MECHANISMS OF HEPATOCELLULAR APOPTOSIS INDUCED BY TROVAFLOXACIN-TUMOR NECROSIS FACTOR-ALPHA INTERACTION: AN IN VITRO MODEL OF IDIOSYNCRATIC DRUG-INDUCED LIVER INJURY

By

Kevin Michael Beggs

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology – Environmental Toxicology – Doctor of Philosophy

2013

ABSTRACT

MECHANISMS OF HEPATOCELLULAR APOPTOSIS INDUCED BY TROVAFLOXACIN-TUMOR NECROSIS FACTOR-ALPHA INTERACTION: AN IN VITRO MODEL OF IDIOSYNCRATIC DRUG-INDUCED LIVER INJURY

By

Kevin Michael Beggs

Idiosyncratic drug-induced liver injury (IDILI) is an adverse and typically rare response to drugs that are safe in most people. Although infrequent, the response to these reactions can be severe and result in liver failure and death. Despite extensive research, the underlying mechanisms responsible for IDILI are currently unknown. One theory suggests that a modest inflammatory stress can render individuals susceptible to IDILI. Animal models based on this hypothesis demonstrated that administration of a nontoxic dose of lipopolysaccharide (LPS) to induce modest inflammation interacts with drugs associated with causing IDILI to precipitate liver injury in rodents.

Trovafloxacin (TVX) is a fluoroquinolone antibiotic associated with IDILI. In mice, TVX was not hepatotoxic but became injurious during concomitant inflammation induced by LPS. The pathogenesis of this liver injury was critically mediated by the proinflammatory cytokine tumor necrosis factor-alpha (TNF). The focus of this dissertation was to test the hypothesis that TVX and TNF interact directly to cause hepatocyte cell death, and to identify critical signaling mechanisms involved in cytotoxicity.

In human derived HepG2 cells, neither TVX nor TNF alone induced any cytotoxicity *in vitro*. However, in combination TVX/TNF treatment resulted in cytotoxicity in a concentration-dependent manner. The hepatocellular death was dependent on

activated caspases and involved a prolonged activation of c-Jun N-terminal kinase (JNK).

TVX has the potential to inhibit mammalian topoisomerase enzymes, which can lead to DNA damage. In hepatocytes, TVX produced DNA damage and resulted in cell cycle arrest and decreased cell proliferation. TVX also activated extracellular signal-regulated kinases (ERK) and ataxia telangiectasia and Rad3 related (ATR), both of which can be activated in response to genotoxic stress. However, none of the above events evoked cytotoxicity unless cells were also exposed to TNF. Activated ERK and ATR promoted cytotoxicity in hepatocytes exposed to both TVX and TNF.

Collectively, these results contribute to increased understanding of the molecular mechanisms underlying the cytotoxic interaction of TVX and TNF. If similar mechanisms are involved in response to other IDILI associated drugs interacting with TNF to kill hepatocytes, this information could be used to develop high-throughput screening assays capable of discerning the potential of new drug candidates to cause IDILI.

ACKNOWLEDGEMENTS

There is a long list of people I would like to thank for helping me with everything I have accomplished in graduate school. Although I will not be able to address every person individually, I trust that anyone who might read this will know how much he or she assisted me, even if it wasn't with the science directly. I had a very good support system that helped me every step of the way, and without the aide of my friends, family and colleagues I don't know what would have become of this project.

First, I have to thank my mentors, Dr. Robert Roth and Dr. Patricia Ganey. I had expressed interest in joining their lab early during my time at Michigan State University. Although funding wasn't readily available for me at first, Bob kept me informed to the status of their latest grant application and held a spot for me to rotate when the time came. Bob and Patti have both worked hard to acquire funding from the National Institutes of Health to provide me with a stipend for all these years. Bob also worked to get an extension on the grant and allowed me more time to do experiments, which was very beneficial, and for this I'd like to thank the NIH as well. For me, the wait to rotate in the lab was worth it as the research had peaked my scientific curiosity. What I didn't expect after joining the lab was the sense of feeling like I was part of a family. I've had the pleasure of working with a lot of great scientists in the lab over the years, and managed to have a surprising amount of fun with them while doing it.

I joined the lab with very little experience in biology and biological lab techniques. Bob and Patti displayed incredible patience with me, and helped my progression as a biological scientist. They also helped me to develop into a critical thinking experimenter

who could feel (more) comfortable presenting and talking about my findings in front of an intimidating audience. The weekly lab meetings and triweekly meetings with Bob and Patti were instrumental in developing my ability to think critically about my data, and helped give focus and direction to the project. Additionally, I've enjoyed my time spent with Bob and Patti and the rest of the lab in social settings. Although I missed a lot of them, the Friday evening happy hours were a lot of fun. I also enjoyed the lab lunch/dinner we had each year, as well as the farewell parties and general get-togethers at their house. Bob and Patti have been very generous to me and concerned about the well being of my family and me, and I am truly grateful to have had such a wonderful experience working with these two mentors.

There are a number of other people at MSU that I need to thank, including my other committee members, Dr. Kaminski and Dr. Pestka. Although they were both very busy with other obligations, they were always able to make time for me when I asked, and their guidance was helpful in giving direction to a sometimes-confusing project. I also would like to thank Dr. Lookingland and Dr. Dorrance for convincing me that as long as I kept working hard, I could succeed in graduate school. As a whole, the Department of Pharmacology and Toxicology also deserves my thanks. It provided me the opportunity to advance my education while working toward my degree. The administrative assistants helped by handling a lot of the paper work involved, and making sure everything was in order so that I could focus on my experiments.

