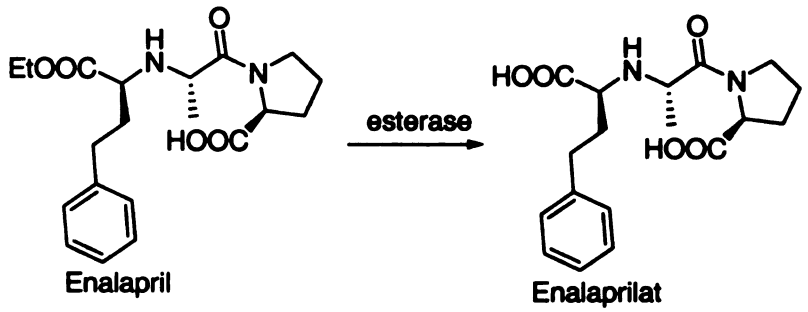



Figure **VI-2**. Conversion of drug (enaliprat) from prodrug (enalapril)

Among anti-tumor agents, nitrogen mustards have gained maximum importance and have been used extensively for DNA cross- linking.[12]

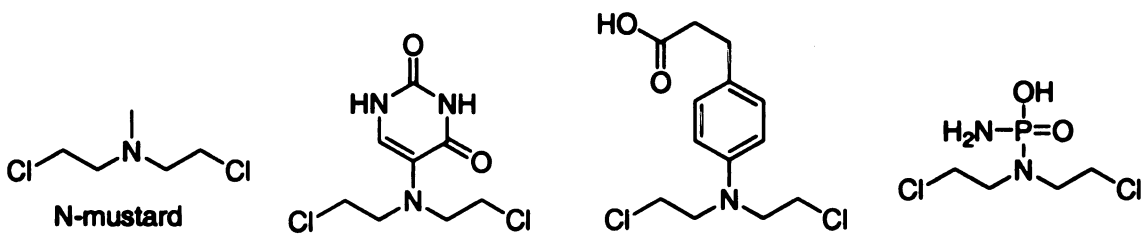



**Figure VI-3**. Commonly known nitrogen mustards.

After much dilemma pertaining to the mechanism of interstrand cross-linking, research from Hopkin's and Gruenberg's groups independently ascertained the covalent cross-linking with DNA via aziridinium formation followed by alkylation to afford the N7 to N7 interstrand cross-linking ( Figure VI-4). [13]

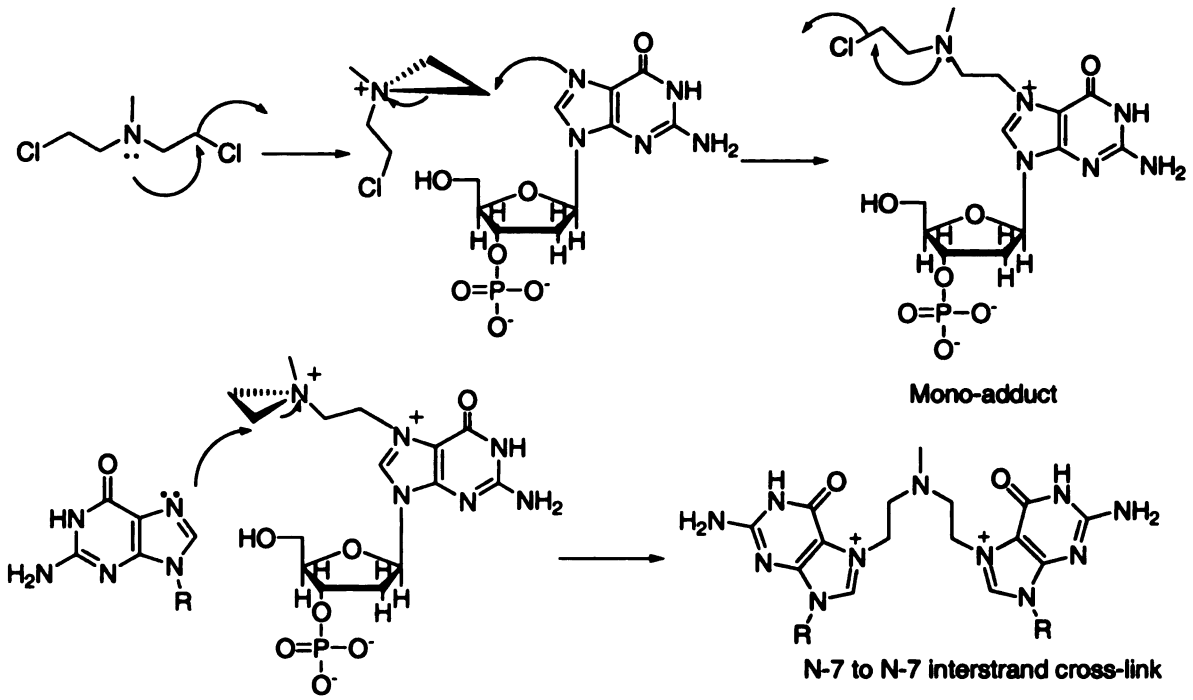



**Figure VI-4.** Proposed mechanism of DNA-DNA cross linking with N-mustards.

### B. Hypoxia and Prodrugs

Tumor hypoxia, a deficiency of oxygen due to an inefficient vasculature, is a limiting factor in both the radiotherapy and chemotherapy of solid tumors.[14] Paradoxically, it is also an attractive therapeutic target, because severe hypoxia occurs only in solid tumor tissue. The hypoxic environment causes resistance due to various reasons. Firstly, the cells are distant from the blood vessels, hence drugs that are administered intravenously or orally can't reach the site of action (Figure VI-5).[15] Secondly, cellular proliferation decreases inside the solid tumor.[16] Thirdly, hypoxia selects for p53 mutated cells and finally, it also up-regulates anti-apoptotic factors via activation of the HIF protein.[17] Thus, in response to cytotoxic therapy, a tumor selects for the malignant type,[18] increased expression of tumor invasion[19] and could even compromise treatment by surgery. [20]

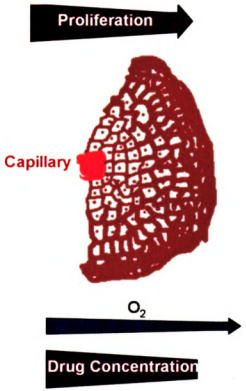



**Figure VI-5**. Depletion of oxygen, drug concentration in solid tumor cells.

Since the oxygen-depleted reductive microenvironment of hypoxic cells is unique to solid tumors, it can be exploited for therapy by non-toxic, hypoxia-activated prodrugs. Conceptually, 'trigger' units in these drugs are selectively activated in hypoxic cells to release or activate a toxic 'effector', capable of killing surrounding oxygenated tumor cells. Useful triggers include nitroaromatics, quinones, N-oxides, and transition metals.[21] Figure VI-6 illustrates the current examples using reducing environment to target DNA and cause genotoxic burden to tumor cells.

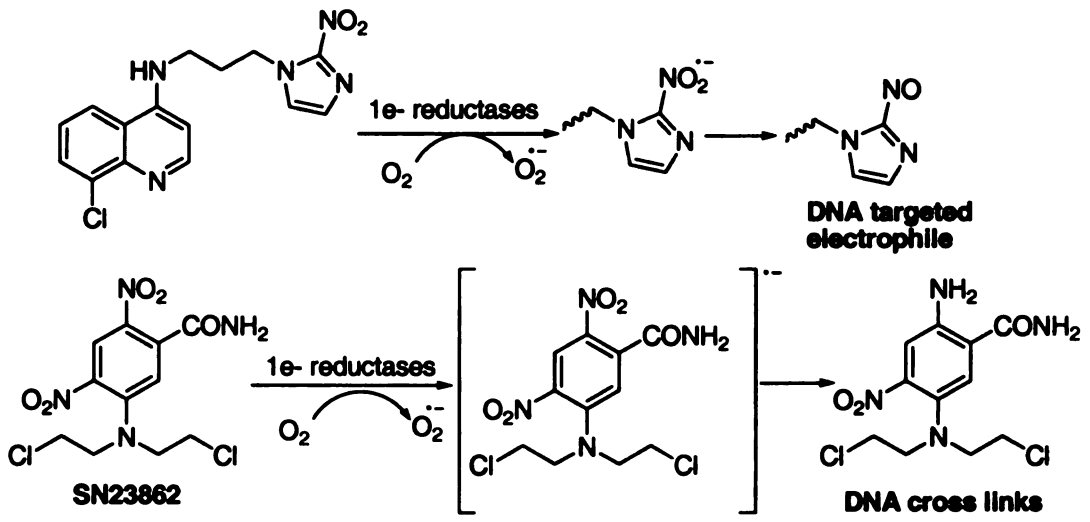



**Figure VI-6.** Use of nitroimidazoles and masked N-mustards for targeting DNA.

Denny *et al* describe nitro group as an electronic switch, [21b] redistributing the electron density to activate the N-mustard upon reduction. They found that the 2-amine (*ortho* to the amide) was the metabolite and about 2000 folds stronger than the inactive prodrug moiety.[21b] Another prodrug, N-oxide tirapazamine (TPZ) discovered by Brown and Lee[22] two decades ago is now in phase III clinical trials. Figure VI-7 illustrates the reduction mechanism of TPZ leading to topoisomerase-2 poisoning.

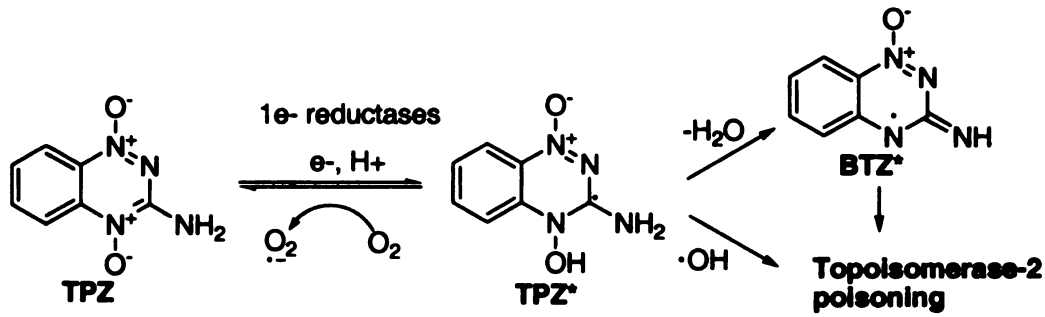



**Figure VI-7.** Conversion of prodrug TPZ into active BTZ* for topoisomerase-2 poisioning.

Concurrent to our studies, Niculescu-Duvaz *et al* [23]described self- immolative nitrogen mustard prodrugs cleavable by carboxypeptidase CPG2 (Figure VI-8) .

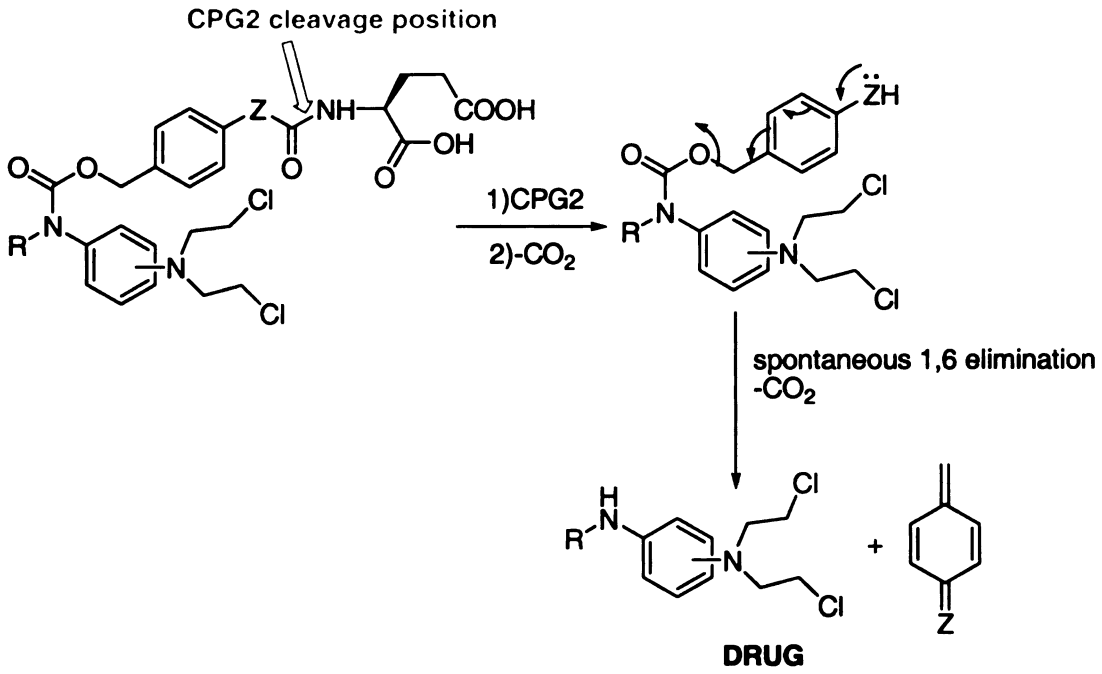



**Figure VI- 8.** Self- immolation prodrug generates DNA cross linker upon CPG2 activtiy.

### C. Reductive activation of DNA cross-linkers.

Mitomycin C, (Figure VI-9) a quinone antibiotic that requires reductive metabolism for activity, is the prototype anticancer drug of this class of drugs that get activated upon hypoxic conditions.[24]

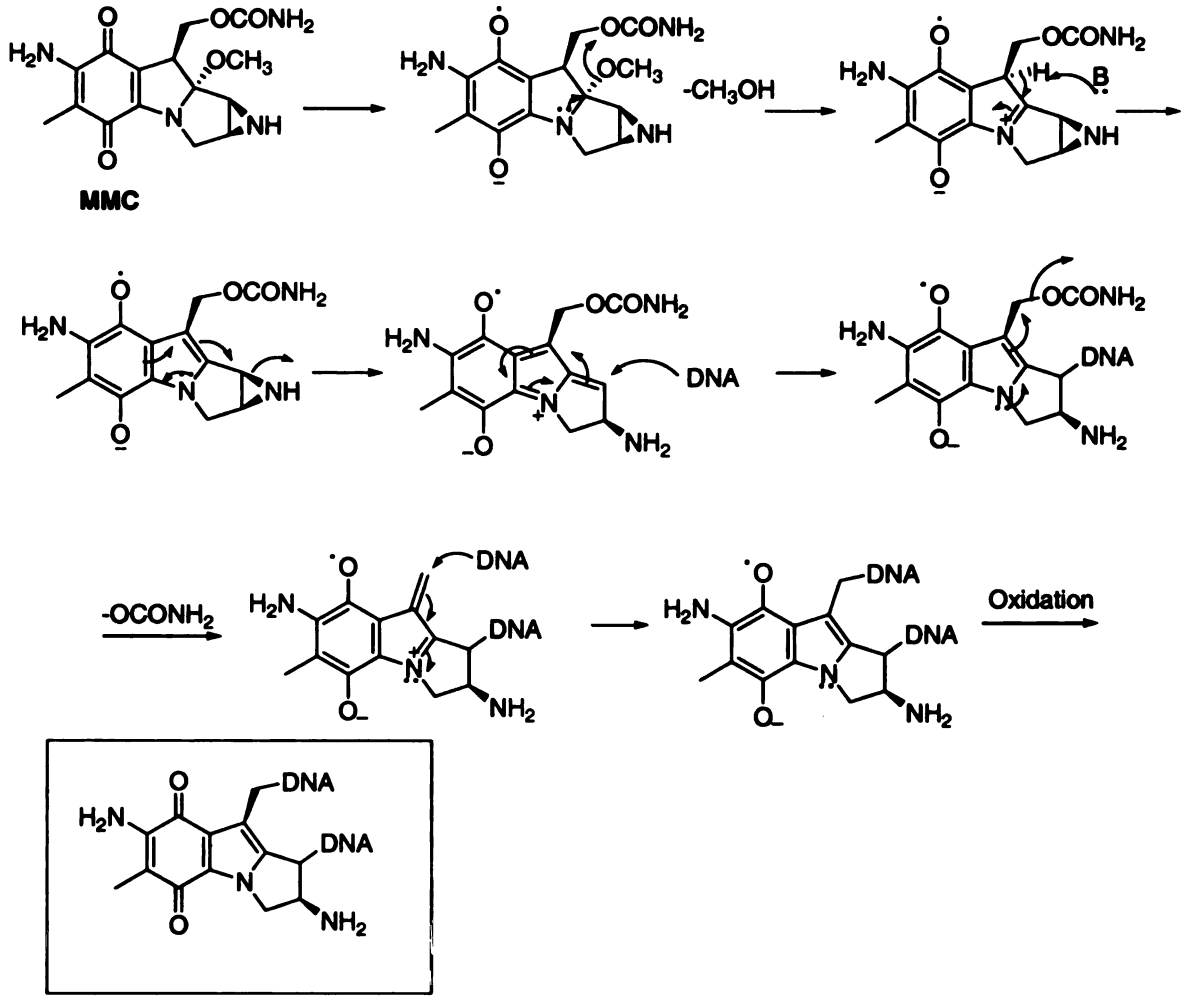



**Figure VI-9.** Activation of mitomycin C ( MMC) under reducing conditions.

Some important bio-reductively activated drugs belong to the FR-series-FR66979 and FR900482, which undergo N-O bond cleavage under reducing conditions to yield mitosene-like intermediates. Goto and Fukuyama[26] were the pioneers to propose the reductive activation of the FR900482 before cross linking to DNA. MMCs undergo electrophilic activation via a one or two-electron reduction on the quinine moiety (Figure VI-9). Deriving from this model, they proposed the reductive cleavage of N-O bond to generate aniline derivative which could cyclize and dehydrate to give aziridinomitosene-like intermediates responsible for their DNA damaging activity.[25-27]

Further, Williams and Rajski, demonstrated the reduction with $Fe^{3+}$, and proposed a mechanism for the reductive activation of FR099482 in presence of thiols and Fe (III) salts (Figure VI-10).[27]

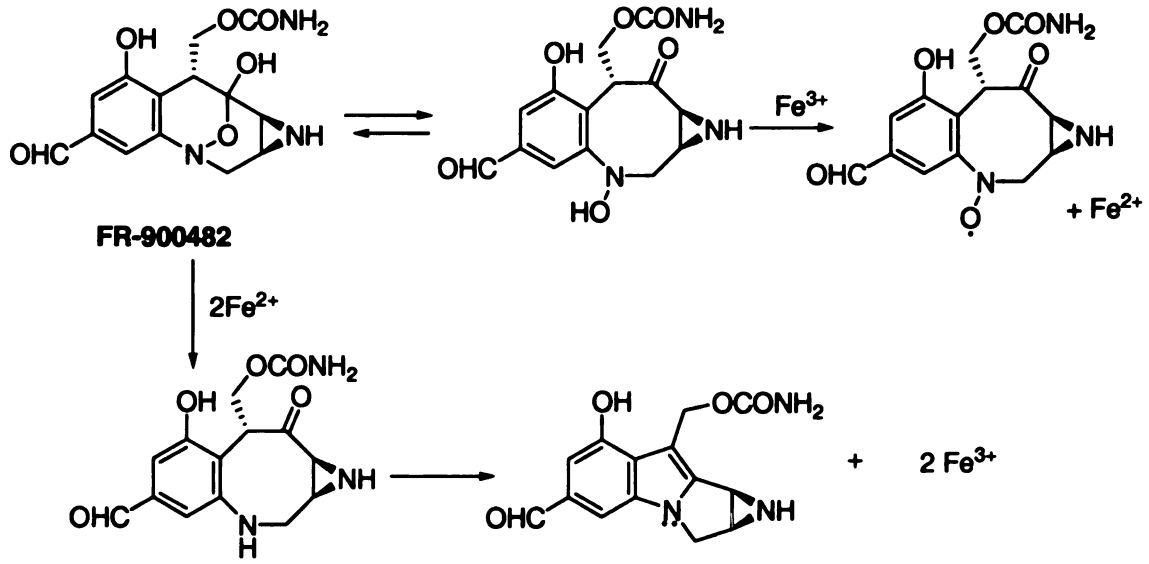



**Figure VI-10.** Proposed model for Fe (III)-mediated reductive activation of FR900482.

Encouraged by the ability to selectively use hypoxic conditions, we designed prodrug molecules for N-mustard (drug). Figure VI-11 shows the rationale and mechanism for generation of N-mustard from the drug.