Dr. Pat Shaw and Dr. Wei Zou provided my initial training and helped me become familiar with the projects being investigated. Dr. Erica Sparkenbaugh taught me proper sterile techniques and how to work with cultured cells, and was always

someone I could count on to answer any of a number of random questions I might have over the course of a day. Nicole Crisp was very helpful in ordering laboratory equipment and reagents for experiments in a timely manner, and Dr. Christine Dugan and Dr. Jingtao Lu also helped me think critically about my project. Allen MacDonald and Ryan Albee, a couple of hardworking undergraduates, helped with aspects of my project. Dr. Kaz Miyakawa has been instrumental in all things involving fixation, permeabilization and imaging.

Much credit needs to be given to Dr. Aaron Fullerton and Dr. Kyle Poulsen. These two contributed greatly to experimental design and development, as well as the generation of ideas that resulted in studies providing fruitful data. Additionally, both of them (along with their better halves, Sarah and Jessica) provided a place for me to stay on those late nights in the lab, and spared me the 45-minute drive home in the middle of the night. They also helped me progress as a writer by reviewing my manuscripts and helping me outline my dissertation. Lastly, Ashley Maiuri has been a pleasure to have in the lab. Her work has helped reaffirm my belief that the results Kyle and I have generated are important, and I will be curious to see how her project develops.

I also am grateful to the Michigan Regional Chapter for the Society of Toxicology, as well as the Society of Toxicology. My membership in both organizations has provided me the opportunity to attend meetings and present my work to other scientists. Both organizations provided funding that allowed me to travel to different meetings. My involvement in these organizations also has allowed me to network and meet other toxicologists. I want to thank the Center for Integrative Toxicology and the Environmental and Integrative Toxicological Sciences program at MSU, for providing

further funding opportunities to travel to meetings, as well as interact with a number of highly skilled toxicologists from diverse backgrounds, and other toxicology graduate students from different programs.

My family has been very supportive of me over the years. My parents have always encouraged my academic pursuits, and helped drive me to pursue an advanced degree. So thank you Mom and Dad, for your support over the years. I would like to thank my sisters, Jen and Michelle, for supporting me and making me laugh when we are together. Although I don't see my extended family very often, when we are together they have been encouraging at every step. Also, my wife's family has been encouraging and reassuring of me from the beginning of my time in graduate school. They have all kept my spirits up over the years and believed I could succeed even when I didn't. To Dana and Nancy, I want to thank you for treating me as one of your own children, and for providing me with a place to finish my writing.

Finally, I have to give the most credit for this accomplishment to my wife Carolyn. She has been unbelievably patient throughout this whole ordeal. I know I have missed out on a lot with you over the years, and I hope to be able to make it up to you someday. You have given me joy even during my worst days, and reassurance when I needed it the most. I look forward to moving on to the next stage of our lives together, and can't wait to start a family with you. I love you Carolyn, and want you to know how important your support is to my success.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ALT	alanine aminotransferase
AnnV	annexin V
AP-1	activator protein-1
ATM	ataxia telangiectasia-mutated
ATR	ataxia telangiectasia-mutated and Rad3-related
CYP	cytochrome P450
DCF	2',7'-dichlorofluorescein
DDR	DNA damage response
DILI	drug-induced liver injury
DNA-PK	DNA-dependent protein kinase
Doxo	doxorubicin
DSB	DNA double-strand break
ERK	extracellular regulated kinase
FADD	Fas-associated death domain
FDA	Food and Drug Administration
gH2AX	phosphorylated histone 2AX
IDILI	idiosyncratic drug-induced liver injury
IkB	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase

LPS	bacterial lipopolysaccharide
LTA	lipoteichoic acid
LVX	levofloxacin
MAPK	mitogen activated protein kinase
MKK	MAPK kinase
MKP	mitogen activated protein kinase phosphatase
NAC	N-acetyl-L-cysteine
NF-kB	nuclear factor-kappa B
NIH	National Institutes of Health
PBS	phosphate buffered saline
PGN	peptidoglycan
PI	propidium iodide
RIP1	receptor-interacting protein 1
TAB2/3	TAK1-binding protein 2/3
TAK1	transforming growth factor beta-activated kinase 1
TLR	Toll-like receptor
TNF	tumor necrosis factor-alpha
TNFR1	TNF receptor 1
TNFR2	TNF receptor 2
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
Toco	a-tocopherol
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

TVX	trovafloxacin

CHAPTER 1

General Introduction and Specific Aims

1.1 Idiosyncratic drug-induced liver injury

1.1.1 Overview of idiosyncratic drug-induced liver injury

Acute liver failure (ALF) is characterized by severe liver cell dysfunction, jaundice, coagulopathy, and hepatic encephalopathy (O'Grady, et al., 1993). If left untreated, ALF can result in multi-organ failure and mortality (Larsen and Bjerring, 2011). It has been estimated that acute liver failure affects about 2000 people each year in the United States. Before the availability of liver transplantation, the mortality rate of patients with ALF was greater than 90%. Currently, there is an absence of curative medical therapy, making liver transplantation the only reliable, life saving treatment for patients with ALF. Survival rates after liver transplant are greater than 60%, but ALF continues to be a significant cause of morbidity and mortality in both children and adults. An incomplete understanding about the pathogenesis of ALF, as well as the scarcity of livers available for transplantation, requires that more knowledge about the treatment and prevention of ALF be obtained (Lee, 2012, Nguyen and Vierling, 2011).