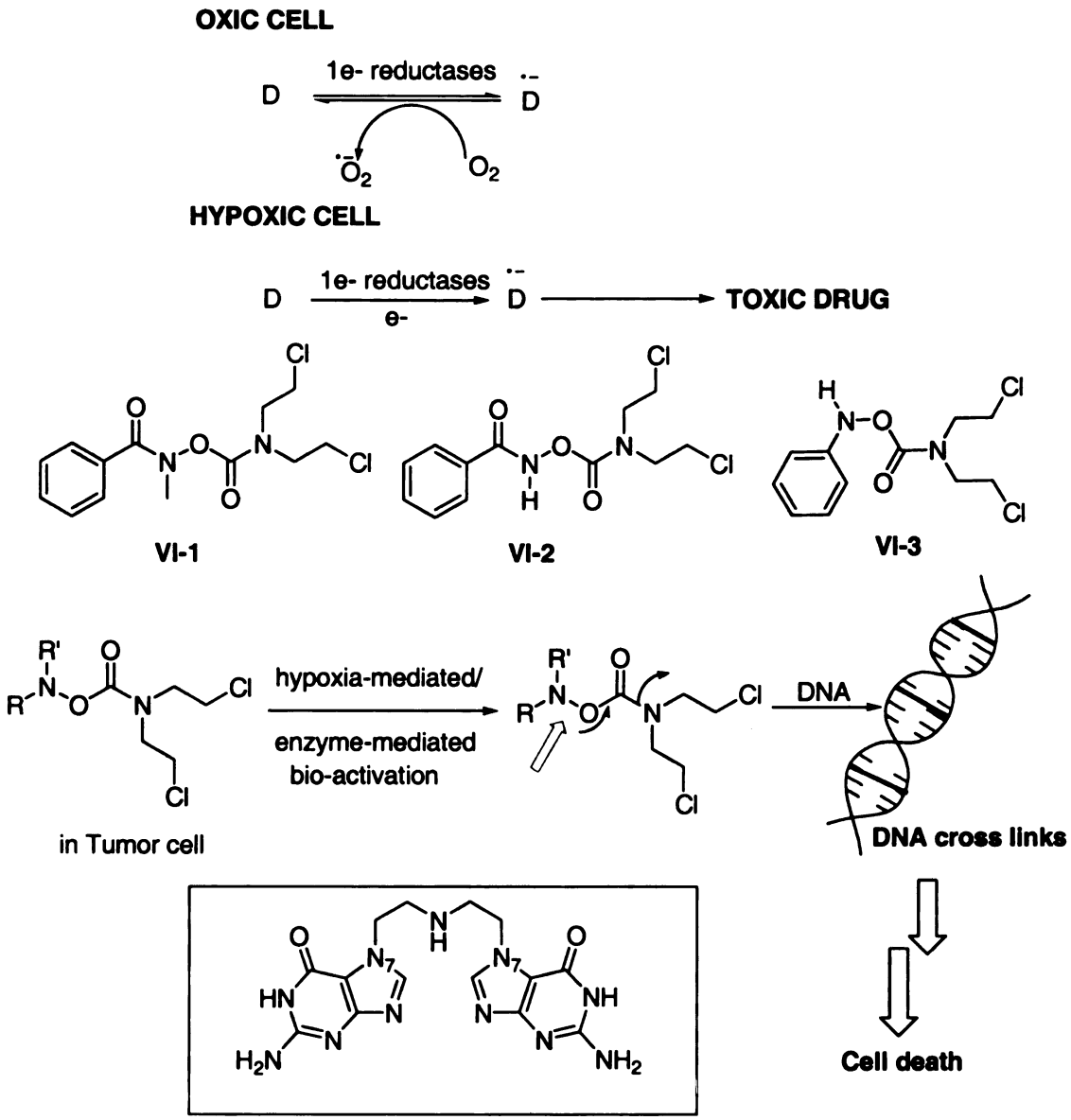



**Figure VI-11.** (Top). Rationale for the design of the prodrug. (Middle) Structure of the prodrug model system. (Bottom) mechanism for prodrug reductive activation.

Encouraged by the reductive activation studies on molecules such as mitomycin C and FR series of DNA cross linking agents, we designed DNA cross linkers (Figure VI-11) which would undergo cleavage at the N-O bond. This would lead to the decarboxylation and liberation of the N-mustard to cross-link DNA.

These were treated as a model system for identification of tumor selective NF-κB "prodrugs" through the incorporation of a reductive mode of activation of known NF-κB inhibitors. As described earlier (Chapters1-5) extracellular stimuli such as TNF-α, LPS, UV light, hypoxia as well as anti-neoplastic agents activate NF-κB.[28] NF-κB activation leads to inhibition of apoptosis and activation of proto-oncogenes which indirectly inhibit tumor repressor proteins.[29] Thus, chemotherapeutic treatment often fails as a result of NF-κB double stimulus causing resistance and unfavorable cell growth.[28, 29] Inhibition of NF-κB has been identified as a potential therapeutic target in diseases such as cancer and inflammation. The impediment that has obviated its clinical utility as a target protein in cancer treatment is wide variety of NF-κB mediated gene products. An alternative to this could be a tumor selective prodrug moiety to circumvent the side effects.

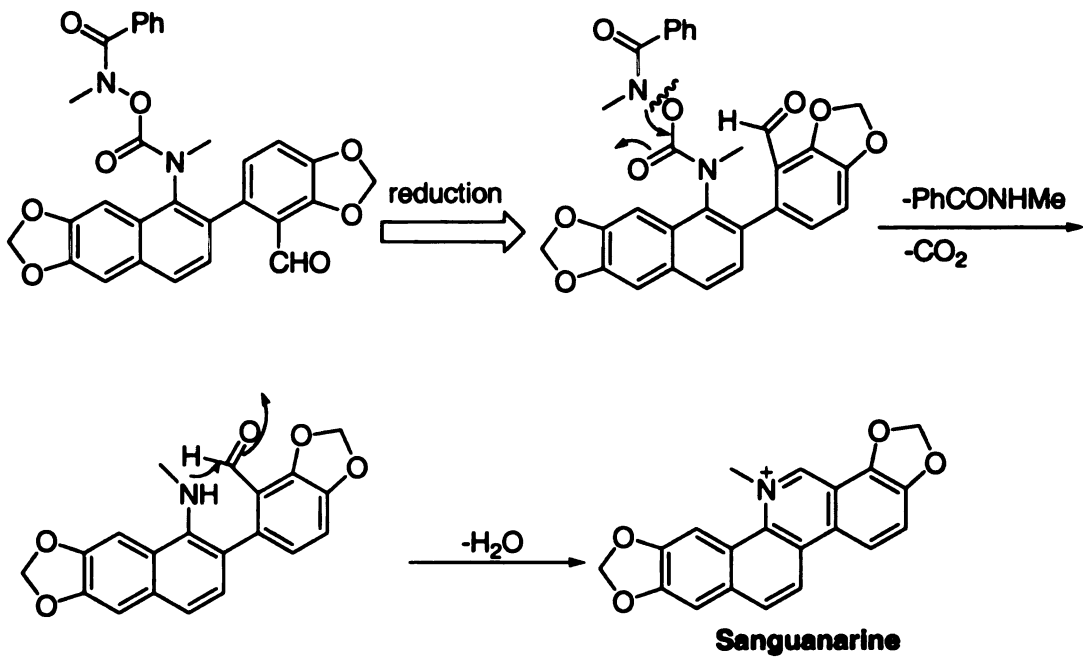



**Figure VI-12.** Reductive activation of NF-κB by sanguanarine prodrugs

Figure VI-12 shows the prodrug for the I-κB-α phosphorylation inhibitor sanguanarine [30] To quickly determine the proof of principle and check if our design for the prodrug

moiety would work, we focused on nitrogen mustards as the 'drug', for *in vitro* analysis of the reductive cleavage of the N-O bond and planned for *in vivo* studies in cell lines such as murine leukemia cell line, L210 and P388, colorectal carcinoma cell lines Colon26 and melanoma cell line B16.[31] The N-mustard released in the tumor cells can cross link the DNA strands by alkylation, and hence prevent the indefinite cellular growth. DNA cross-linkers proved to be a good model for the prodrug reductive activation because the synthesis requires limited number of steps and is time-efficient.

**D. Retro-synthetic analysis for the prodrug**

The retro-synthetic analysis for **VI-1** was based on formation of hydroxamic acid from benzoic acid (Figure VI-13). This could then be converted to a carbonate which would undergo a nucleophilic substitution (as illustrated in Scheme VI-1) and finally a substitution of hydroxyl groups by Cl via an $SN_2$ mechanism.

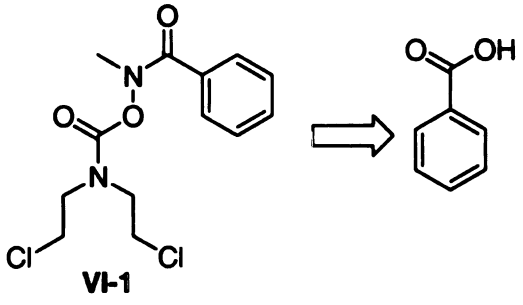
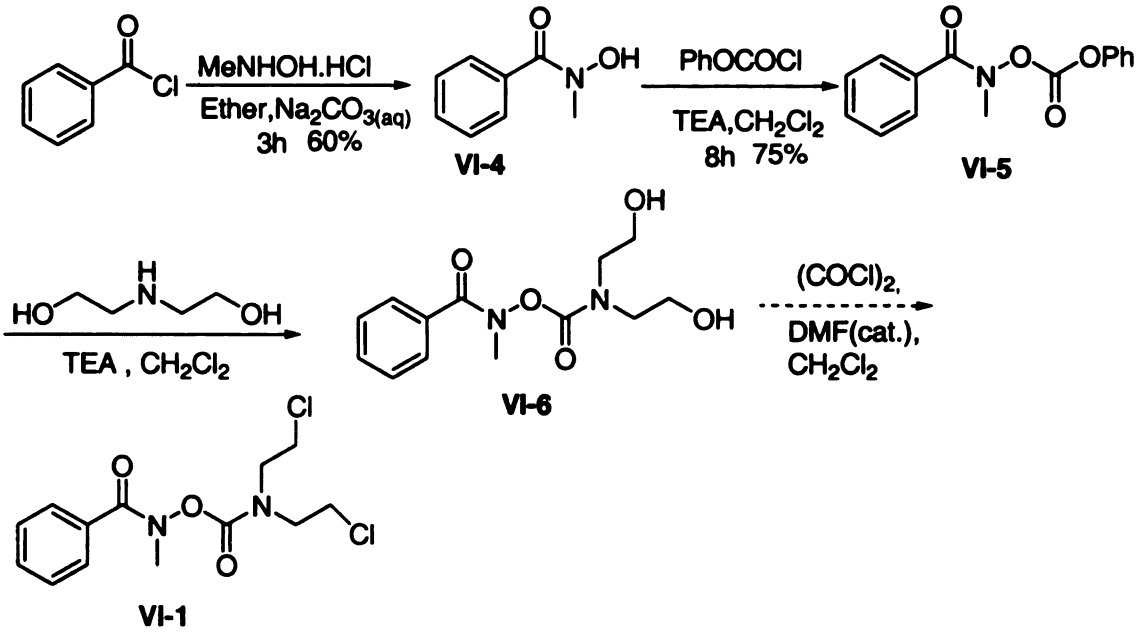



**Figure VI-13.** Retrosynthetic analysis of **VI-1**

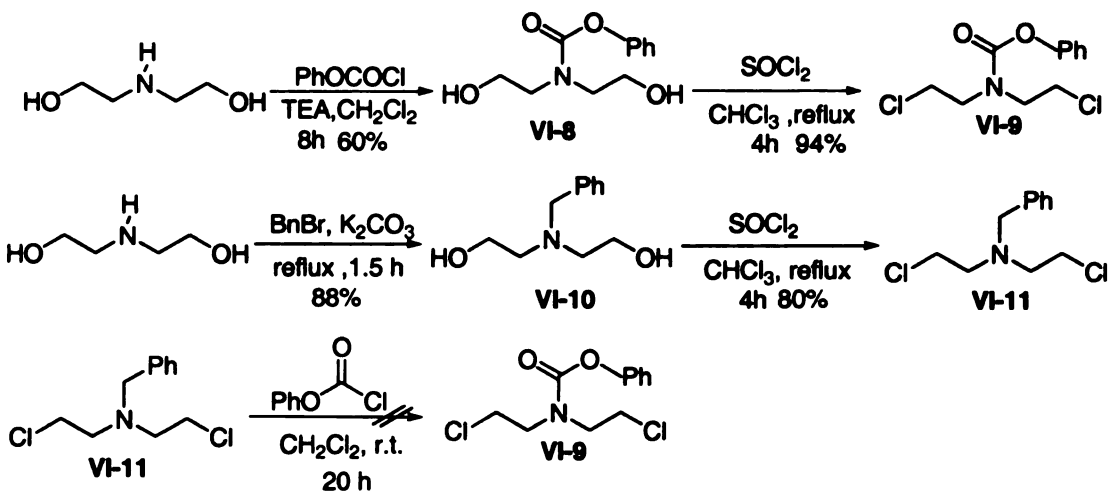



**Scheme VI-1.** Synthesis of **VI-1** from benzoic acid.

This synthetic route suffered low yield due to nucleophilic substitution on **VI-5** to yield **VI-4** as a by-product indicating the cleavage of both the C-O bonds of the carbonate **VI-5**. We modified the route by employing a symmetrical carbonate **VI-7**, which could be condensed with diethanolamine. However, upon purification, the product **VI-6** decomposed.

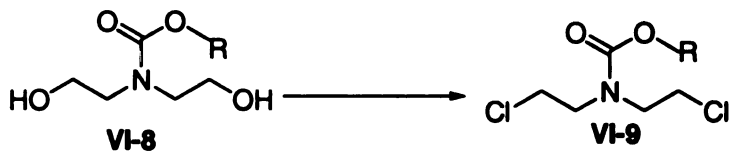
We further modified our synthetic route to take advantage of the nucleophilicity of N in diethanolamine. Commercially available diethanolamine was protected using phenylchloroformate under mild basic conditions to give **VI-8** in moderate yields.[32] This protected alcohol was then subjected to substitution with $SOCl_2$ in $CHCl_3$[33] to give a protected N-mustard **VI-9** in 94% yield. (Scheme VI-2) Diethanolamine was also subjected to N-benzylation to give **VI-10** in modest yields. This was converted into a N-mustard **VI-11** (*caution: DNA cross linker!!*) in 80% yield. However, conversion of **VI-**

11 to **VI-9** via a von Braun reaction[34] in $CH_2Cl_2$ as well as $CH_3CN$ did not work (Scheme VI- 2).




**Scheme VI-2.** Conversion of diethanolamine into protected and unprotected N-mustards (negative and positive controls for *in vitro* studies)

The conversion of the protected alcohols into alkyl halides was tried under various conditions as shown in Table VI-1. $SOCl_2$ in chloroform under reflux conditions provided most satisfactory results.

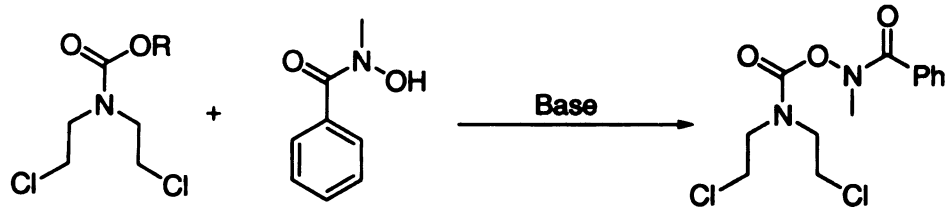



| Entry | R | Conditions | Yield |
|---|---|---|---|
| 1 | $CH_2Ph$ | $SOCl_2$, 0°C→r.t. | 60% |
| 2 | $CH_2Ph$ | $SOCl_2$, dry DMF | 0% |
| 3 | TROC | $SOCl_2$, reflux, 2h | 93% |
| 4 | Ph | $(COCl)_2$, DMF(1drop) $CH_2Cl_2$ | 44% |
| 5 | Ph | $SOCl_2$, $CHCl_3$, reflux | 94% |

**Table VI-1.** Conversion of protected alcohol to the alkyl halide.

**VI-9** was then condensed with **VI-4** in presence of a base to for **VI-1**. [35]

When the leaving group was a phenyl, this conversion was not facile under TEA/$CH_2Cl_2$ or KH/THF. We modified the protecting group to p-nitrophenyl and/or Cl (**VI-13**) and coupled the resulting carbamate with the hydroxamic acid in presence of a base. Table VI-2 summarizes the reaction under various basic conditions. The reaction yields depended on the choice of leaving group and the base required.



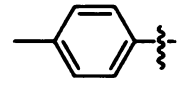
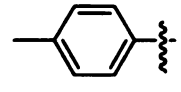
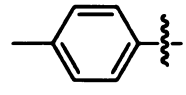
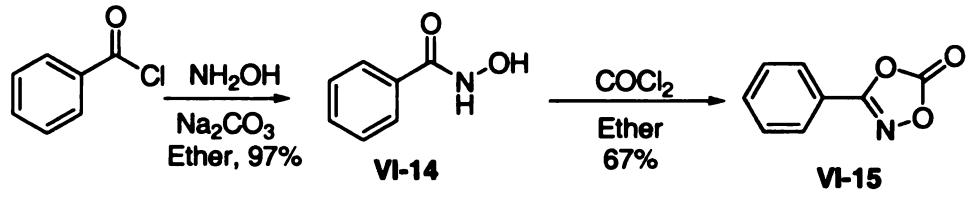

| R | Base | Solvent/catalyst | Temperature | Yield |
|---|---|---|---|---|
| Ph | NaH | THF | -42°C | 0 % |
| Ph | KH | THF / 18-crown-6 | 0°C | 0 % |
| Cl | KH | THF / 18-crown-6 | 0°C-> r.t. | 80 % |
| Cl | TEA | $CH_2Cl_2$ | 0°C ->r.t. | 91 % |
| $NO_2$  | TEA | $CH_2Cl_2$ | 0°C ->r.t. | 0 % |
| $NO_2$  | pulv. $K_2CO_3$ | $CH_2Cl_2$ | 0°C ->r.t. | 0 % |
| $NO_2$  | KH | THF / 18-crown-6 | 0°C ->r.t. | 79 % |

**Table VI-2.** Conversion of the carbamate into the prodrug moiety **VI-1**.

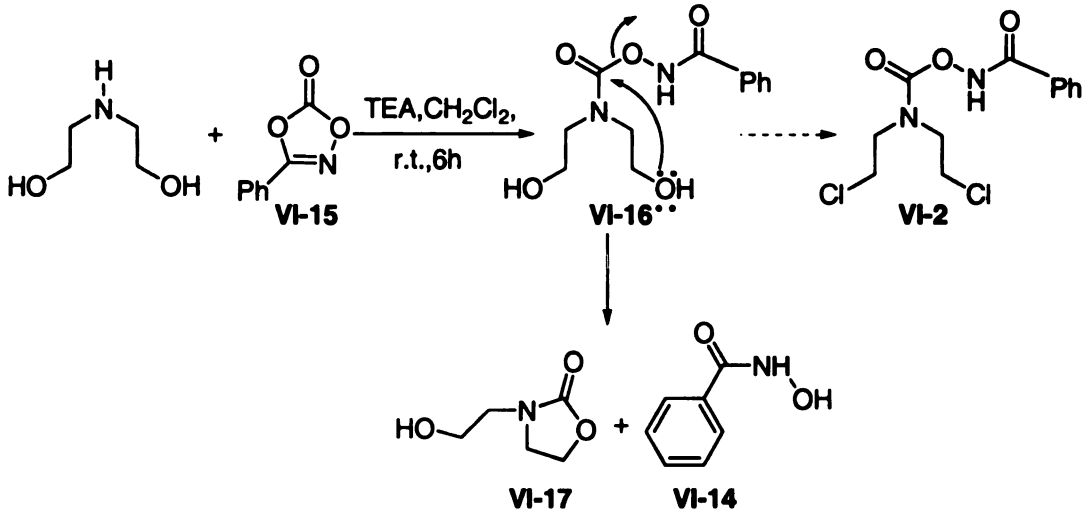
A similar route for **VI-2** did not afford the product and decomposed. Another route for the synthesis of **VI-2** was tried using dioxazalone as shown in Scheme VI-4. **VI-14** was synthesized by condensation of hydroxylamine to benzoyl chloride. The dioxazalone was

synthesized from **VI-14** by condensation with $COCl_2$ (phosgene) as described previously by Overberger *et al.*[36]



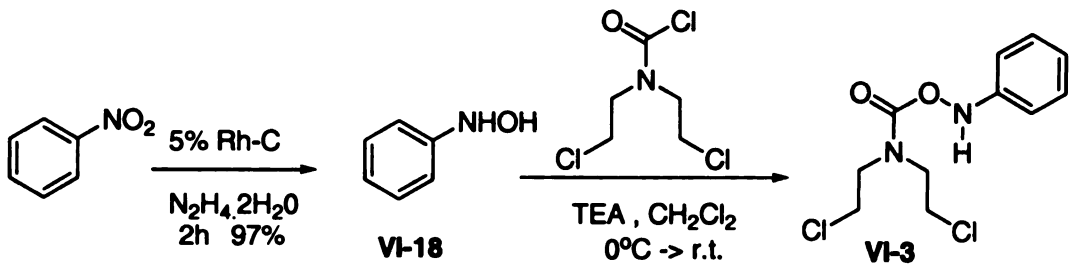

**Scheme VI-3.** Conversion of **VI-14** into the dioxazalone **VI-15.**

The dioxazalone was then condensed with the commercially available diethanolamine in presence of TEA at room temperature. We expected to convert the protected diol into the di-alkyl halide using $SOCl_2$. However, the diol **VI-16** cyclized intramolecularly to yield the 5-membered ring following Baldwin's rule (5-exo trig, favored), and yielded **VI-17** and **VI-14.**




**Scheme VI-4.** Formation of 2-oxazolidinone ring **VI-17.**

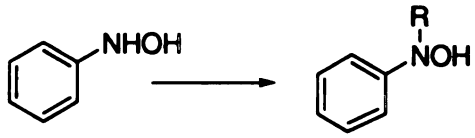
Further, **VI-3** was synthesized starting from commercially available nitrobenzene.




**Scheme VI-5.** Synthesis of VI-**18** and conversion to **VI-3.**

Commercially available nitrobenzene was treated with 5%Rh-C and hydrated hydrazine[37] for 2h at room temperature to afford **VI-18** which was then condensed with commercially available carbamoyl chloride to form **VI-3.** The product was purified but even after several columns, an unknown impurity co-eluted with the product and could not be purified any further (Scheme VI-5).

We further focused on the development of analogs of this prodrug moiety for which a number of hydroxamic acids and hydroxylamines were prepared [38]




| Entry | R | Reaction Conditions | Yield |
|---|---|---|---|
| **VI-19** | $CH_3$ | $CH_3I/CH_3OH$ r.t. 8h | 40% |
| **VI-20** | Bn | BnBr, Py, ether, r.t. 48h | 35% |

**Table VI-3.** Synthesis of N-alkylated hydroxyl amine.

Both **VI-19** and **VI-20** were very unstable. **VI-19** was isolated as its oxalate salt; however, all efforts to isolate **VI-20** were futile.

Before we progressed any further, we wanted to determine if our design of the pro-drug moiety would show differential activity under oxic and hypoxic conditions. For this, we

first calculated the reduction potential values for the N-O bond using cyclic voltammetry experiments.

**E. Electrochemical data for VI-1, VI-4 and VI-14:** [39]

For all these prodrugs, a major concern for us was to determine the reduction potential values for these compounds. We addressed this problem using cyclic voltammetry. All experiments were performed in non-aqueous solvent. Anhydrous $CH_3CN$ that was passed through alumina and stored with molecular sieves was used as the solvent. Ferrocene and 1, 4-benzoquinone were used as standards to standardize the working electrode and also calibrate the reference electrode, $Ag/AgNO_3$ (in $CH_3CN$). Since the prodrug decomposes on reduction, only the reduction half cycle was seen. The reduction potential found for the different compounds are as shown below in Table VI- 4.

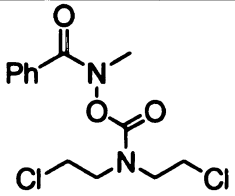
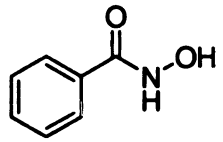
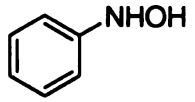
| Entry | Reduction Potential(V) |
|---|---|
|  | -0.301 |
|  | -0.265 |
|  | -0.254 |

**Table VI-4.** Reduction potential of prodrug **VI-1**, **VI-4**, and **VI-14**.

Figure VI-14 shows the cyclic voltametry curve for the compound **VI-1**. It is clearly evident from the curve, that once, reduced the compound can't go through the reverse cycle due to its decomposition into $CO_2$ and the mustard gas.

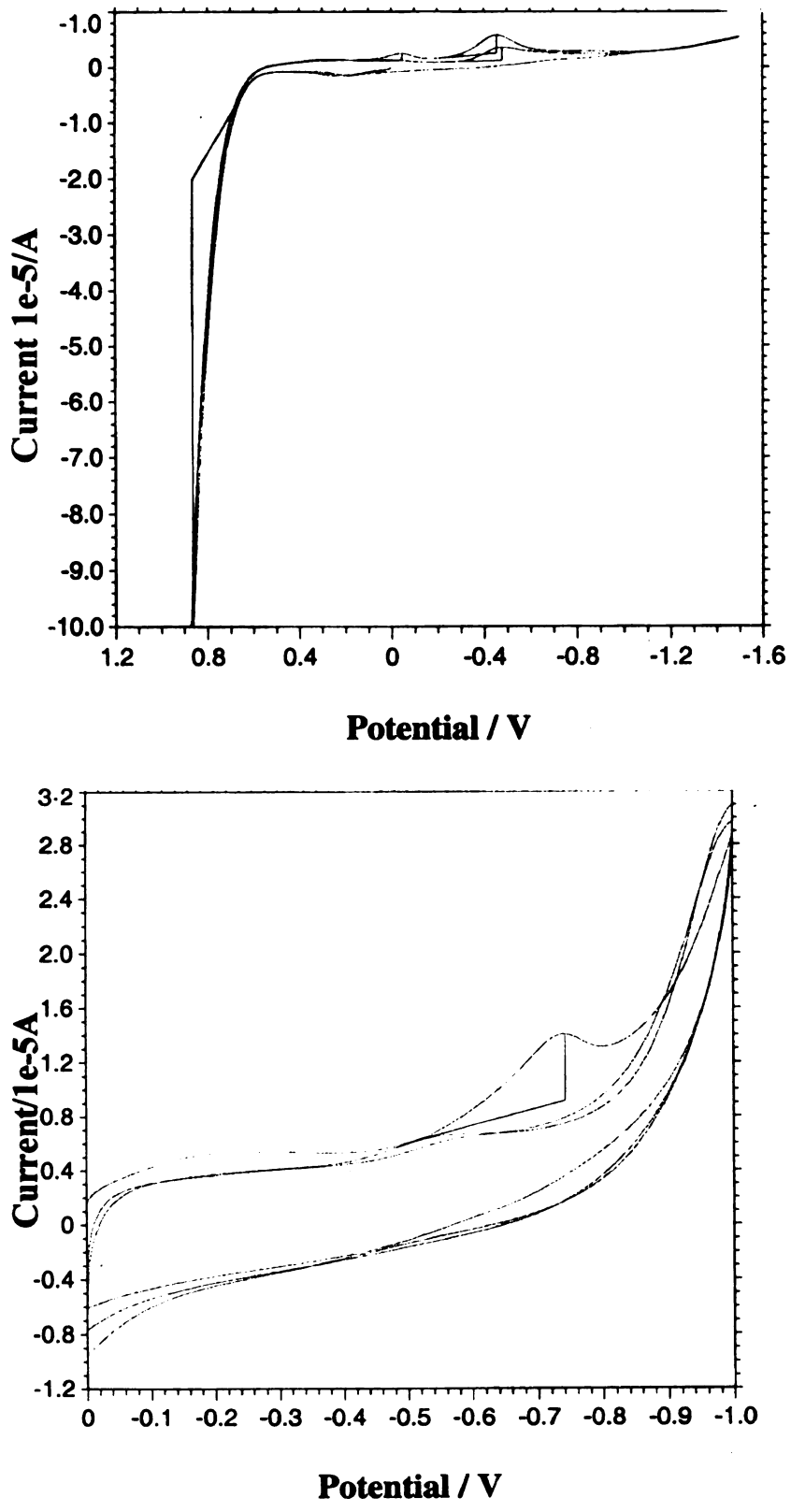



**Figure VI-14.** Cyclic voltametry for **VI-1.** (TOP): Blank control (BOTTOM): **VI-1** in $CH_3CN$

In a tumor cell, the redox potential varies between 0.3-0.45 V [39] indicating that the prodrug should undergo reduction readily. Denny *et al* have shown various analogs of nitro benzyl quarternary salts with reduction potentials in the range of -0.3 to -0.46 to undergo reduction in tumor cells (Figure VI-15).[39b]

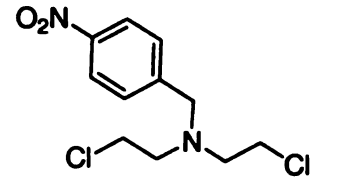
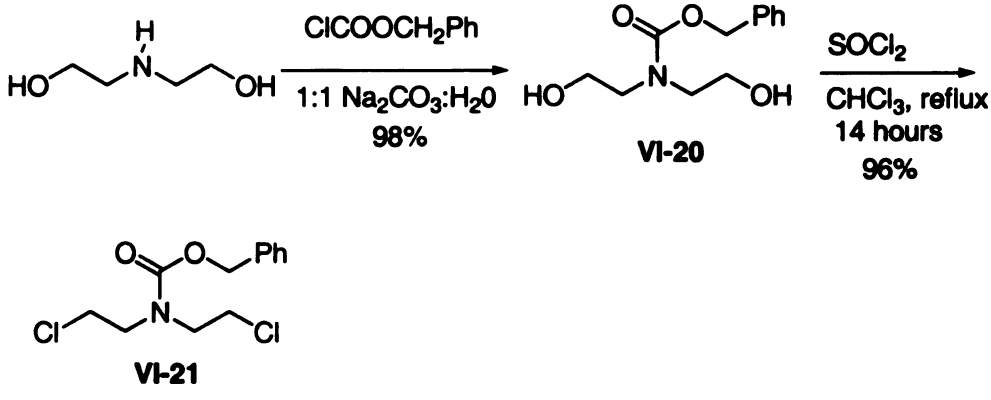



**Figure VI-15.** Denny's nitro benzyl quarternary salts as a prodrug for N-mustard.

**F. In-vitro bio-reductive activation studies**

The prodrug **VI-1** was now subjected to reductive activation strategy, which would liberate the DNA cross linker and can be visually seen on a denaturing alkaline agarose gel.[25] We synthesized a negative control, Cbz-protected N-mustard (**VI-21**) for this experiment. The C-O bond can undergo reductive cleavage at a potential of ~ 2.2 V, a value much higher than the reduction potential provided by the reductive environment in a tumor cell intrinsically or that provided by most reducing agents in *in vitro* experiments




**Scheme VI-6.** Synthesis of **VI-20.**

**G. Cross- linking studies with the pro-drug**

The prodrug was now subjected to reductive activation strategy using a wide variety of reducing agents, which could possibly mimic the tumor cell. Supercoiled plasmid DNA pBR322 was linearized using EcoR1 restriction enzyme. The linearity and concentration of the linear DNA was determined by carrying out densitometry experiments on a neutral agarose gel (Figure VI-16).



1 2

Lane1: *λHIND-III* marker, Lane 2: Linearized pBR322 run on a neutral agarose gel for 1 h. Densitometry calculations show the size of the linearized plasmid to be 5.1 kb.

**Figure VI-16**. Neutral agarose gel to check the linearity, concentration and size of the plasmid.

Encouraged by the complete digestion of the DNA, we proceeded to attempt a denaturing alkaline agarose gel to separate the single stranded DNA from the double stranded cross-linked DNA.

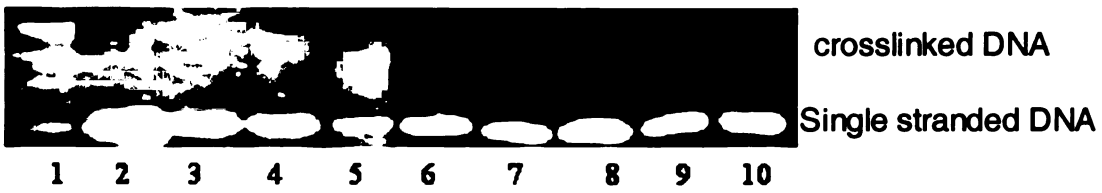
For this, the prodrug was incubated with the linearized DNA, in presence and absence of the reducing agent. In the presence of the reducing agent the N-O bond in the prodrug was expected to get reduced leading to decarboxylation and liberation of the free N-

mustard which would then cross link across the DNA molecule. The reaction mixture was run on a denaturing gel to separate single stranded DNA from the cross linked DNA. Mitomycin C, which gets reductively activated was used as a positive control. However, some of the reducing agents did not activate mitomycin C. Thus, we optimized the reducing power of various reducing agents, like DTT, $Na_2S_2O_4$, β-mercaptoethanol, Cytochrome C reductase for mitomycin C.

| Reducing Agent | Effect on Mitomycin C |
|---|---|
| Dithiothreitol (DTT) 2mM | reduced MMC in presence and absence of $Fe^{2+}$ |
| $Na_2S_2O_4$, 10 mM | reduced MMC in presence and absence of $Fe^{2+}$ |
| $Fe^{2+}$/EDTA, 100 mM | No reduction in MMC |
| Cytochroome C reductase | Crude enzyme cleaved DNA |

**Table VI-5**. Optimizing the reducing agent for MMC reductive activation.

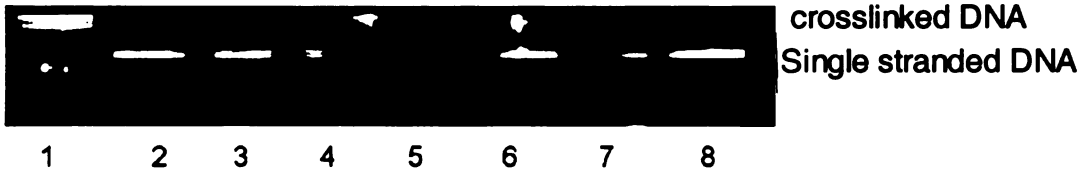
*λHINDIII* was employed as a molecular weight standard (lane 1, Figure VI-16). Control reactions were performed linearized DNA only (lane 2, Figure VI-16) and with an authentic sample of mitomycin C (1 mM, 2 mM DTT). As illustrated in Figure VI-16, incubation of mitomycin C with the DNA duplex in absence of a reducing agent leads to no detectable cross-linked product (lane 3). As shown, only the reactions depicted in lanes 4, 5, 7, 9 and 10 produced the interstrand DNA-DNA cross-link.