ALF can occur as a result of a variety of conditions, including drug-induced liver injury (DILI), viral hepatitis, Wilson's Disease, and cancer. Between 1998 and 2001, a cohort study among 17 tertiary care centers in the United States revealed that DILI accounted for 52% of all ALF cases, indicating that DILI had surpassed viral hepatitis as the leading cause of ALF (Ostapowicz, et al., 2002). DILI also is one of the most common adverse effects preventing regulatory approval for the marketing of new drugs (Watkins, 2005), as well as post-marketing actions resulting in warnings or withdrawal of

drugs from the marketplace (Temple and Himmel, 2002, Watkins, 2005). Between 1975 and 1999, a total of 548 new chemical entities were approved for use. After regulatory approval, 56 of these drugs (10.2%) either acquired a black box warning, severely restricting their use, or were removed from the market completely due to adverse drug reactions (Lasser, et al., 2002). Although antibiotics, analgesics, and non-steroidal anti-inflammatory drugs are still the leading causes of DILI, emerging data suggest that herbal and dietary supplements also contribute significantly to the overall number of hepatotoxic incidences. As more data are collected on these supplements, the prevalence of DILI could become event greater. DILI continues to be a major concern for pharmaceutical companies and public health worldwide, affecting an estimated 14 people for every 100,000 residents each year (Bell and Chalasani, 2009, Bjornsson, 2010, Rangnekar and Fontana, 2011).

Most of the incidents of DILI were a result of acetaminophen overdose, which accounted for 39% of ALF cases. The remaining 13% of ALF resulting from DILI were a result of adverse drug reactions considered idiosyncratic in nature, also known as idiosyncratic drug-induced liver injury (IDILI). More recent evidence suggests that as much as 17% of all ALF cases can be attributed to IDILI (Hussaini and Farrington, 2007). More than 75% of IDILI cases resulted in liver transplantation or death (Lee, 2003, Ostapowicz, et al., 2002). Although idiosyncratic adverse drug reactions can occur in a number of tissues, the liver often is a target organ (Uetrecht and Naisbitt, 2013).

There are a number of different definitions of IDILI, but among them are some common characteristics. Typically, IDILI occurs in only a small proportion of individuals

exposed to a drug, ranging from 1 in 500, to 1 in 50,000 (Kaplowitz, 2005). These adverse reactions occur at doses of a drug that are considered therapeutic in humans. Since the same dose only causes toxicity in some patients exposed to a drug and not others, there appears to be a lack of obvious dose-dependence. The onset of liver injury relative to the exposure of a drug is variable. It can occur as soon as after the first administration of a drug, and as late as months after drug therapy has ended, further adding to the complexity of these responses (Zimmerman, 1993). More recently however, a positive correlation between the daily dose of oral medicine and the incidence of IDILI was observed (Ballet, 2010, Lammert, et al., 2008). This suggests that at some level, drugs with idiosyncratic liability have a dose-dependent relationship in promoting liver injury.

Although current preclinical and clinical safety testing prevents many potential hepatotoxicants from entering the market, the ability to detect drugs capable of causing IDILI is inadequate. Because the incidence of an idiosyncratic reaction is roughly 1 in 10,000, the sample size of standard preclinical and clinical trials of new drug candidates, which typically involves a couple thousand subjects, is not large enough to reliably detect drugs with idiosyncratic liability. It has been estimated that as many as 30,000 patients would need to be tested to predict accurately whether or not a drug will cause idiosyncratic hepatotoxicity. Unfortunately, this is not a feasible means of studying IDILI, as it is both cost- and time-prohibitive (Lee, 2003). The ramifications of idiosyncratic hepatotoxicity are widespread and have resulted in restriction or removal of otherwise highly effective pharmaceuticals, liver failure, death, litigations, and a loss of confidence in the Food and Drug Administration's (FDAs) ability to identify drugs that

are safe for public use (Watkins, 2005). More reliable screening protocols need to be devised and implemented to detect new drug candidates with idiosyncratic liability. Ideally, these screening protocols would be high-throughput, cost-effective, and able to identify consistently a drug candidate with the potential to cause IDILI. In order for this to be achieved, a better understanding of the mechanism(s) underlying IDILI is required.

1.1.2 Hypotheses for the etiology of idiosyncratic drug-induced liver injury

Currently the mechanism(s) underlying IDILI are not fully characterized and require further investigation. This is due, in part, to the rarity with which these adverse reactions occur, as well as a perceived lack of dose-responsiveness and variation in timing between drug exposure and the onset of liver injury. Several theories have been proposed to explain the pathogenesis of IDILI. These theories involve both genetic and environmental factors, and how they might make a patient susceptible to rare liver injury from a drug (Corsini, et al., 2012, Li and Uetrecht, 2010, Roth and Ganey, 2011, Uetrecht and Naisbitt, 2013, Watkins and Seeff, 2006). Although there is supporting evidence for each of these hypotheses, none is able to explain fully the occurrence of IDILI for all drugs with idiosyncratic liability. It is likely that a combination of susceptibility factors is involved in precipitating IDILI in humans, and that different drugs can cause IDILI by different mechanisms. Despite extensive research, animal models that adequately reproduce idiosyncratic hepatotoxicity in the laboratory are lacking.

1.1.2.1 Metabolic polymorphism hypothesis

The metabolic polymorphism hypothesis suggests that biotransformation of a drug into a reactive metabolite, capable of covalently binding to cellular

macromolecules, could interfere with the homeostatic regulation of cells or tissue, resulting in toxicity (Walgren, et al., 2005). For example, the antidiabetic drug troglitazone, which caused idiosyncratic hepatotoxicity in humans (Murphy, et al., 2000), can be bioactivated by cytochrome P450 3A4 (CYP3A4) to form a reactive metabolite (He, et al., 2004). Glutathione conjugates of troglitazone also have been detected in the bile of rats that were administered the drug, indicating the reactive potential of the drug to bind covalently to thiol groups (Kassahun, et al., 2001).