Lane 1: Molecular weight standard λ*HIND*III (0.5 μg) Lane 2: DNA (0.5μg) (negative control) Lane 3: DNA (0.5 μg) + 1mM mitomycin C Lane 4: DNA (0.5 μg)+ 1mM mitomycin C+ 2mM DTT (positive control for reductive activation) Lane 5: DNA (0.5μg)+ 1 mM mitomycin C+ 2mM DTT + $Fe^{2+}$(0.5 μM)(positive control for reductive activation) Lane 6: DNA (0.5 μg) + $Fe^{2+}$ (0.5 μM) Lane 7: DNA (0.5 μg)+ 1mM mitomycin C+ 100mM $Na_2S_2O_4$ (positive control for reductive activation) + $Fe^{2+}$(0.5 μM) Lane 8: DNA (0.5 μg)+ 1 mM mitomycinC+ $Fe^{2+}$(0.5 μM) Lane 9: DNA (0.5μg) + 1.0 mM **VI-1** Lane 10: DNA (0.5 μg)+ 1.0 mM **VI-1** + 2mM DTT.

**Figure VI-17.** Reductive activation of **VI-1**. Mitomycin C used as a positive control for reductive activation.

We also employed the N-methyl mustard and N-benzyl mustard. However, both compounds did not give a crisp band for double stranded DNA (data not shown). We observed that the prodrug group was able to cross-link DNA even without reduction (Lane 9, Figure VI-17; Lane 6, Figure VI-18).

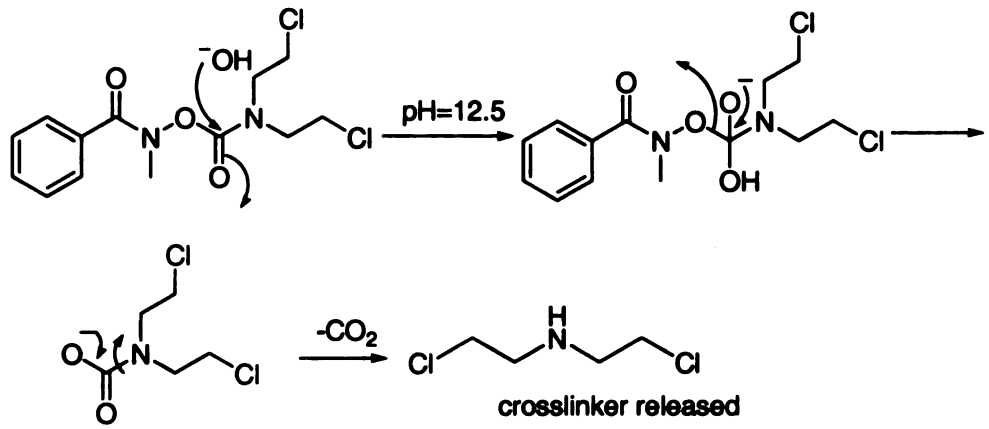



Lane 1: *λHINDIII* marker (5 μL) + 5 μL water Lane 2: pBR322 (0.5 μg) Lane3: pBR322 (0.5 μg) + mitomycin C (1mM) Lane4: pBR322 (0.5 μg) + mitomycin C (1mM) + $Fe^{2+}$/EDTA (100 mM) Lane5: pBR322 (0.5 μg) + **VI-1** (1mM) Lane6: pBR322 (0.5 μg) + **VI-1** (1mM) Lane7: pBR322 (0.5 μg) + **VI-1** (1mM) + $Fe^{2+}$/EDTA (100 mM) Lane8: pBR322 (0.5 μg) + $Fe^{2+}$/EDTA (100 mM).

**Figure VI-18.** Reductive activation of **VI-1** in presence of $Fe^{2+}$/ EDTA.

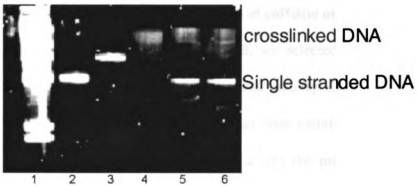
We propose that this could be due to hydrolysis of the prodrug moiety under alkaline pH. The pH of the denaturing gel is 12.8-12.9 (experimentally determined). At this pH, in

accordance with Scheme VI-7, the prodrug **VI-1** undergoes hydrolysis and liberates the N-mustard which cross links the DNA. This observation can be supported by observations made by Gruenhert *et al*[40] where they found a pH dependence for the stability of the MMC crosslinked and N-mustard–cross linked DNA. They could observe cross linked double stranded DNA at pH=12.3 but not at pH=14. Since, we have an intermediate pH range; we probably have some hydrolyzed double stranded DNA-MMC complex for the positive control as well.




**Scheme VI-7.** Hydrolysis of the prodrug **VI-1**

To ascertain this hypothesis, we ran a gel using CDDP as the positive control. CDDP is a clinically used DNA damaging agent which causes DNA damage by cross-linking at N7 of dG.[2] The prodrug **VI-1** was not activated under reducing conditions (Figure VI-19) but was only incubated with DNA. Though, this gel was overloaded with the molecular weight marker, the gel clearly shows cross linked DNA similar to that from N-mustard (lane 4, 5, 6 , Figure VI-19).




Lane 1: *λ HINDIII* marker (5 μL) + 5 μL water Lane 2: pBR322 (0.5 μg) Lane3: pBR322 (0.5 μg) + Cis-Platin (1mM) Lane4: pBR322 (0.5 μg) + N-carbamoyl chloride 2, 2'-bischloroethyl amine (1mM) Lane5: pBR322 (0.5 μg) + **VI-1** (1mM) Lane6: pBR322 (0.5 μg) + **VI-1** (1mM).

**Figure VI-19. VI-1** undergoes hydrolysis under alkaline conditions.

In a tumor cell, the physiological pH is 7.4, and hence, the prodrug is not expected to undergo hydrolysis *in vivo*. As an alternative to the alkaline agarose gel, we set up a denaturing polyacrylamide- urea gel (DPAGE). No denaturation could be seen on a 5% TBE/ 6M DPAGE or a 25% TBE/6M DPAGE gel.

The following proposed alterations might address the above problem:

- Since, the DNA used for a polyacrylamide gel is a 10 base pair oligonucleotide, it may not be able to provide the actual binding site for the N-mustard and CDDP. In order to overcome this, we could synthesize our own dodeca or trideca nucleotide sequence.
- Other analytical tools such as UV absorption spectrophotometry could also be used to detect and analyze the DNA cross linking.[41]

These studies on DNA cross linkers were initially started as model studies for pro-drug activated NF-κB inhibitors. Unfortunately, the optimization for detection of the DNA-cross links did not prove to be successful.

**H. Bio-reductively activated NF-κB inhibitors of caffeine and theophylline**

Among the various known inhibitors of NF-κB, we selected the xanthine derivatives, because of their easy availability and low/minimal toxicity. Our initial studies included theophylline and its derivatives. Theophylline has been identified as an NF-κB inhibitor by preventing the translocation of the protein across the nuclear membrane.[42] Earlier studies reveal that theophylline, aminotheophylline and bromotheophylline have been used as against asthma and inflammation, though, the complete mode of action is not known. Since NF-κB promotes chemoresistance and is also involved in inflammation in cells, [43] we sought to synthesize a small molecule that would exploit the reducing conditions of the tumor cells to generate an NF-κB inhibitor *in vivo*.

Denny *et al* have emphasized that nitroimidazoles can behave as an electronic switch for bio-reductive activation.[21] They observed that when nitroimidazoles were reduced in a tumor cell, they became more cytotoxic. This indicates that the nitro group could act as a prodrug moiety for amino/hydroxylamino groups, which are cytotoxic in nature. In the light of the above facts, we initially hypothesized that nitrotheophylline (**II-1**) could act as a prodrug to undergo reduction under hypoxia in the tumor cell and generate aminotheophylline (**II-3**). Encouraged by these facts, we synthesized and tested these small molecules for NF-κB activity (Chapter 2).

However, we successfully synthesized both **II-1** and **II-3** among many other 7 and 8-substituted theophyllines and caffeines. However, when these were tested for NF-κB activity, both **II-1** and **II-3** were found to modestly inhibit NF-κB translocation across the nuclear membrane. Hence, we deduced that this may not find a clinically relevant result due to absence of differential selectivity in NF-κB inhibition. Further, in order to enhance

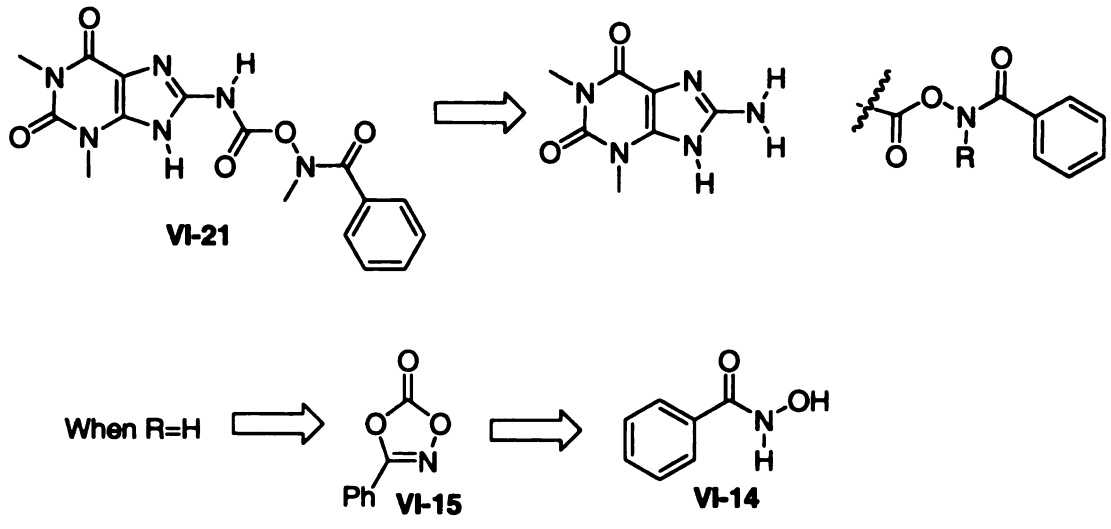
the specificity of the NF-κB inhibitor, we functionalized 8-aminotheophylline with the prodrug group.

**I. Reductive activation of prodrug group on 8-aminotheophylline (II-3), a NF-κB inhibitor**

We have earlier shown 8-aminotheophylline (**II-3**, see chapter 2 page 69) to be an inhibitor of NF-κB among other derivatives of theophylline like 8-nitrotheophylline (**II-1**) and 8-bromotheophylline (**II-2**). In order to exploit the drug activity of aminotheophylline, specifically in tumor cells we functionalized **II-3** with the prodrug group described earlier with the aim to study the effect of the molecule (**VI-24**) in Jurkat cells *in vivo* under aerobic and anaerobic conditions.

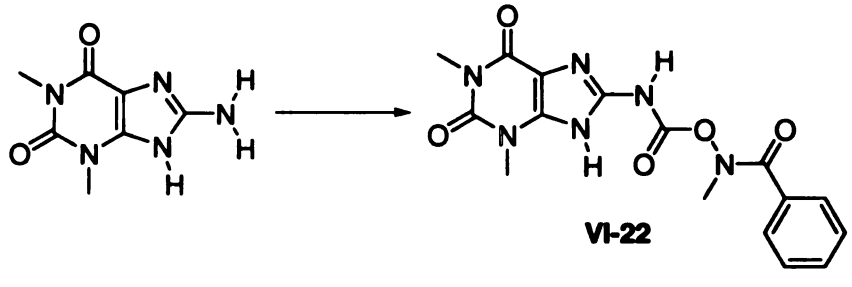
8-aminotheophylline (**II-4**) was found to be a potent inhibitor of NF-κB and inhibited NF-κB-DNA binding even at 0.01 μM (Chapter 2). Zimmer *et al* have prepared 8-substituted caffeines and theophyllines which did not have any carcinogenic activity and in fact were potential anticancer compounds.[44] Cote *et al* 7-substituted derivatives of 8-aminotheophylline can be used as coronary vasodilators and diuretics.[43] Substituted aminopurines have also been shown to have bactericidal activity and may possess central nervous system depressor effect.[45]

Retrosynthetically, the prodrug moiety could be coupled to the aminotheophylline (**II-3**) using the dioxazalone template (Scheme VI-8).




**Scheme VI-8.** Retrosynthetic analysis of **VI-21**.

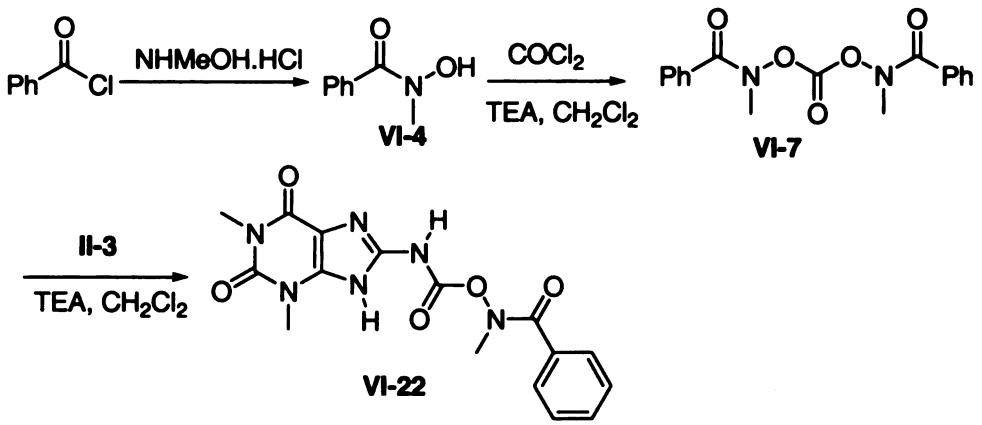
As explained previously, **VI-14** was prepared via condensation of benzoyl chloride and hydroxylamine in presence of mild base $Na_2CO_3$. **VI-14** was then condensed with $COCl_2$ to give the dioxazalone **VI-15**. Efforts towards **VI-15** with carbonyldiimidazole as the condensation agent did not afford the product. **VI-15** was now subjected to condensation with aminotheophylline(**II-3**) (Table VI-6). The reaction proceeded sluggishly only in the presence of a mild base.




| Entry | Reaction Conditions | Yield |
|---|---|---|
| 1 | VI-15, $CH_2Cl_2$ | 0% |
| 2 | VI-15,$CH_2Cl_2$, TEA | 10% |

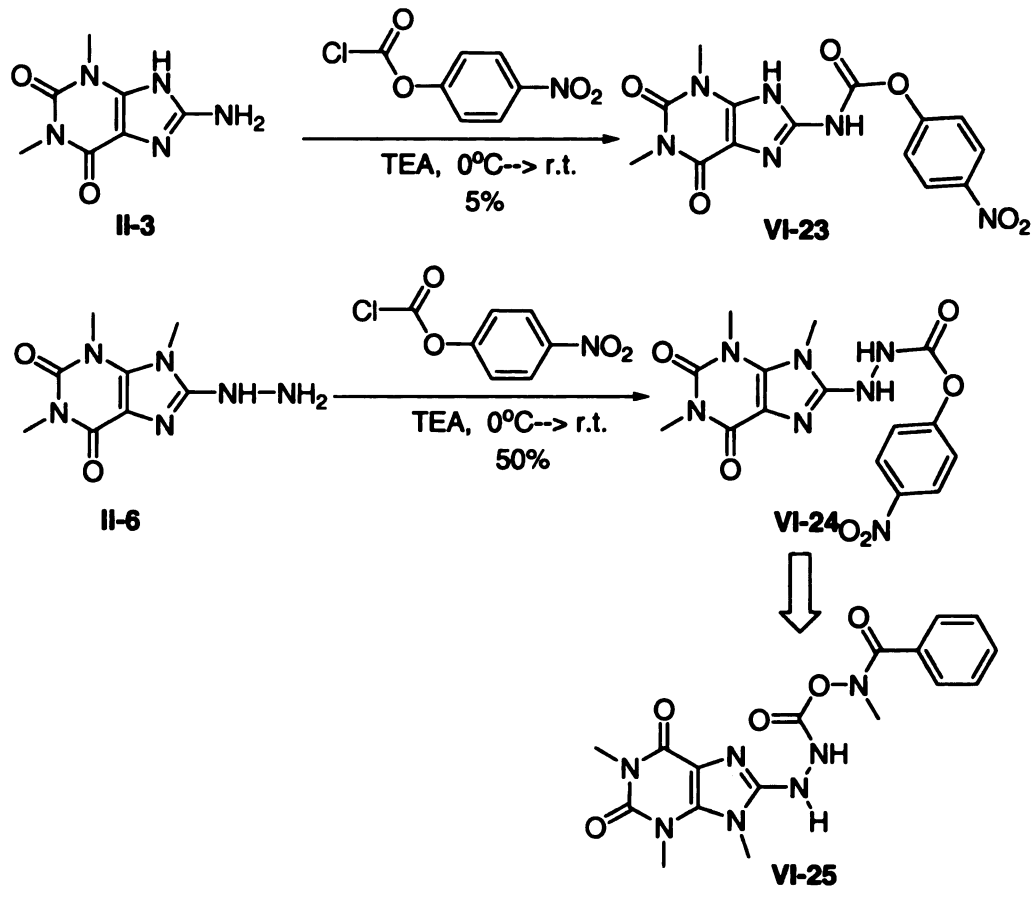
**Table VI-6.** Towards functionalization of NF-κB inhibitor, **II-3**.

Since the yield of the reaction was low it was difficult to completely characterize it. Hence, to ascertain the presence of the compound, a comparative study would be done via a different synthetic route. As described previously, N-methylhydroxamic acid was synthesized from benzoyl chloride and further condensed with $COCl_2$ to afford the carbonate **VI-7** (Scheme VI-9).




**Scheme VI-9**. Synthesis of functionalized aminotheophylline **VI-22.**

**VI-7** was subjected to treatment with aminotheophylline in presence of TEA to yield the desired functionalized aminotheophylline **VI-22.** Concurrently, we also pursued a condensation reaction between **II-3** and **II-6** with p-nitrophenyl chloroformate under basic conditions which could then be functionalized with the prodrug moiety (Scheme VI-10).




**Scheme VI-10**. Conversion of **II-3** and **II-6** into their chloroformate which would give their functionalized form.

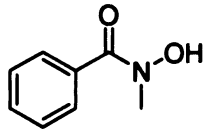
Both **II-3** and **II-6** reacted with p-nitrophenylchloroformate though their reactivity was differential. This is probably due to the fact that the amino group in **II-3** participates in resonance and hence is not as strongly nucleophilic as the terminal amino group in **II-6**.

**J. Conclusion.**

We successfully synthesized **VI-1** prodrug. We further successfully functionalized aminotheophylline, shown to be a potent NF-κB inhibitor in Chapter 2. Unfortunately, the commonly used DNA cross-linking experiments hydrolyzed the prodrug, indicating that either the method of detection has to be altered or the design of the pro-drug would have to be changed.

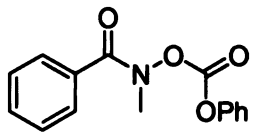
**K. Experimental Section**

**N-methyl N-hydroxy benzamide , VI-4**



A suspension of 1.8 GR N-methyl hydroxylamine.HCl (1.948 g, 23.4 mmol) and $Na_2CO_3$ (2.488 g, 23.48 mmol) in 125 ml ether was cooled at 0°C. Added benzoyl chloride (2.477 ml, 21.3 mmol) and 5 ml $H_2O$ to the reaction mixture. The reaction was allowed to warm to room temperature and stirred for 3h. Filtered off the solid and extracted the reaction mixture in EtOAc. Collected the organic layer , dried over $Na_2SO_4$. Filtered and evaporated the solvent *in vacuo*. Purified the product by column chromatography with EtOAc/Hexane(1:1). Collected the fractions, dried *in vacuo* to get a waxy low-melting solid. Yield : 60% (2.87 g) Rf: 0.25; $^1H$ NMR (300 MHz) ($CDCl_3$) δTMS: 3.36(s, 3), 7.46(m,5), 8.9(s broad,1) $^{13}C$ NMR (74.49MHz) ($CDCl_3$) δ: 38.36, 127.88, 128.28, 130.66, 132.76, 167.65 IR($CHCl_3$): 3167.52, 2924.46, 1601.12, 1574.11, 1219.17. EI(LRMS)m/z 151(M+).

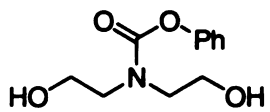
**O-(N-methyl N-benzoyl) O'- Phenyl carbonate, VI-5**



A solution of **VI-4** (60 mg, 0.39 mmol) in 10 ml $CH_2Cl_2$ was maintained at 0°C. TEA (0.06ml, 0.44 mmol) was added to the reaction flask. PhOCOCl (0.055ml, 0.44 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 2 h. The reaction was monitored by TLC to go to completion. The

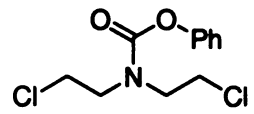
reaction mixture was extracted in $Et_2O$ and saturated $NH_4Cl$. The organic layer was collected, dried over $Na_2SO_4$ and dried *in vacuo*. The product was purified by column chromatography using 10% EtOAc in hexane. Yield: 75%, (0.14 g) $R_f$ =0.25. $^1H$ NMR (300 MHz) ($CDCl_3$) δTMS: 3.53(s, 3H), 7.46(m, 10H); $^{13}C$ (NMR) (74.49MHz) ($CDCl_3$) δ: 37.52, 120.70, 126.97, 128.32, 128.33, 128.63, 129.95, 131.64, 133.10, 150.88, 152.79, 171.27; IR ($CDCl_3$): 2924.46, 1797.89, 1672.50, 1591.48 $cm^{-1}$ EI (LRMS) (m/z):177 (M+) HRMS ($C_{15}H_{13}NO_4$) Calcd.(M+H, ES) 272.0923, found:272.0931.

**Phenyl bis(2-hydroxyethyl)carbamate, VI-8**



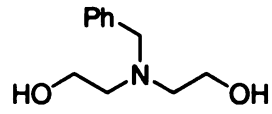

A solution of diethanolamine (500 mg, 4.76 mmol) in 10 ml $CH_2Cl_2$ was maintained at 0°C. TEA (728.9 μl, 5.23 mmol) was added to the reaction mixture. PhOCOCl (657.2μl, 5.23mmol) was added dropwise and allowed to stir at room temperature for 2 h. Extracted in $Et_2O$ and saturated $NH_4Cl$. The organic layer was collected, dried over $Na_2SO_4$ and dried *in vacuo*. The product was purified by column chromatography using 20% acetone in $CH_2Cl_2$. Yield : 60% (0.64 g) $^1H$ NMR (300 MHz) ($CDCl_3$) δTMS: 3.53(m, 4H), 3.7(s, 4H), 4.4(s, br, 2H), 7.05-7.25(m, 5H); $^{13}C$ NMR (74.49MHz) ($CDCl_3$) δ: 52.42, 52.72, 61.01, 61.60, 121.63, 125.48, 129.29, 150.99, 155.293; IR ($CDCl_3$):3400.9, 2932.2, 1701.4, 1207.6 $cm^{-1}$ EI (LRMS) (m/z): 226 (M+H); HRMS ($C_{11}H_{15}NO_4$)Calcd.(M+Na, ES) 248.0899, found:248.0906.

**Phenyl bis(2-chloroethyl)carbamate, VI-9**



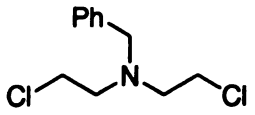
A solution of **VI-8** (0.6 g, 2.64 mmol) in $CHCl_3$ (10 mL) was refluxed with $SOCl_2$ (2.31 ml, 31.68 mmol) for 4 h. The reaction mixture was evaporated *in vacuo*. The reaction mixture was evaporated *in vacuo*. Yield : 94% ( 0.66 g) $^1H$ NMR (300 MHz) (d6-$CDCl_3$) δ TMS: 3.75-3.86(m, 8H), 7.14-7.42(m, 5H);$^{13}C$ (74.49 MHz) ($CDCl_3$) δ: 42.12, 42.31, 51.29, 51.57, 121.90, 126.00, 129.70, 151.16, 154.54 IR ($CHCl_3$): 2963.04 , 1722.65, 1410.14,1205.66 $cm^{-1}$ EI (LRMS) (m/z): 261(M+) HRMS ($C_{11}H_{13}O_2Cl_2N$) Calcd.(M+) 261.0323, found:261.0320.

**N-benzyl-diethanolamine, VI-10.**



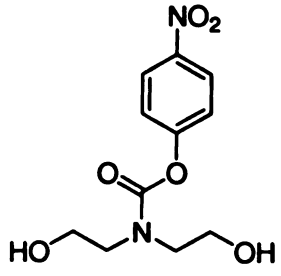
A solution of diethanolamine (350 mg, 3.32 mmol) and $K_2CO_3$ (459.3 mg, 3.32 mmol) in 10 ml water was maintained at room temperature. Benzyl bromide (0.51 g, 2.99 mmol) was added dropwise and the reaction mixture was refluxed for 1 h. The reaction mixture was cooled and extracted with ether. The organic layer was collected, dried over $Na_2SO_4$ and dried *in vacuo*. The product was purified by column chromatography using 15% ethanol in $CHCl_3$. Yield : 88% (0.57 g) $^1H$ NMR (300 MHz) ($CDCl_3$) δTMS: 2.68(m, 4H), 3.49(s, 24H), 3.61(m, 4H), 3.72(s, , 2H), 7.15-7.34(m, 5H); $^{13}C$ NMR (74.49MHz) ($CDCl_3$) δ: 55.97, 59.43, 59.75, 127.53, 128.68, 129.28, 138.79; IR ($CDCl_3$):3364.3, 2928.5, 1452.6, 1026.3 $cm^{-1}$ EI (LRMS) (m/z): 164.2 (M-31); HRMS ($C_{11}H_{17}NO_2$)Calcd.(M+) 195.1259, found:195.1252.

**N-benzyl-2-chloro-N-(2-chloroethyl) ethanamine (N-Benzyl 2, 2,'bis (dichloroethanamine), VI-11.**




A solution of **VI-10** (125 mg, 0.64 mmol) in 10 ml $CHCl_3$ was refluxed with $SOCl_2$ (0.19 ml, 2.56 mmol) for 7 hours under nitrogen. The solvent was dried *in vacuo*. Yield: 80% (0.12 g) $^1H$ NMR (300 MHz) ($CDCl_3$) δTMS: 3.54(s, 4H), 4.07(s, 4H), 4.46(s, 2H), 7.5(m, 5H); $^{13}C$ NMR (74.49 MHz) ($CDCl_3$): δ36.90, 54.17, 58.85, 127.93, 129.96, 130.87, 131.60 IR ($CHCl_3$): 2924.14, 2430.07, 1652.9, 1457.42 $cm^{-1}$ EI (LRMS) (m/z): 231 (M+); HRMS ($C_{11}H_{15}NCl_2$)Calcd.(M+) 231.0579, found:231.0582.

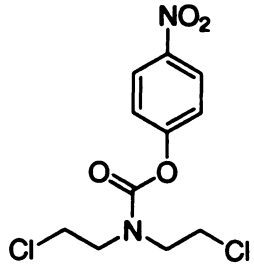
**4-nitrophenyl bis(2-hydroxyethyl)carbamate, VI-12.**




A solution of diethanolamine (100 mg, 0.952 mmol), p-nitrophenylchloroformate (211 mg, 1.047 mmol) and TEA (0.145 mL, 1.047 mmol) in $CH_2Cl_2$ (15 mL), was stirred for 8h. Evaporated the solvent and extracted in EtOAc/$NH_4Cl$. Collected the organic layer, dried over $Na_2SO_4$ and evaporated the solvent. Purified with column chromatography by eluting with EtOAc. $R_f$:0.3 $^1H$ NMR (300 MHz) ($CDCl_3$) δ TMS: 3.1(s, broad, 2H), 3.64 (t, 2H, $J$ = 4.65 Hz), 3.71(t, 2H, $J$ = 4.8 Hz), 3.97 ( m , 4H) ,7.34(d, 2H, $J$ = 9 Hz), 8.28(d, 2H, $J$=9Hz) $^{13}C$ (74.49 MHz)

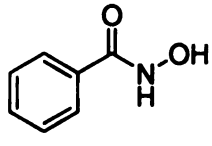
($CDCl_3$) δ: 52.25, 61.11, 122.27, 125.05, 145.06, 153.96, 155.96; IR($CHCl_3$): 3373.93, 2961.11, 1713.01, 1522.03, 1348.41 $cm^{-1}$ EI(LRMS) 239 (M-31).

**4-nitrophenyl bis(2-chloroethyl)carbamate, VI-13**



A solution of **VI-12** (350 mg, 1.29 mmol) in $CHCl_3$ (15 mL) was refluxed with $SOCl_2$ (0.945 ml, 12.9 mmol) for 7 h. The reaction mixture was evaporated *in vacuo*. A white solid crystallized which was further purified with 60% $Et_2O$/hexane. Rf: 0.6. Yield : 75% ( 0.29 g) $^1H$ NMR (300 MHz) (d6-$CDCl_3$) δ TMS: 3.9(m, 8H), 7.34(d, 2H, *J* = 9 Hz), 8.28(d, 2H, *J*=9Hz) $^{13}C$ (74.49 MHz) ($CDCl_3$) δ: 41.43, 51.01, 122.19, 125.07, 145.017, 152.92, 155.48 IR ($CHCl_3$): 2963.11 , 1726.51, 1522.03, 1346.41 $cm^{-1}$ EI (LRMS) (m/z): 168, 170 (M-OAr($pNO_2$)).

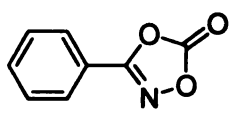
**N-hydroxy benzamide VI-14**



A suspension of 1.8 GR hydroxylamine.HCl (53.176 mg, 0.782 mmol) and $Na_2CO_3$ (82.8 mg, 0.782 mmol) in 75 ml ether was maintained at $0^oC$ . Benzoyl chloride (0.0825 mL, 0.711 mmol) was added dropwise. The reaction was cooled further and 3.5 mL $H_2O$ was added to it. The reaction was warmed to room temperature and stirred for 3h. Filtered off the solid and extracted the reaction

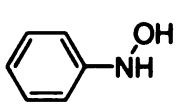
mixture in EtOAc. Collected the organic layer and dried it over $Na_2SO_4$. Evaporated off the organic solvent *in vacuo*. Recrystallised in EtOAc. Yield : 97% (0.95 g) M.P. : 121 °C $^1$H NMR (300 MHz) ($CD_3COCD_3$) δTMS: 7.45(t, 2H, *J*= 4.5 Hz), 7.54(t, 1H, *J* =2.7 Hz), 7.84(d, 2H, *J* =4.8 Hz), 8.3(broad, s, 1H), 10.2 (broad, s, 1H); $^{13}$CNMR (74.49 MHz) ($CD_3COCD_3$): 127.82, 129.31, 132.29, 133.231, 166.13 IR: 1575.29, 1621.73, 1645.69, 3297.32 cm$^{-1}$ EI (LRMS): 119(M-H).HRMS ($C_7H_7NO_2$) Calcd.(M+) 137.0477, found:137.0472.

**3-Phenyl 1, 4, 2-dioxazol-5-one , VI-15**



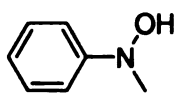
A solution of **VI-14** (200 mg, 1.458 mmol) in 100 mL ether was maintained at -78°C for 10 minutes. Phosgene (158.77 mg, 1.6043 mmol) was added drop wise with continuous stirring until the solid dissolved. Stirred the reaction mixture at room temperature overnight. Evaporated the solvent *in vacuo*. $^1$H NMR (300 MHz) ($CDCl_3$) δTMS: 7.53(t, 2H, *J*= 6.3 Hz), 7.63(t, 1H, *J* = 2.4 Hz), 7.84(d, 2H, *J*= 7.2 Hz) $^{13}$C (NMR) (74.49 MHz) ($CDCl_3$): 120.27, 126.63, 129.38, 133.74, 153.79, 163.56 IR: 2924.46, 1865.4, 1819.1, cm$^{-1}$ EI (LRMS): 163(M+), 119, 105.Anal. ($C_7H_5NO_3$) Calcd: C, 58.6; H, 3.09; N, 8.59; O, 29.42; found: C, 59.66; H, 3.31; N, 8.22; O, 28.81.

**N-hydroxybenzenamine VI-18**



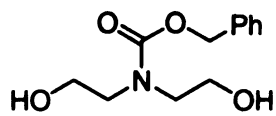
Nitrobenzene (100mg, 0.8 mmol) was dissolved in 2 ml THF. 5% Rh-C (10 mg) was added to the reaction mixture. Hydrazine hydrate (79.2 μl , 65% aqueous solution, 1.62 mmol) was added dropwise and the reaction mixture was allowed to stir at room temperature. Fitered the reaction through celite, and extracted in $Et_2O$/water. Collected the organic layer, dried over $Na_2SO_4$ and evaporated *in vacuo.* A pale yellow solid was obtained. Yield: 70% (60 mg).M. P. 71 °C. $^1H$ NMR (300 MHz) ($CDCl_3$) δTMS: 6.65-7.15(m, 5H), 8.2(s, 1H, exchangeable),8.23(s, 1H, exchangeable) $^{13}C$ (NMR) (74.49 MHz) (DMSO-*d6*): 112.86, 119.10, 129.29, 151.99 ; IR: 3244.46, 1496.9 $cm^{-1}$ EI (LRMS): 109.1(M+).

**N-hydroxy-N-methylbenzenamine, VI-19**



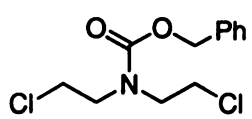

A solution of **VI-18** (50 mg, 0.46 mmol) in $CH_3OH$ (5 ml) was maintained in dark at 0°C. $CH_3I$ (1 ml, 6.6 mmol) was added and the reaction was stirred at room temperature for 14h. The solvent was evaporated and a saturated solution of oxalic acid (0.7 M, 3 ml) was added. The reaction was dried under vacuum (freeze-dry). Note : *this is a very unstable hydroxyl amine and should be used immediately after isolation.* Yield : 40% $^1H$ NMR (300 MHz) ($CD_3OD$) δ TMS: 3.11(s, 3H), 7.56-7.61 (m, 5H); $^{13}C$ (74.49 MHz) ($CDCl_3$) δ: 38.06, 123.29, 124.19, 130.93, 131.47, 138.20; EI(LRMS) 121, 107(M-$CH_3$).

**Benzyl bis(2-hydroxyethyl)carbamate, VI-20**



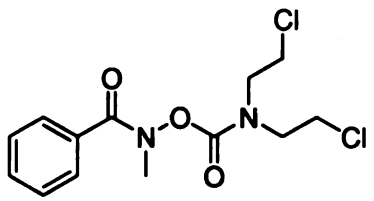
A solution of diethanolamine (100 mg, 0.952 mmol), benzylchloroformate (243 mg, 1.43 mmol) and $Na_2CO_3$ (0.53 g, 1M, 10 ml, ) in dioxane (10 ml), was stirred for 14h in a 50 ml round bottom flask. Added $NH_4Cl$ to neutralize and extracted in EtOAc. Collected the organic layer, dried over $Na_2SO_4$ and evaporated *in vacuo*. Purified with column chromatography by eluting with EtOAc. $R_f$:0.3 Yield : 97% (0.242 g) $^1H$ NMR (300 MHz) ($CDCl_3$) δ TMS: 3.49(s, 2H), 3.76(s, 2H), 3.83(s, 2H), 4.14(s, 2H), 5.14(s, 2H), 7.32-7.37 (m, 5H); $^{13}C$ (74.49 MHz) ($CDCl_3$) δ: 52.05, 52.56, 61.39, 61.68, 67.33, 127.84, 128.08, 128.51, 136.29, 156.80; IR($CHCl_3$): 3387.43, 2961.11, 1701.4, 1684.6, 1348.41 $cm^{-1}$ EI(LRMS) 239.2(M+). HRMS ($C_{12}H_{17}NO_4$) Calcd.(M+) 239.1158, found:239.1148.