Similarly, if a polymorphism existed in a critical gene involved in the disposition of a drug, such as a cytochrome P450, the resultant enzyme might metabolize the drug differently than in people not expressing the polymorphism, possibly promoting the formation of a reactive metabolite. Another drug with idiosyncratic liability, the anti-tuberculosis medication isoniazid, is metabolized into acetylhydrazine and hydrazine, which are thought to be toxic metabolites responsible for causing hepatotoxicity. This process occurs through acetylation by N-acetyltransferase enzymes, as well as oxidized by CYP2E1. There are noted correlations between polymorphisms of these metabolizing genes and the incidence of liver injury in patients taking isoniazid (Huang, 2007), however a conflicting report states that there is no relationship apparent between acetylator phenotype and isoniazid-induced liver injury (Gurumurthy, et al., 1984). Also, several drugs form reactive intermediates without causing IDILI, providing further evidence opposing the metabolic polymorphism hypothesis (Uetrecht, 2006). Lastly, several genome-wide association studies have failed to find dispositional polymorphisms that associate with IDILI. Together, these findings suggest that

metabolic polymorphisms might play a role in promoting IDILI but cannot solely explain the mechanisms of toxicity.

1.1.2.2. Hapten hypothesis

One of the more accepted theories pertaining to the mechanism of IDILI suggests that reactive metabolites that covalently bind to macromolecules are capable of evoking a damaging adaptive immune response. Antibodies recognize the drug-protein complex as foreign and bind to the hapten, initiating an autoimmune response that ultimately causes liver injury (Uetrecht, 2007). In support of this theory is the observation that autoantibodies were detected in the sera of patients exposed to drugs with idiosyncratic liability including diclofenac, tienilic acid, and halothane (Aithal, et al., 2004, Kitteringham, et al., 1995, Obermayer-Straub, et al., 2000). However, the presence of these antibodies did not always coincide with IDILI, as autoantibodies were also detected in patients that did not develop liver injury after being exposed to these drugs.

It has also been hypothesized that individuals who express certain human leukocyte antigen (HLA) genotypes might be more susceptible to an immunological reaction towards haptens. Examples for this are described in patients who developed liver injury from flucloxacillin or amoxicillin-clavulanate treatment. In these studies a correlation between expression of particular HLA genotypes and liver injury was observed in patients (Daly, et al., 2009, Hautekeete, et al., 1999). However, the association of certain HLA genotypes to promote liver injury has only been demonstrated for a few drugs, and many patients that expressed a particular HLA

genotype associated with IDILI did not develop liver injury after drug treatment (Uetrecht, 2007).

Lastly, the hapten hypothesis cannot explain the development of liver injury in patients who have experienced only a single exposure to a drug. For an adaptive immune response to occur, there must first be sensitization to a hapten, whereby antigen presentation causes memory T cell clonal expansion. Upon subsequent exposure to a drug, the memory T cells recognize the resulting hapten as foreign and promote a robust immune response. This response cannot occur without more than one exposure, or continuous exposure, to a drug. However, there have been incidents of drugs associated with IDILI causing liver injury after just one exposure in humans (Clay, et al., 2006, Eghtesadi-Araghi, et al., 2008). Although the hapten hypothesis seems like a logical explanation for the etiology of IDILI, the evidence from animal models is lacking, and clinical data are incomplete.

1.1.2.3 Danger hypothesis

An extension to the hapten hypothesis, the danger hypothesis suggests that immunogenicity towards a hapten will not occur unless the binding of the reactive metabolite to a protein initiates a stress response in cells that results in a danger signal that can be recognized by the immune system. These danger signals are thought to act as adjuvants that activate antigen-presenting cells and promote the upregulation of costimulatory molecules. The interaction of the T cell receptor with the major histocompatibility complex between T cells and antigen-presenting cells composes one signal. To activate T lymphocytes and produce an effective immune response, the interaction of costimulatory molecules is also required (Kaplowitz, 2005, Liu and

Kaplowitz, 2002, Shi, et al., 2003, Uetrecht, 2007). This suggests that the binding of a reactive metabolite to a protein to form a hapten is, by itself, insufficient to cause toxicity. If the drug-protein adduct results in some form of cellular stress, this required secondary signal is able to promote activation of the adaptive immune system and injury. However, the list of defined danger signals is not well characterized, and these could arise from independent events such as other xenobiotics or an infection activating the innate immune system, creating an inflammatory stress.

1.1.2.4 Inflammatory stress hypothesis

Inflammation is a complex biological response that results from a multitude of stimuli including infection, tissue damage, diet, alcohol consumption, xenobiotics, and other irritants. Classically, inflammation is characterized by redness, swelling, heat and pain at the site of occurrence. Cellular and molecular studies have revealed that these processes are the result of complex signaling networks involving cytokines and chemokines, the coagulation system, and the activation of leukocytes. Typically, inflammation is a protective response that eliminates microbial pathogens and damaged tissue from the body. However, an inflammatory response that is too robust can result in host tissue injury (Ganey, et al., 2004). Inflammation has been implicated in the pathogenesis of diabetes, cardiovascular disease, stroke, and cancer to name a few (Gonzalez and Selwyn, 2003, Platz and De Marzo, 2004, Price, et al., 2003, Tracy, 2003).

Inflammation also has worsened the liver injury induced by xenobiotics known to cause hepatotoxicity. This was observed in animal models investigating intrinsic hepatotoxicants. In these studies, classic hepatotoxicants cause liver injury in a

predictable manner at sublethal doses. As the dose of a hepatotoxicant is increased, there is a corresponding increase in liver injury. A modest inflammatory stress induced by administration of lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, can cause a leftward shift in the dose-response curve for hepatotoxicity (Roth, et al., 1997). This was observed in animals treated with aflatoxin B(1), galactosamine, monocrotaline, carbon tetrachloride, and acetaminophen (Barton, et al., 2000, Chamulitrat, et al., 1995, Czaja, et al., 1994, Maddox, et al., 2010, Yee, et al., 2003). A diagram depicting the inflammation-induced enhancement of liver injury in response to intrinsic hepatotoxicants is shown (Figure 1).