**Benzyl bis(2-chloroethyl)carbamate, VI-21**




A solution of **VI-20** (100 mg, 0.42 mmol) in 15 ml $CHCl_3$ was refluxed with $SOCl_2$ (0.091 ml, 1.25 mmol) for 3 hours under nitrogen. Cooled the reaction mixture and dried the solvent *in vacuo*. Yield: 93% (0.12 g) $^1H$ NMR (300 MHz) ($CDCl_3$) δTMS: 3.42(m, 2H), 3.73-3.89(m, 4H), 4.61(m, 2H),5.19(s, 2H), 7.33-7.39(m, 5H); $^{13}C$ NMR (74.49 MHz) ($CDCl_3$): δ49.32, 49.84, 60.66, 60.87, 54.17, 67.95, 128.25, 128.51, 128.86, 136.46, 155.70 IR ($CHCl_3$): 2918.67, 2851.15, 1701.43, 1462.23 $cm^{-1}$ EI (LRMS) (m/z): 176 (M-$N(CH_2)_4Cl_2$)

**N- (O-(N-methyl phenyl hydroxamic acid))2,2'-bis-(chloroethylamine) carbamate VI-1**

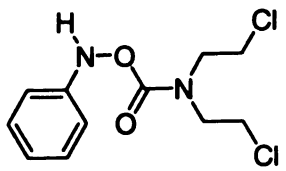


In a 50 ml round bottom flask sufficiently charged with $N_2$, 2,2'-bis-(chloroethylamine )carbamoyl chloride(100 mg, 0.49 mmol) was stirred at 0°C in $CH_2Cl_2$ (7 ml). TEA was added (137.02 $\mu$l, 0.97 mmol) dropwise. **VI-4** (95.4 mg, 0.58 mmol) dissolved in 3 ml $CH_2Cl_2$ was added and the reaction was allowed to stir at room temperature for 12 h. The solvent was removed and the reaction was extracted with ether and $NH_4Cl$. The organic layer was collected and dried over $Na_2SO_4$ .The compound was purified over silica column chromatography eluting with 1:1 EtOAc/ hexane. Yield: 0.142 g, 90 %

Alternatively, in a 50 ml round bottom flask sufficiently charged with $N_2$, KH ( 42.6 mg, 0.64 mmol) and 18-crown-6 (10 mg, 0.03 mmol) was maintained in THF (10 ml) at 0°C. N- (p-nitrophenyl) carbamate, **VI-13** (100 mg, 0.32 mmol) and **VI-4** (104.4 mg, 0.64 mmol) in 10 mL THF were added and the reaction was allowed to stir at room temperature overnight. The solvent was evaporated and the reaction mixture was extracted in EtOAc/$NH_4Cl$. The product was purified on silica gel column chromatography in 1:1 EtOAc/Hexane. $R_f$:0.5 Yield : 80% (0.083 g) $^1$H NMR (300 MHz) ($CDCl_3$) δ TMS: 3.28(m, 2H), 3.38(s, 3H), 3.40-3.55(m, 6H), 7.28(m, 5H); $^{13}$C (74.49 MHz) ($CDCl_3$) δ: 37.37, 41.15, 41.24,

50.33, 51.71, 127.39, 128.25, 130.84, 133.61, 153.61, 171.33; IR($CHCl_3$): 2924.5, 1747.73, 1668.6 $cm^{-1}$ EI(LRMS) 168(M—N(Me)OCOPh).

**N- (O-(phenyl hydroxyl amine)) 2, 2'-bis-(chloroethylamine) carbamate VI-1**

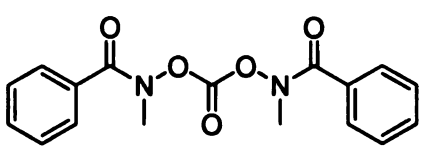


In a 50 ml round bottom flask sufficiently charged with $N_2$, 2,2'-bis-(chloroethylamine )carbamoyl chloride(100 mg, 0.49 mmol) was stirred at 0°C in $CH_2Cl_2$ (7 ml). TEA was added (68.4 $\mu$l, 0.49 mmol) dropwise. **VI-18** (58.5 mg, 0.54 mmol) dissolved in 3 ml $CH_2Cl_2$ was added and the reaction was allowed to stir at room temperature for 8 h. The solvent was removed and the reaction was extracted with ether and $NH_4Cl$. The organic layer was collected and dried over $Na_2SO_4$.The compound was purified over silica column chromatography eluting with $CH_2Cl_2$. Note: two doublet between δ 8-8.5 (not exchangeable) coeluted with the product. Further, purification attempts started decomposing the product.

**Partial characterization of VI-3:**

$^1H$ NMR (300 MHz) ($CDCl_3$) δ TMS: 3.66-3.93(m, 8H), 7.47-7.551(m, 5H); IR($CHCl_3$): 3067.9, 2924.6, 1749.6, 1481.6 $cm^{-1}$.

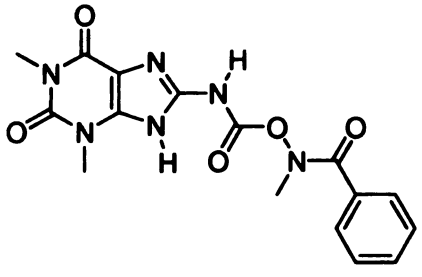
**Bis-(O, O'-(N-methylbenzamide))carbonate VI-7**



A solution of N-methyl phenyl hydroxamic acid (300 mg, 1.84 mmol) and TEA(0.57 ml, 4.04 mmol) in 12 ml $CH_2Cl_2$ was maintained at 0°C. $COCl_2$ (428.6

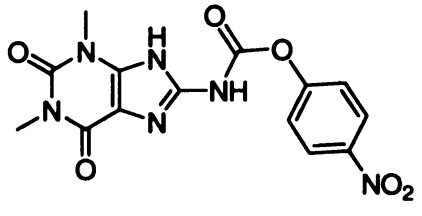
μL, 4.04 mmol) was added dropwise and stirred overnight. The solvent was dried *in vacuo* and characterized. Partial characterization of **VI-7:** $^1$H (NMR) (300 MHz) (CDCl3) δTMS: 3.32(s, 9)*, 7.45 (m, 15) IR($CHCl_3$):2926.39 $cm^{-1}$,1817.1 $cm^{-1}$,1676.3 $cm^{-1}$ *can't purify the compound due to stability reasons and was used immediately.

**Reaction of Aminotheophylline with carbonyl bis-(O-(N-methylbenzamide)), VI-22.**



A solution of 394.9 mg (1.122 mmol) **6** and 0.157 mL TEA in 15 mL $CH_2Cl_2$ was stirred overnight with 200 mg (1.02 mmol) of Aminotheophylline. A red solid separated in about an hour though it was not identified to be the product. Allowed the reaction to continue. Extracted in EtOAc/$NH_4Cl$. Collected the organic layer. Purification and identification required.

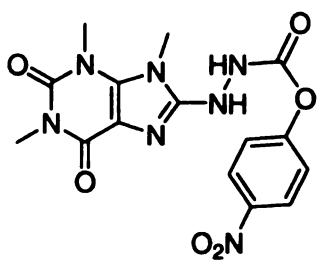
**4-nitrophenyl 2, 3, 6, 9-tetrahydro-1, 3-dimethyl-2, 6-dioxo-1H-purin-8-ylcarbamate, VI-23**



A solution of **II-3.HCl** (150 mg, 0.65 mmol), p-nitrophenylchloroformate (261 mg, 1.29 mmol) and TEA (0. 45 mL, 3.23 mmol) in $CH_3CN$(10 mL), was stirred for 18h. Evaporated the solvent and extracted in EtOAc/$Na_2CO_3$. Collected the organic layer, dried over $Na_2SO_4$ and evaporated the solvent. Yield: 5% (12.02 mg)

**Partial characterization of VI-23.:** $^1$H NMR (300 MHz) ($CDCl_3$) δ TMS: 3.42(m, 6H), 7.30(d, 2H, *J* = 8.4 Hz), 8.24(d, 2H, *J* =9Hz)

**4-nitrophenyl 2, 3, 6-tri hydro-1, 3, 7-trimethyl-2, 6-dioxo-1H-purin-8-hydrazinyl carbamate, VI-24**



A solution of **II-6**(50 mg, 0.223 mmol), p-nitrophenylchloroformate (49.4mg, 0.24 mmol) and TEA (34.8 μl, 0.24 mmol) in $CH_2Cl_2$ (15 ml), was stirred for 18h. Evaporated the solvent and extracted in EtOAc/$Na_2CO_3$. Collected the organic layer, dried over $Na_2SO_4$ and evaporated the solvent. Yield: 50% (43.5 mg)

**Partial characterization of VI-24:** $^1$H NMR (300 MHz) ($CDCl_3$) δ TMS: 3.38(s, 3H), 3.56(s, 3H), 3.96(s, 3H), 7.26(d, 2H, *J* = 9.3 Hz), 7.48(s, 1H), 8.20(d, 2H, *J* =9.6 Hz) .

**Linearising the plasmid using EcoR1 restriction enzyme:**

Supercoiled pBR322 (30 μL, 30μg) was incubated with EcoR1 (new England Biolabs, 6 μl), EcoR1 buffer(10X, 20 μl)and 144 μl $H_2O$ for 2h at 37°C in a water bath. NaOAc(20 μl, 3M) and ethanol (440 μl) were added and the solution was cooled at -70 °C for 10 minutes. The mixture was centrifuged at 14000 rpm for 15 minutes and ethanol was decanted off. The remainder residue was evaporated *in vacuo* and resuspended in 60 μl TE buffer.

Note: Volume of EtOH= (Reaction volume + Volume of NaOAc) * 2

The concentration of DNA was then checked by UV spectroscopy at 260 nM on a UV spectrometer Spectronic Instruments Genesys 5.

Prepared a 0.8% agarose gel (0.2 g agarose molecular biology grade (Fisher Scientific) in 25 ml 1X TBE). Heated in a microwave to dissolve (32'') and poured. Allowed to cool for an hour. *λHIND–III* fragments were used as molecular weight marker. For lane1, 1.0 μl *λHIND–III* was mixed with 9 μl $H_2O$ and warmed to 60 °C. It was slowly cooled to room temperature. This step is required for to separate bands 2 and 4 in the marker fragments (9416 and 2322 kb respectively). For lane 2, 1μl linearized plasmid was mixed with 9μl $H_2O$. Both the samples were mixed with 2 μl tracking dye (40% glycerol, 0.02% bromophenol blue, 250 mM Tris HCl (pH 6.8) in nuclease free water) and loaded on the gel. The gel was run at 80V for 1 h in 1X TBE buffer with10 μL Ethidium bromide. The amount of linearized pBR322 was quantitated by UV analysis as described by P. N. Borer in the *Handbook of Biochemistry and Molecular*

*Biology*, CRC Press, 1975, in conjunction with the optical density measurements obtained by UV absorption at 260 nm. For this plasmid DNA substrate the conversion factor was determined to be an OD260 of 1) 50$\mu$g/mL.

**Denaturing Alkaline agarose gel electrophoresis.**

Supercoiled plasmid pBR322 was linearised using the procedure described above. The DNA was subjected to ethanol precipitation, centrifugation at 14000 rpm, and the resulting DNA was dissolved in sterile $H_2O$ or TE buffer. The concentration of the resulting DNA was found out. Reactions were prepared as follows Lane 1, 0.5 $\mu$g $\lambda$HIND–III marker, lane 2, 0.5 $\mu$g linearised pBR322, lane3-9, 0.5 $\mu$g DNA + treatment for 10 $\mu$L reaction volume. The agarose gels were prepared by adding 50 mL of a 50 mM NaCl/2 mM EDTA (at pH ) 8) to 0.6 g of agarose. The suspension was heated in a microwave oven until all of the agarose was dissolved (45 s). The gel was poured and was allowed to cool and solidify for 1 h at room temperature. After 1 h the gel was soaked in an alkaline running buffer (25 mL of 2 N NaOH, 2 mL of 0.5 M EDTA in 1 L of $H_2O$) for an additional hour. The comb was removed, and the buffer was refreshed prior to electrophoresis. Agarose loading dye (5 $\mu$L, 300 mM NaOH, 6 mM EDTA, 18% Ficoll type 400 in water, 0.15% bromocresol green, 0.25% xylenecyanol FF) was added to the samples (10 $\mu$L), and the samples were loaded into the wells. The gel was run for 4 h at 40 mA/90 V which resulted in a traveling distance of approximately 8 cm. The gel was then neutralized for 45 min in a 1 M Tris pH=7 / 1.5 M NaCl solution, which was refreshed every 15 min. The gel was subsequently stained in an ethidium bromide solution (200$\mu$L of a 10 mg/mL ethidium bromide solution

in 1 L of 1 M Tris/ 1.5 M NaCl buffer at pH = 7.5) for 1 h. The background staining was then removed by soaking the gel in a solution of 50 mM NH4OAc and 10 mM β-mercaptoethanol. Gels were visualized on a UV transilluminator and photographed using Polaroid black and white film No. 667 and scanned on Biorad Foxproplus phosphor imager.

Note: An alternative protocol could be used--The agarose gels were prepared by adding 25 mL of a 50 mM NaOH/2 mM EDTA (at pH=8) to 0.3 g of agarose. The suspension was heated in a microwave oven until all of the agarose was dissolved (45 s). The water lost was balanced by weight. The gel was poured and was allowed to cool and solidify for 1 h at room temperature. After 1 h the gel was soaked in an alkaline running buffer (25 mL of 2 N NaOH, 2 mL of 0.5 M EDTA in 1 L of $H_2O$) for an additional hour. The comb was removed, and the buffer was refreshed prior to electrophoresis. Agarose loading dye (5 μL, 300 mM NaOH, 6 mM EDTA, 18% Ficoll type 400 in water, 0.15% bromocresol green, 0.25% xylenecyanol FF) was added to the samples (10 μL), and the samples were loaded into the wells. The gel was run for 4 h at 250 mA/60 V which resulted in a traveling distance of approximately 7 cm. The gel was then neutralized for 45 min in a 100 mM Tris pH=7.9 solution, which was refreshed every 15 min. The gel was subsequently stained in an ethidium bromide solution (100 μL of a 10 mg/mL ethidium bromide solution in 100 mL of 100 mM Tris buffer at pH = 7.9 for 20'. The background staining was then removed by soaking the gel in a solution of 50 mM NH4OAc and 10 mM β-mercaptoethanol. Gels were visualized on a UV

transilluminator and photographed using Polaroid black and white film No. 667 and scanned on Biorad Foxproplus phosphor imager.

**Cyclic voltammetry**

Prepared a 0.1 M blank solution of t-Butyl ammonium bromide in HPLC grade dry $CH_3CN$. Prepared the analyte solution in dry $CH_3CN$. Sparged the solutions with nitrogen and started the redox cycle to get the background. The working electrode used was gold electrode, counter electrodewas Pt electrode and the reference electrode was $Ag/AgNO_3$ in $CH_3CN$ (non-aqueous conditions). The value obtained was $E^0_{cell}$ which was then corrected with respect to the hydrogen electrode. The prodrug decomposed upon reduction , hence only the reduction half cycle was seen. The measurements were performed on C. H. Instruments Electrochemical Bench Model CH I650A and data acquired using workstation C H I650 version 4.0

## L. Reference


1. (a) Thomas, C. J.; Rahier, N. J. ; Hecht, S. M. *Bioorg. Med. Chem.* **2004**, *12*, 1585-604. (b) Pommier, Y.; Pourquier, P.; Fan, Y. ; Strumberg, D. *Biochim. Biophys. Acta* **1998**, *1400*, 83-105. (c) Macdonald, T. L.; Labroli, M. A.; Tepe, J. J. in *Comprehensive natural products chemistry* (ed. Kool, E. T.) 593-614 (Elsevier-North Holland, Amsterdam, 1999).

2. (a) Wang, D. ; Lippard, S. J. *Nat. Rev. Drug Discov.* **2005**, *4*, 307-20.(b) Barnes, K. R. ; Lippard, S. J. *Met. Ions Biol. Syst.* **2004**, *42*, 143-77. (c) Cohen, S. M.; Lippard, S. J. *Prog. Nucleic Acid Res. Mol. Biol.* **2001**, *67*, 93-130. (d) Jamieson, E. R.; Lippard, S. J. *Chem. Rev.* **1999**, *99*, 2467-98. (e) Sedletska, Y., Giraud-Panis, M. J. & Malinge, J. M. *Curr. Med. Chem. Anti-Canc. Agents* **2005**, *5*, 251-65.

3. (a) Dwarakanath, B. S.; Khaitan, D.; Mathur, R. *Indian J. Exp. Biol.* **2004**, 42, 649-659. (b) Holden J. A. *Ann. Clin. Lab Sci.* **1997**, *27*, 402–412.

4. Poddevin, B; Orlowski, S.; Belehradek, J., Jr.; Mir, L. M. *Biochem. Pharmacol.* **1991**, *42*, suppl S67-75.

5. (a) Wang, C. Y.; Mayo, M. W.; Baldwin A. S. Jr., *Science* **1996**, 274, 784–787. (b) Sharma, V.; Lansdell, T.; Peddibhotla, S.; Tepe, J. J. *Chem. Biol.* **2004**, 11, 1689-1699.

6. (a) Levav-Cohen, Y.; Haupt, S.; Haupt, Y. Growth Factors **2005**, *23*, 183-192. (b) Klein, C.; Vassilev, L. T. *Br. J. Cancer* 2004, 91(8), 1415-1419.

7. Cole, S. P. C.; Bhardwaj, G.; Gerlach, J. H.; Mackie, J. E.; Grant, C. E.; Almquist, K.C.; Stewart, A. J.; Kurz, E.U.; Duncan, A. M. V.; Deeley, R. G. *Science* **1992**, *258*, 1650-1654.

8. Hecht, J. R. *Oncology* **1998**, *12*, 72-78.

9. (a) Hyacinthe, M.; Jaroszeski, M. J.; Dang, V. V.; Coppola, D.; Karl, R. C.; Gilbert, R. A.; Heller, R. *Cancer* **1999**, *85*, 409-417. (b) Berg, K.; Selbo, P. K.; Prasmickaite, L.; Tjelle, T. E.; Sandvig, K.; Moan, J.; Gaudernack, G.; Fodstad, O.; Kjolsrud, S.; Anholt, H.; Rodal, G. H.; Rodal, S. K.; Hogset, A. *Cancer Res.* **1999**, *59*, 1180-1183.

10. Boger, D. L.; Johnson, D. S. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1439.

11. Wolkenberg, S. E.; Boger, D. L. *Chem. Rev.* **2002**, *102*, 7, 2477-2495.

12. Lawley, P. D. *BioEssays* **1995**, *17*, 561.

13. (a) Hopkins, P. B.; Raucher, S.; Millard, J. T. *J. Am. Chem. Soc.* **1990**, *112*, 2459.(b) Ojwang, J. O.; Grueneberg, D. A.; Loechler, E. L. *Cancer Res.* **1989**, *49*, 6529. (c) Rink, S. M.; Solomon, M. S.; Taylor, M. J.; Sharanabasava, R. B.; McLaughlin, L. W.; Hopkins, P. B. J. Am. Chem. Soc. **1993**, *115*, 2551.

14. Brown, J. M. *Mol. Med. today* **2000**, 6, 157-162.

15. Tannock, I. F. *Lancet* **1998**, *351*, suppl. 2 (9-16).

16. Tannock, I. F.*Br. J. Cancer* **1968**, *22*, 258-273.

17. Wartenberg, M.; Ling, F. C.; Müschen, M.; Klein, F.; Acker, H.; Gassmann, M.; Petrat, K.; Pütz, V.; Hescheler, J.; Sauer, H. *FASEB J.* **2003**, *17*, 503-505.

18. Harris, A. L. *Nat. Rev. Cancer* **2002**, *2*, 38-47.

19. Subarsky, P.; Hill, R. P. *Clin .Exp. Metastasis* **2003**, *20*, 237-250.

20. Hockel, M.; Schlenger, K.; Aral, B.; Mitze, M.; Schaffer, U.; Vaupel, P. *Cancer res.* **1996**, *56*, 4509-4515.

21. (a) Siim, B. G.; Denny, W. A.; Wilson, W. R. *Oncol. Res.* **1996**, *9*, 357–369. (b) Helsby, N. A.; Wheeler, S. J.; Pruijn, F. B.; Palmer, B. D.; Yang, S. Denny, W. A.; Wilson, W. R. *Chem. Res. Toxicol.* **2003**, *16*, 469-478.

22. (a) Zeman, E. M.; Brown, J. M.; Lemmon, M. J.; Hirst, V. K. ;Lee, W. W. *Int. J. Radiat.Oncol. Biol. Phys.* **1986**, *12*, 1239–1242. (b) Brown, J. M. & Lemmon, M. J. *Cancer Res.* **1990**, 745–7749. (c) Brown, J. M. *Br. J. Cancer* **1993**, *67*, 1163–1170.

23. Niculescu-Duvaz, D.; Niculescu-Duvaz, I.; Friedlos, F.; Martin, J.; Lehouritis, P.; Marais, R.; Springer, C. J. *J. Med. Chem.* **2003**, *46*, 1690-1705.

24. (a) Pan, S. S.; Andrews, P. A.; Glover, C. G. *J. Biol. Chem.* **1984**, *259*, 2, 959-966. (b) Gruenhert, D.C.; Cleaver, J. E. *Biochem. Biophys. res. Comm.* **1984**, *123*, 2, 549-554.

25. (a) Paz, M. M.; Hopkins, P. B. *Tetrahedron Lett.***1997**, *38*, 3, 343-346. (b) Cech, T. R. *Biochemistry* **1981**, *20*, 1431-1437. (c) Paz, M.M.; Hopkins, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 5999-6005. (d) Williams, R. B.; Rajski, S. R. *Tetrahedron Lett.* **1992**, *33*, 21, 2929-2932. (e) Williams, R. B.; Rajski, S. R. *Tetrahedron Lett.* **1993**, *34*, 44, 7023-7026. (f) Tepe, J. J.; Williams, R.B. *Angew. Chem. Int. Ed.* **1999**, *38*, 3501-3503. (g) Naoe, Y.;

Inami, M.; Kawamura, I.; Nishigaki, F.; Tsujimoto, S.; Matsumoto, S.; Manda, T.; Shimomura, K. *Jpn. J. Cancer Res.* **1998**, *89*, 666-672.

26. Fukuyama, T.; Goto, S. *Tetrahedron Lett.* **1989**, *30*, 6491.

27. Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723-2795.

28. (a) Das, K. C.; White, C. W. *J. Biol. Chem.* **1997**, *272*, 14914-14920. (b) Huang, T. T.; Wuerzberger-Davis, S. M.; Seufzer, B. J.; Shumway, S. D.; Kurama, T. Boothman, D. A.; Miyamoto, S. *J. Biol. Chem.* **2000**, *275*, 9501-9509.

29. Sherr, C. J. *Science*, **1996**, *274*, 1672-1677.

30. Chaturvedi, M. M., Kumar, A.; Darnay, B. G.; Chainey, B. N.; Agarwal, S.; Aggarwal, B. B. *J. Biol. Chem.* **1997**, *272*, 30129-30134.

31. Matsuba, Y.; Edatsugi, H.; Mita, I; Matsunaga, A.; Nakanishi, O. *Cancer Chemother. Pharmacol.* **2000**, *46*, 1-9.

32. (a) Hobson, J. D.; Mc Cluskey, J. G. *J. Chem. Soc. (C)* **1967**, 2015-2017.(b) Adamaik, R. W.; Stawinski, J. *Tetrahedron Lett.* 22, 1935-1936.

33. (a) Saraf, S.; Edun, M.; Khan, M. A. *Heterocycles* **1985**, *23*, 5, 1173-1180. (b) Jones, G. B.; Matthews, J. E. *Tetrahedron* **1997**, *53*, **43**, 14599-14614. (c) Chen; Zhi-Long; Wei-Qin, W.; Fang, Z.; De-Yu, X. *Heterocycles* **1998**, *48*, **9**, 1739-1745. (d) Bianchini, C.; Farnetti, E.; Glendenning, L.; Graziani, M.; Nardin, G.; Peruzzini, M.; Rocchini, E.; Zanobino, F. *Organometallics*, **1993**, *14*, 1489-1502.

34. Cooley, J. H.; Evain, E. J. *Synthesis*, **1988**, 1-7.

35. Comins D.; Weglarz M. .A. *J. Org. Chem.* **1988**, *53*, 4437 – 4442.

36. Mitra, S.; Overberger, C. G. *J. Org. Chem.* **1988**, *53*, 2674 –2677.

37. Entwistle, I. D.; Gilkerson, T.; Johnstone, R. A. W. ; Telford, R. P. *Tetrahedron*, **1978**, *34*, 213-215.

38. (a) King, R. S.; Sharma, V.; Pederson, L. C.; Kakuta, Y.; Negishi, M.; Duffel, M. W. *Chem. Res. Toxicol.* **2000**, *13*, 1251-1258. (b) Bordwell, F. G.; Liu, W. B. *J. Am. Chem. Soc.* **1996**, *118*, 8777-8781.

39. (a) Tannock, I. F. *Br. J. Radiol.* **1972**, *45*, 515-424.(b) Tercel, M.; Wilson, W. R.; Anderson, R. F.; Denny, W. A. *J. Med. Chem.* **1996**, *39*, 1084-

1094.(c) Foye, W. O. In: Cancer Chemotherapeutic Agents, American Chemical Society, Washinton, DC. Chapter 15, 500-523.

40. Gruenert, D. C.; Cleaver, J. E. *Biochem. Biophy. Res. Comm.* **1984**, *123*, 549-554.

41. Hartwig, J. F.; Pil, P. M.; Lippard, S. J. *J. Am. Chem. Soc.* **1992**, 114, 8292-8293.

42. Sagara, H.; Makino, S.; Chibana, N.; Ota, M.; Holgate, S. T.; Church, M. K.; Fukuda, T. *Int. Arch. Allergy Immunol* **2001**, *124*, 371-376.

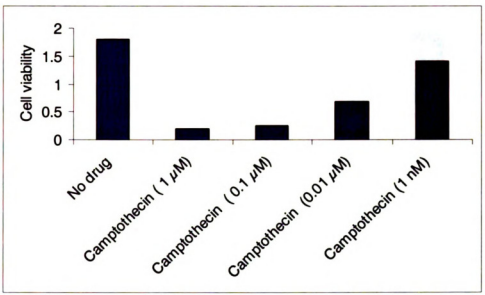
43. Cote, R. FRM2105 19631126, **1963.**

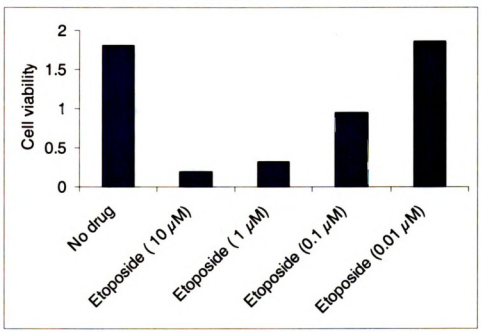
44. Zimmer, H.; Matallia, J. B.; Atchley, R. *Ohio J. Science* **1963**, *63*, 97-102.

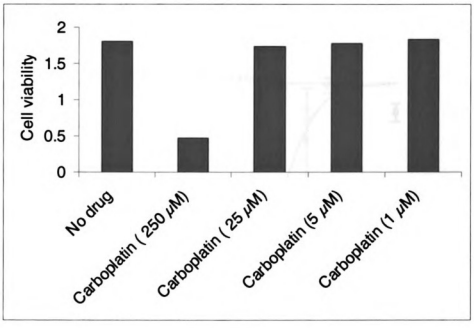
45. Glushkov, R. G.; Ovcharova, I. M.; Koptenkova, V. A.; Nikitin, V. B.; Kaminka, M. E.; Mashkovskii, M. D. *Khim-Farm. Zh.* **1987**, *21*, 55-59.

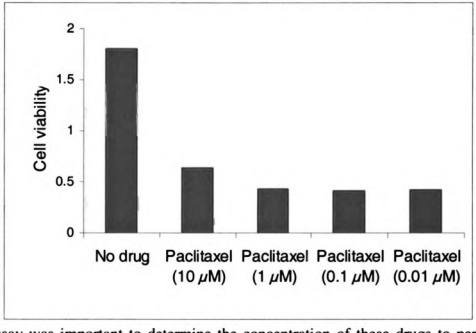
# APPENDIX

1) **Optimization of cytotoxicity of various chemotherapeutic agents in CEM cells. Measuring cell viability at 48h with MTS assay.**



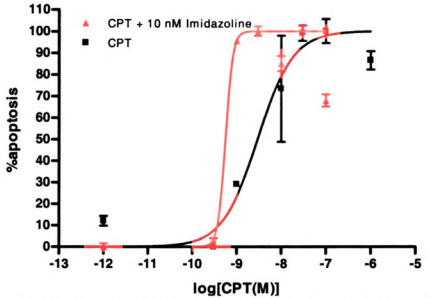





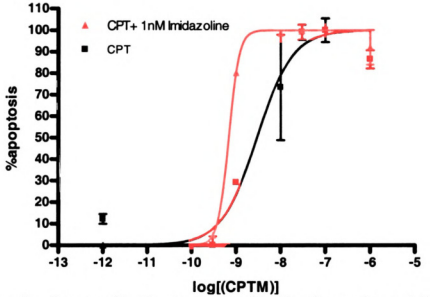




This assay was important to determine the concentration of these drugs to perturb the cellular system but not cause extensive apoptosis.

**2) Measure of Induction of apoptosis through caspase activity using Apo-One Assay™**



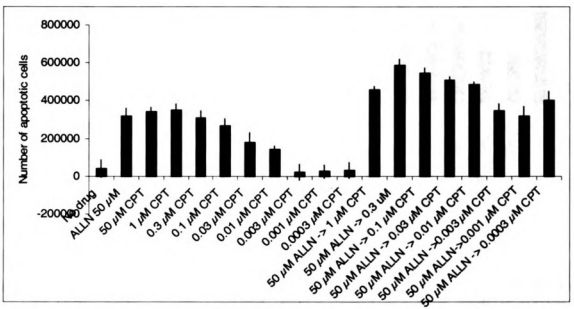

The red triangles show combination treatment of imidazoline and camptothecin. Blue squares are camptothecin alone. Values are averages of two independent experiments. P=0.0051



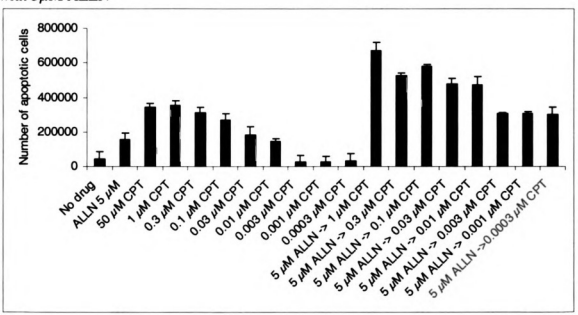

The red triangles show combination treatment of imidazoline and camptothecin. Blue squares are camptothecin alone. Values are averages of two independent experiments. P=0.0004

3) **Effect of various concentrations of ALLN, and lactacystin (proteasomal inhibitors) on apoptotic induction by Camptothecin.**

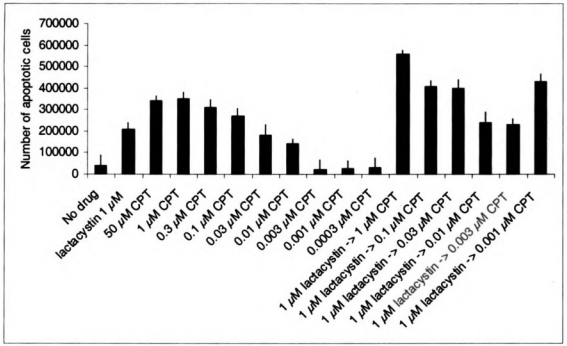
with 50 $\mu$M ALLN




with 5$\mu$M ALLN

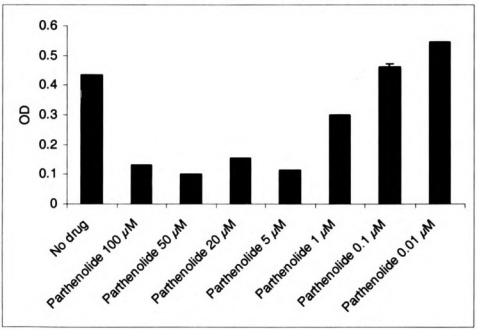


1 μM Lactacystin

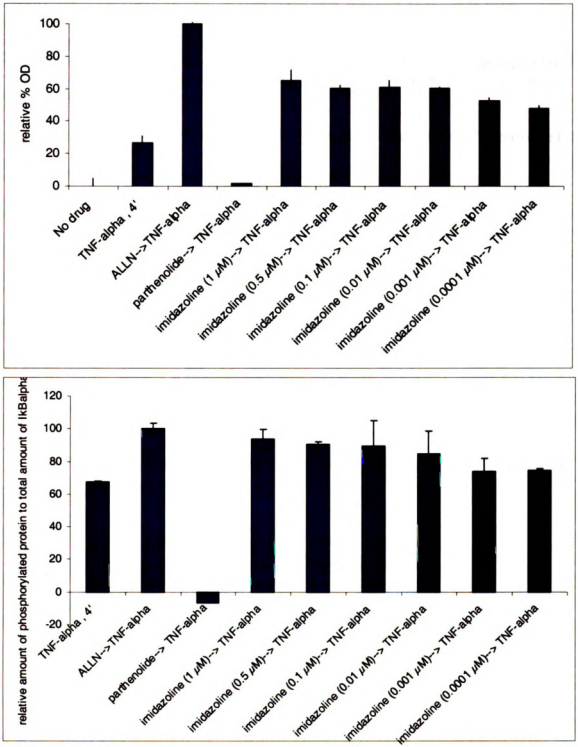



Proteasomal inhibitors such as ALLN and lactacystin proved to be cytotoxic at timepoints beyond 12 hours; hence, caspase-3 induction measured may be a result of cytotoxic effects of the drug by themselves.

**4) Cytotoxicity of Parthenolide: Dose response of parthenolide upto 24h**



**5) Imidazoline inhibit the degradation of I-κBα induced by TNF-α in CEM cells**



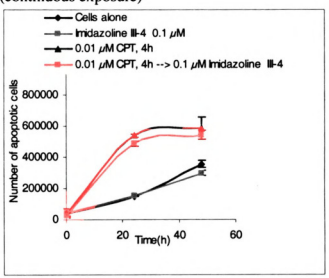

Relative ratio of phosphorylated I-κB (at ser32-and 36) to the total amount of I-κBα. All experiments were run in duplicate and data reported as the average of the two independent experiments. ALLN (50 μM) and parthenolide (50 μM) were used as controls for proteasomal and IKK inhibition respectively. Activation of NF-κB was

initiated using TNF-α (0.4 pg). The control for parthenolide shows a negative bar because it is cytotoxic at the exposure-time at which the experiment was performed (data not shown).

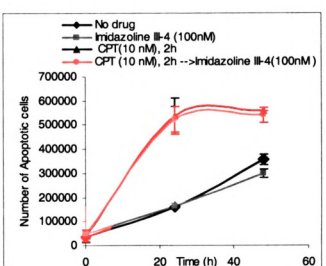
**6) Post-treatment of CEM cells with imidazoline III-4.**

Cells were treated with 0.01 μM CPT for 1, 2 and 4h and post-treatment performed with each 0.1 μM and 0.01μM imidazoline **III-4** in continuous exposure as well as with cell-wash and supplementing with fresh media.