Episodes of inflammation are commonplace and occur intermittently throughout the life of people and animals. Often, these episodes are minor and go unrecognized. The erratic occurrence of inflammatory responses might coincide with the seemingly chance occurance of IDILI. This, along with the knowledge that inflammation can interact with xenobiotics to promote liver injury suggests that inflammation might be an environmental factor that contributes to IDILI. The liver is composed of a multitude of inflammatory cells and offers a unique environment conducive to a localized inflammatory response (Racanelli and Rehermann, 2006). The unpredictable nature of IDILI, regarding sporadic timing and an apparent lack of dose-dependence, might coincide with individuals experiencing an inflammatory stress in the liver. Therefore, it has been hypothesized that a modest inflammatory stress can render the liver sensitive to an otherwise nontoxic dose of a drug and precipitate hepatotoxicity (Ganey, et al., 2004, Roth, et al., 2003).

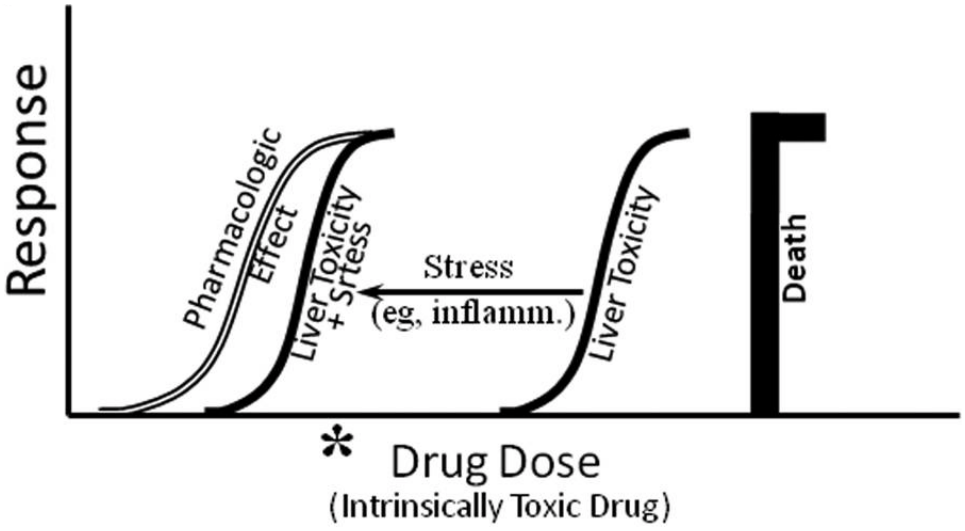


Figure 1: LPS-induced sensitization to intrinsic hepatotoxicants. Intrinsic liver injury occurs in response to hepatotoxicants in a dose-dependent manner at sublethal doses. A therapeutic dose of a xenobiotic, such as acetaminophen causes a pharmacologic effect. Increasing the dose of the xenobiotic or hepatotoxicant gradually results in adverse effects such as liver injury. In the presence of an inflammatory stress the dose of xenobiotic or hepatotoxicant required to cause liver injury can be reduced (Adapted from Roth and Ganey, 2010).

Drugs with idiosyncratic liability do not appear to cause hepatotoxicity in a dose-dependent manner. However, more recent evidence suggests that there is a correlation between dose and the occurrence of IDILI (Ballet, 2010, Lammert, et al., 2008). This perceived lack of dose-dependence of drugs that cause IDILI might be a consequence of the dose required to cause liver injury being greater than the observed lethal dose. In this model, liver injury caused by drugs with idiosyncratic liability occurs at doses greater than the lethal dose, and therefore is not apparent because mortality occurs at smaller doses. The inflammatory stress hypothesis suggests that inflammation might lower the toxic threshold for these drugs with idiosyncratic liability to cause liver injury, causing a leftward shift in the dose-response curve. This would result in observable liver injury at doses smaller than doses required to cause lethality (Figure 2).

Congruent with the inflammatory stress hypothesis is the observation that antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) are the most common classes of drugs that cause idiosyncratic liability (Bjornsson, 2010). NSAIDs are used to treat symptoms of inflammation, and antibiotics are prescribed to combat bacterial infection, which is a potent inducer of inflammation. It might not be merely coincidental that antibiotics and NSAIDs are more frequently associated with IDILI, as they are often administered during times of concurrent inflammatory stress.

1.1.3 Animal models based on the inflammatory stress hypothesis

To test the inflammatory stress hypothesis, LPS was administered to rodents to induce a modest inflammatory stress that did not result in liver toxicity on its own. An

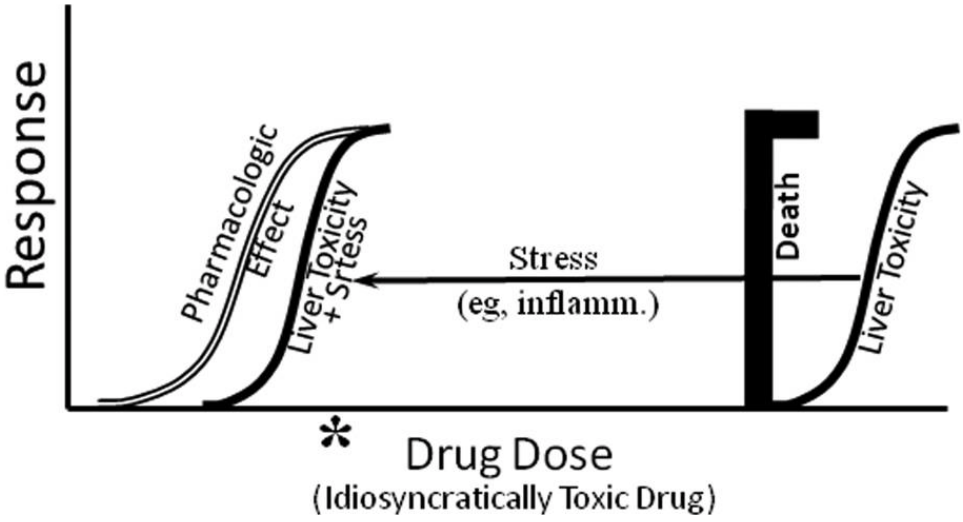


Figure 2: Hypothetical model of dose-responsive idiosyncratic hepatotoxicity. The lack of an apparent dose-response relationship for drugs with idiosyncratic liability could be the result of a xenobiotic causing mortality at a dose that is lower than the theoretical dose required to cause observable liver injury. An inflammatory stress might cause a leftward shift in the liver toxicity dose-response curve, resulting in a xenobiotic causing observable liver injury at a dose smaller than that required to cause lethality (Adapted from Roth and Ganey, 2010).

important clarification is that the LPS administered in these models was not toxic, since LPS itself can produce significant toxicity resulting in multi-organ failure, septic shock, and death (Hewett and Roth, 1993). The activity of LPS in animals is mediated uponbinding to a pattern recognition receptor, toll-like receptor (TLR) 4 (Chow, et al., 1999, Hoshino, et al., 1999). Ligation of the receptor results in a multitude of signaling events, including induction and secretion of chemokines and cytokines that can recruit and activate immune cells (Hewett and Roth, 1993). Liver injury was assessed in these models using light microscopy to examine histopathological lesions in liver sections and by measuring alanine aminotransferase (ALT) activity in the plasma of animals, which is a commonly used biomarker of hepatocellular injury (Bergmeyer and Horder, 1980).

Administration of LPS resulted in liver injury in animals that also were exposed to a drug with idiosyncratic liability. In these studies, treatment with either LPS or drug alone did not cause any liver injury in rats or mice. However, cotreated animals had increased plasma ALT activity and histopathologic lesions in the liver. This phenomenon was demonstrated in rodents that were administered drugs from a variety of drug classes, including chlorpromazine (antipsychotic), amiodarone (antiarrhythmic), doxorubicin (antineoplastic), ranitidine (H_2-receptor antagonist), diclofenac and sulindac (NSAIDs), halothane (volatile anesthetic), and trovafloxacin (antibiotic), all of which are associated with idiosyncratic hepatotoxicity in humans (Buchweitz, et al., 2002, Deng, et al., 2006, Dugan, et al., 2010, Hassan, et al., 2008, Lu, et al., 2012, Luyendyk, et al., 2003, Shaw, et al., 2007, Zou, et al., 2009b). The antibiotic levofloxacin (LVX) and the H_2-receptor antagonist famotidine, members of the same pharmacological classes as trovafloxacin and ranitidine, respectively, are not associated with IDILI and did not

interact with LPS to promote liver injury in rodents. This suggests that models using LPS to induce inflammation are able to distinguish between drugs with idiosyncratic liability and safer alternatives, and also suggests an important role for inflammation in the generation of IDILI (Table 1).

Drug	Causes IDILI in humans?	Hepatotoxic to rodents when administered with LPS?
Chlorpromazine	Yes	Yes
Ranitidine	Yes	Yes
Famotadine	No	No
Diclofenac	Yes	Yes
Sulindac	Yes	Yes
Doxorubicin	Yes	Yes
Halothane	Yes	Yes
Amiodarone	Yes	Yes
Trovafloxacin	Yes	Yes
Levofloxacin	No	No

Table 1: Idiosyncratic-like liver injury in rodents administered a drug and LPS.

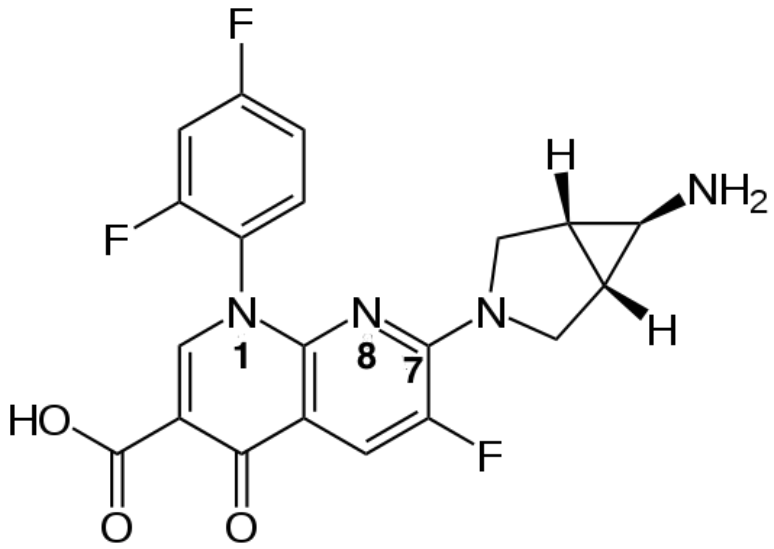
1.2 Trovafloxacin-induced idiosyncratic liver injury

1.2.1 Overview of trovafloxacin pharmacology

Trovafloxacin (TVX) is a broad-spectrum antibiotic in the fluoroquinolone class and was marketed by Pfizer under the brand name Trovan®. In 1998, the FDA approved TVX for distribution after preclinical testing and clinical studies in 7000 patients revealed that it was an effective antibiotic, with no serious adverse effects observed during TVX treatment (Nightingale, 1999, Pannu, et al., 2001). The pharmacological mechanism of action of TVX that accounts for its bactericidal activity is inhibition of the type II topoisomerases, DNA gyrase and topoisomerase IV, which are critical enzymes involved in bacterial DNA replication, recombination and repair (Anderson, et al., 2000, Brighty and Gootz, 1997, Menzel and Gellert, 1994).