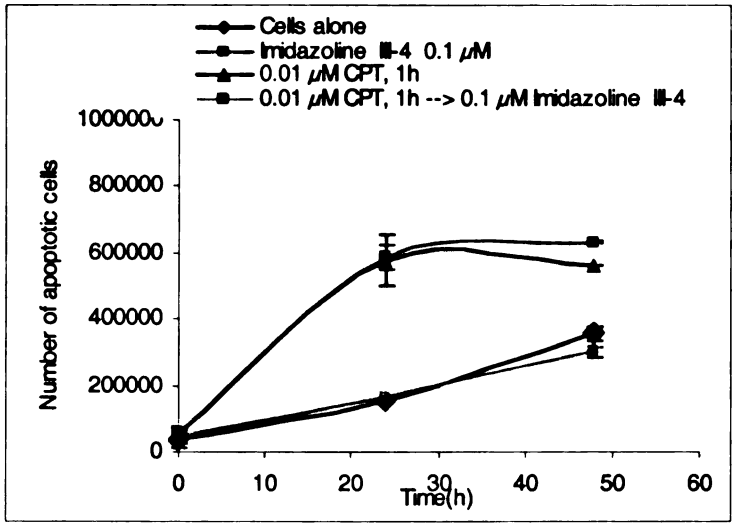
i. CPT incubation (0.01 μM, 4h) followed by treatment with imidazoline 0.1 μM (continuous exposure)



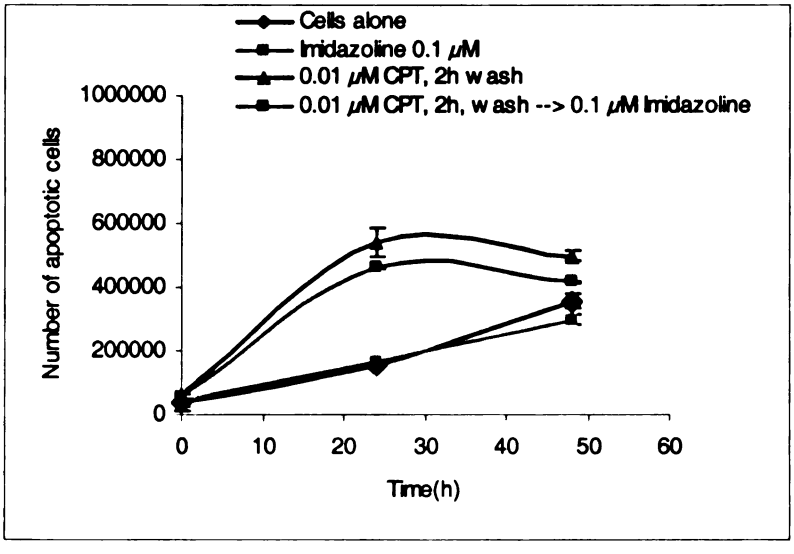

ii. CPT incubation (0.01 μM, 2h) followed by treatment with imidazoline 0.1 μM (continuous exposure)



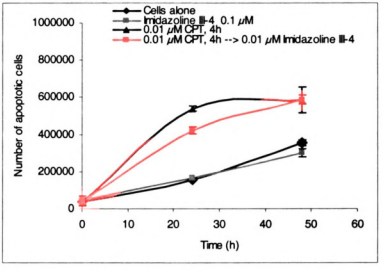
iii. CPT incubation (0.01 $\mu$M, 1h) followed by treatment with imidazoline 0.1 $\mu$M (continuous exposure)



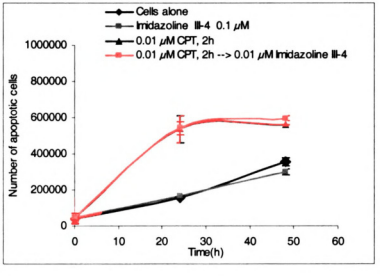

iv. CPT incubation (0.01 $\mu$M, 2h) followed by treatment with imidazoline 0.1 $\mu$M (washed cells)



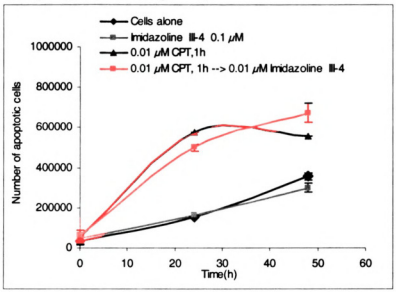
v. CPT incubation (0.01 $\mu$M, 4h) followed by treatment with imidazoline 0.01 $\mu$M (continuous exposure)



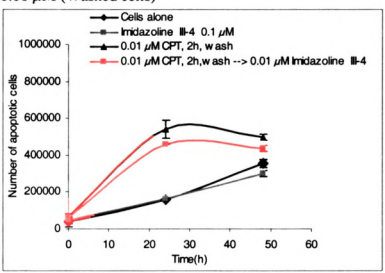

vi. CPT incubation (0.01 $\mu$M, 2h) followed by treatment with imidazoline 0.01 $\mu$M (continuous exposure)



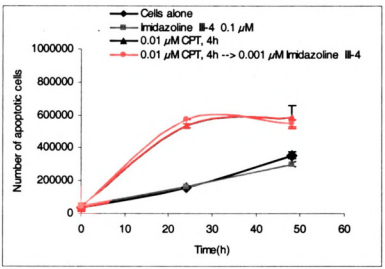
vii. CPT incubation (0.01 $\mu$M, 1h) followed by treatment with imidazoline 0.01 $\mu$M (continuous exposure)



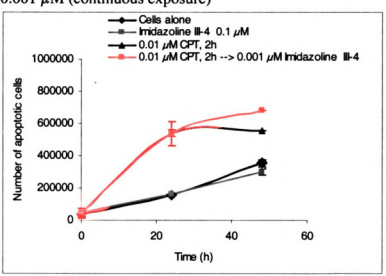

viii. CPT incubation (0.01 $\mu$M, 2h) followed by treatment with imidazoline 0.01 $\mu$M (Washed cells)



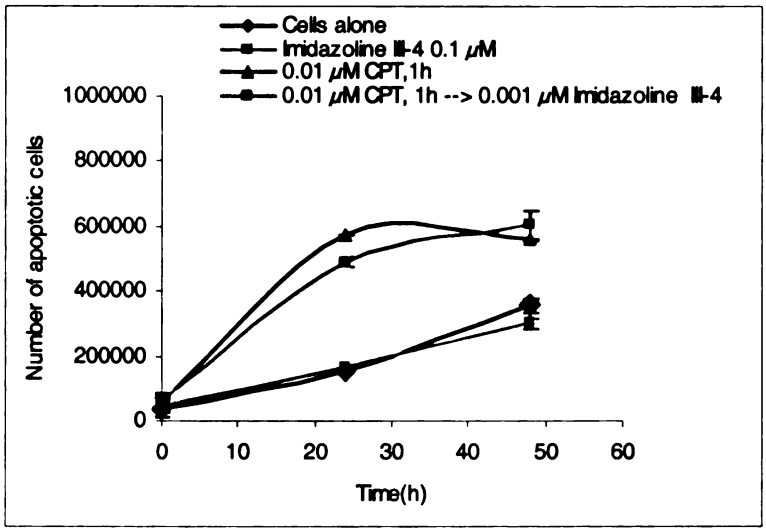
ix. CPT incubation (0.01 $\mu$M, 4h) followed by treatment with imidazoline 0.001 $\mu$M (continuous exposure)



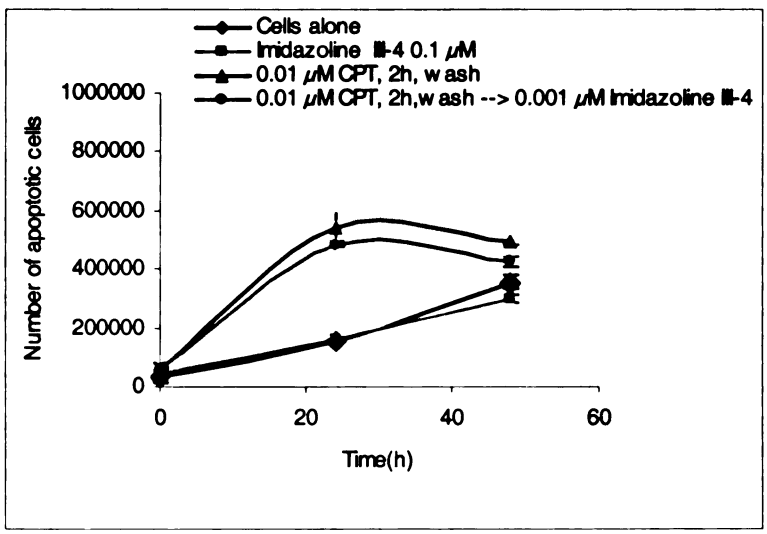

x. CPT incubation (0.01 $\mu$M, 2h) followed by treatment with imidazoline 0.001 $\mu$M (continuous exposure)



xi. CPT incubation (0.01 $\mu$M, 1h) followed by treatment with imidazoline 0.001 $\mu$M(continuous exposure)

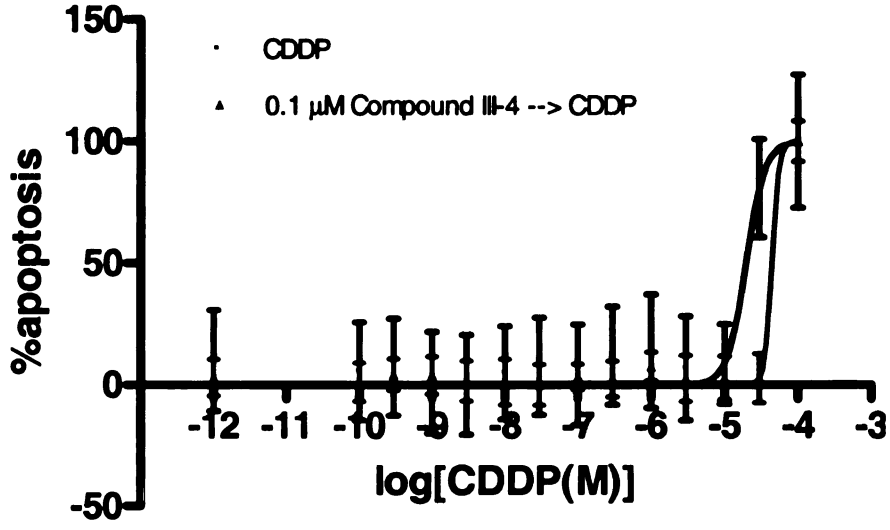



xii. CPT incubation (0.01 $\mu$M, 2h) followed by treatment with imidazoline 0.001 $\mu$M (washed cells).

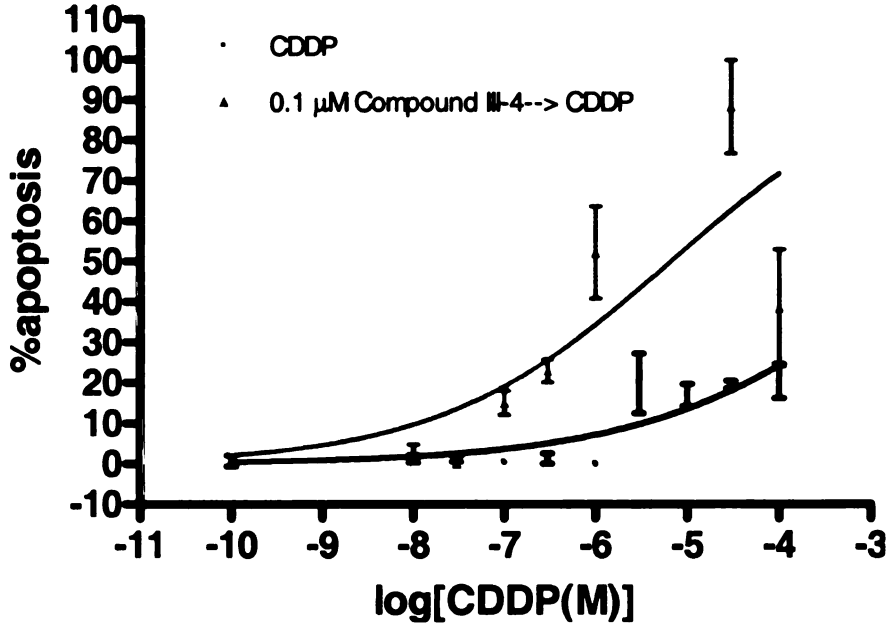


**7) Potentiation of cispatin (CDDP) or camptothecin (CPT) with imidazoline III-4 in different cell types.**

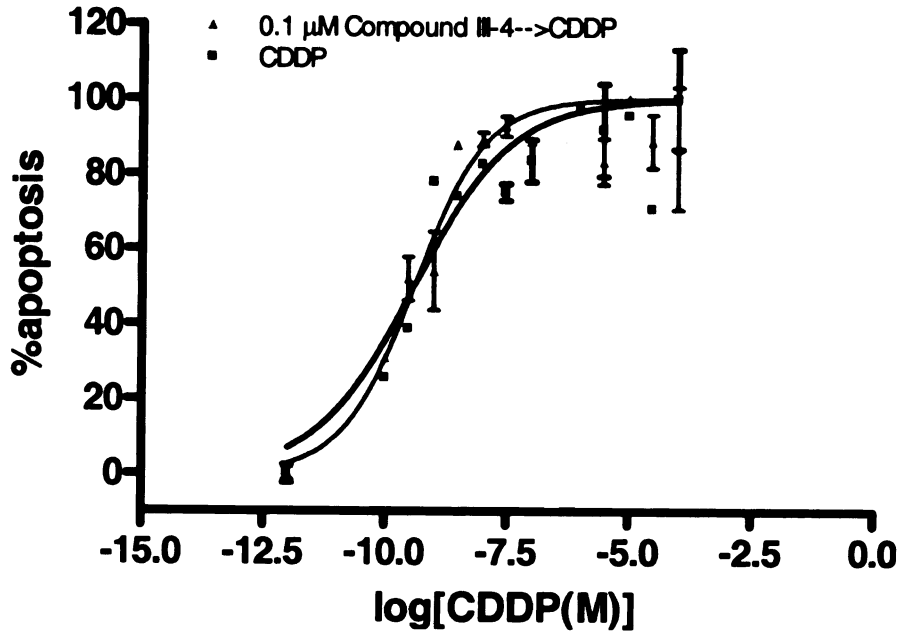
i. SL89 cells with CDDP



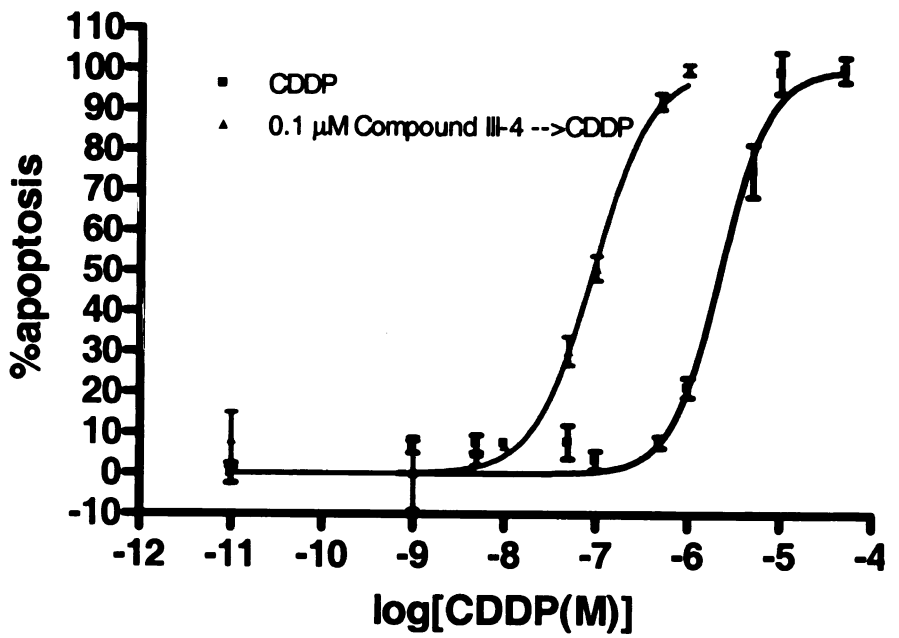

ii. HT1080 with CDDP



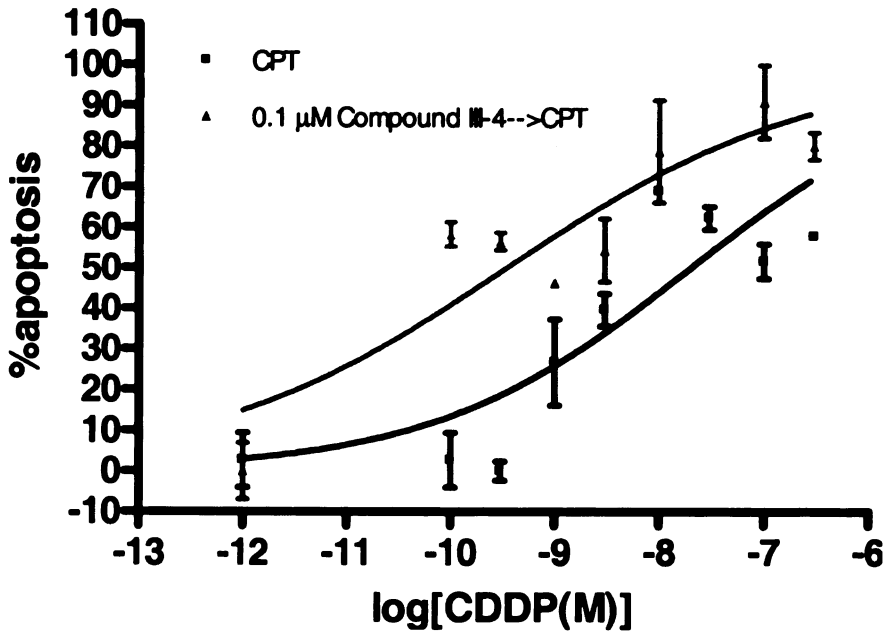
iii. HeLa with CDDP



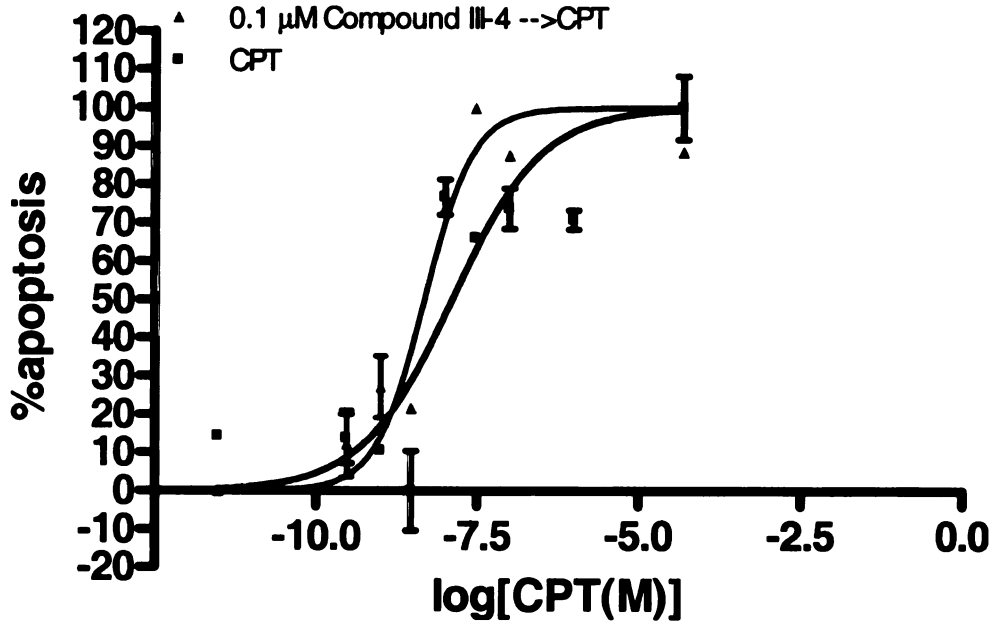

iv. CEM cells with CDDP



v. HT1080 with CPT




vi. HeLa with CPT




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