Similar to other fluoroquinolones, TVX is a potent inhibitor of DNA gyrase found in Gram-negative bacteria. However, compared to other fluoroquinolones, the chemical structure of TVX has some unique components, such as a cyclopropyl-fused pyrrolidine at the C-7 position, as well as a difluorophenyl group at N-1 (Figure 3A). Both of these substituents promote greater affinity towards topoisomerase IV in Gram-positive bacteria that is not observed during treatment with other fluoroquinolones. This ability to target Gram-positive bacteria allows for a broader spectrum of application for TVX-mediated antimicrobial therapy (Brighty and Gootz, 1997). Even though TVX is considered a fluoroquinolone antibiotic, its chemical structure has a nitrogen atom at the 8-position, which makes it a fluoronaphthyridone. This is in contrast to true fluoroquinolones that have a carbon atom at the 8-position like LVX (Figure 3B). This

A



B

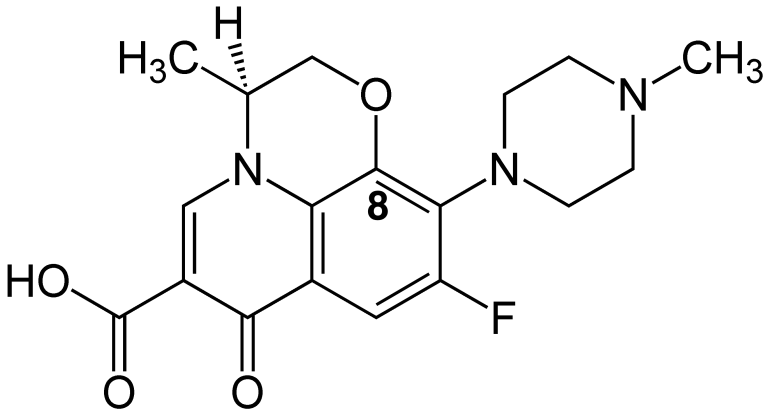


Figure 3: Chemical structure of trovafloxacin and levofloxacin antibiotics. The presence of a nitrogen atom at position 8 makes TVX a fluoronapthyridone (A). LVX has a carbon atom at position 8 making it a fluoroquinolone (B).

subtle difference results in a change in the pharmacokinetic profile of TVX. Greater half-life and bioavailability were observed in cynomolgus monkeys administered TVX versus the quinolone analog with a CH substituted at the 8-position (Brighty and Gootz, 1997). Studies in humans revealed that the half-life of TVX is between 10 and 12 hours (Dalvie, et al., 1997), permitting a dosing regimen of 200 mg tablets once a day for patients on TVX maintenance therapy. These features made TVX a superior antibiotic compared to other fluoroquinolones, with therapeutic advantages that could greatly benefit human health, making it a very marketable drug for Pfizer.

TVX has excellent tissue distribution and penetrates to all peripheral tissues to a significant extent after oral ingestion. Concentrations of TVX in humans were significantly greater in the liver and pancreas compared to all other organs (Fischman, et al., 1998). The primary route of TVX clearance in humans is through phase II conjugation, and oxidative metabolism does not play a significant role. This contrasts with the metabolism of other fluoroquinolones, which were mainly biotransformed through phase I oxidative mechanisms prior to phase II conjugation (Outman and Nightingale, 1989). Most of TVX is eliminated through biliary excretion, and urinary excretion plays a minor role in the clearance. More than half of the drug (55%) is eliminated as the unaltered parent compound, which is responsible for the bactericidal activity (Dalvie, et al., 1997). The remaining 45% of the drug administered undergoes phase II conjugation (Figure 4).

1.2.2 Hepatotoxicity in humans undergoing trovafloxacin therapy

In 1999, TVX received a black box warning after it was associated with causing

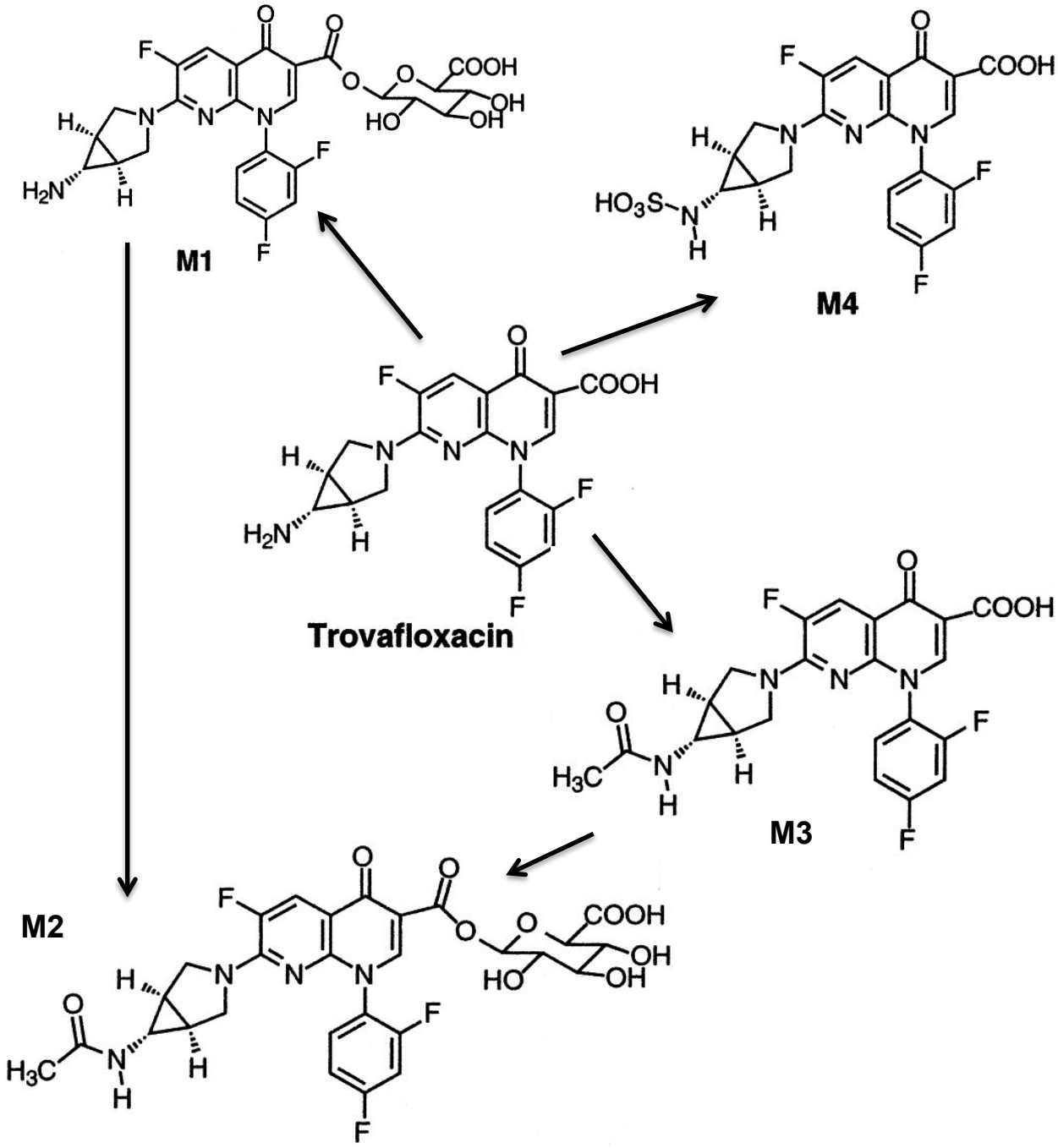


Figure 4: Biotransformation of TVX in humans. Metabolism of TVX involves phase II conjugation reactions, including glucuronidation of the carboxylic acid (M1 and M2), and either sulfonation (M4) or acetylation (M2 and M3) of the amine present on the cyclopropyl-fused pyrrolidine. (Adapted from Dalvie, et al., 1997).

liver injury, and a public health advisory was issued alerting physicians to avoid using TVX if possible. After this announcement, the use of TVX in the United States was severely restricted to cases involving serious life- or limb-threatening infections, complicated intra-abdominal infections, nosocomial or community-acquired pneumonia, gynecological and pelvic infections, and complicated skin infections for which treatment with alternative antibiotics was ineffective (Nightingale, 1999).

In patients suffering adverse hepatic events during TVX therapy, viral, metabolic and autoimmune complications were eliminated as potential causes of liver injury. It was determined that the hepatotoxicity observed in patients coincided with TVX administration. Liver biopsies from patients who had incurred hepatotoxicity due to TVX treatment revealed centrilobular necrosis and signs of inflammation (Chen, et al., 2000, Lazarczyk, et al., 2001, Lucena, et al., 2000, Pannu, et al., 2001). Between February of 1998 and May of 1999, approximately 2.5 million prescriptions were written for TVX, and a total 140 cases of adverse hepatic events occurred (Dembry, et al., 1999). This is an incidence of 5.6 per 100,000 prescriptions, congruent with what is typically defined as an idiosyncratic adverse drug response (Leitner, et al., 2010). These 140 adverse hepatic events resulted in 14 cases of acute liver failure, six of which were fatal.

1.2.3 Murine model of TVX/LPS-induced liver injury

The liver biopsies of patients suffering TVX-mediated liver injury revealed inflammatory cell infiltration at the sites of necrosis (Chen, et al., 2000, Lazarczyk, et al., 2001, Lucena, et al., 2000, Pannu, et al., 2001). This observation suggests that an inflammatory stress might interact with TVX to precipitate liver injury. The

demonstration of other drugs with idiosyncratic liability interacting with an inflammatory stress to cause hepatotoxicity strengthens the rationale that inflammation can be critically involved in liver injury. These animal models based on the inflammatory stress hypothesis have revealed some common factors to be critical to the pathogenesis of liver injury. These factors included the proinflammatory cytokine tumor necrosis factor-alpha (TNF), recruitment and activation of neutrophils, and activation of the hemostatic system (Deng, et al., 2009, Roth and Ganey, 2011). Using this knowledge, the ability of TVX to interact with an inflammatory stress to cause liver injury was investigated in mice.

In mice, a non-toxic dose of TVX, when combined with a modest inflammatory stress induced by LPS, caused liver injury characterized by increased ALT activity in the plasma as well as midzonal and centrilobular hepatocellular necrosis. Neither TVX (up to 500 mg/kg) nor LPS caused liver injury. Similar results were observed in mice treated with both TVX and a mixture of components from Gram-positive bacterial cell walls, peptidoglycan and lipoteichoic acid (PGN-LTA), which is a TLR2 agonist (Shaw, et al., 2009d). This indicates that TVX can interact with an inflammatory stress, regardless of the TLR stimuli, to cause liver injury (Figure 5). An equally pharmacologically efficacious dose of LVX, a fluoroquinolone antibiotic with a much smaller propensity to cause IDILI, did not interact with LPS to cause liver injury (Shaw, et al., 2007).

Treatment with either LPS or PGN-LTA caused inflammatory cell infiltration into the livers of treated mice. Both inflammagens also caused an induction of the chemokines, keratinocyte chemoattractant (KC), and macrophage inflammatory proteins

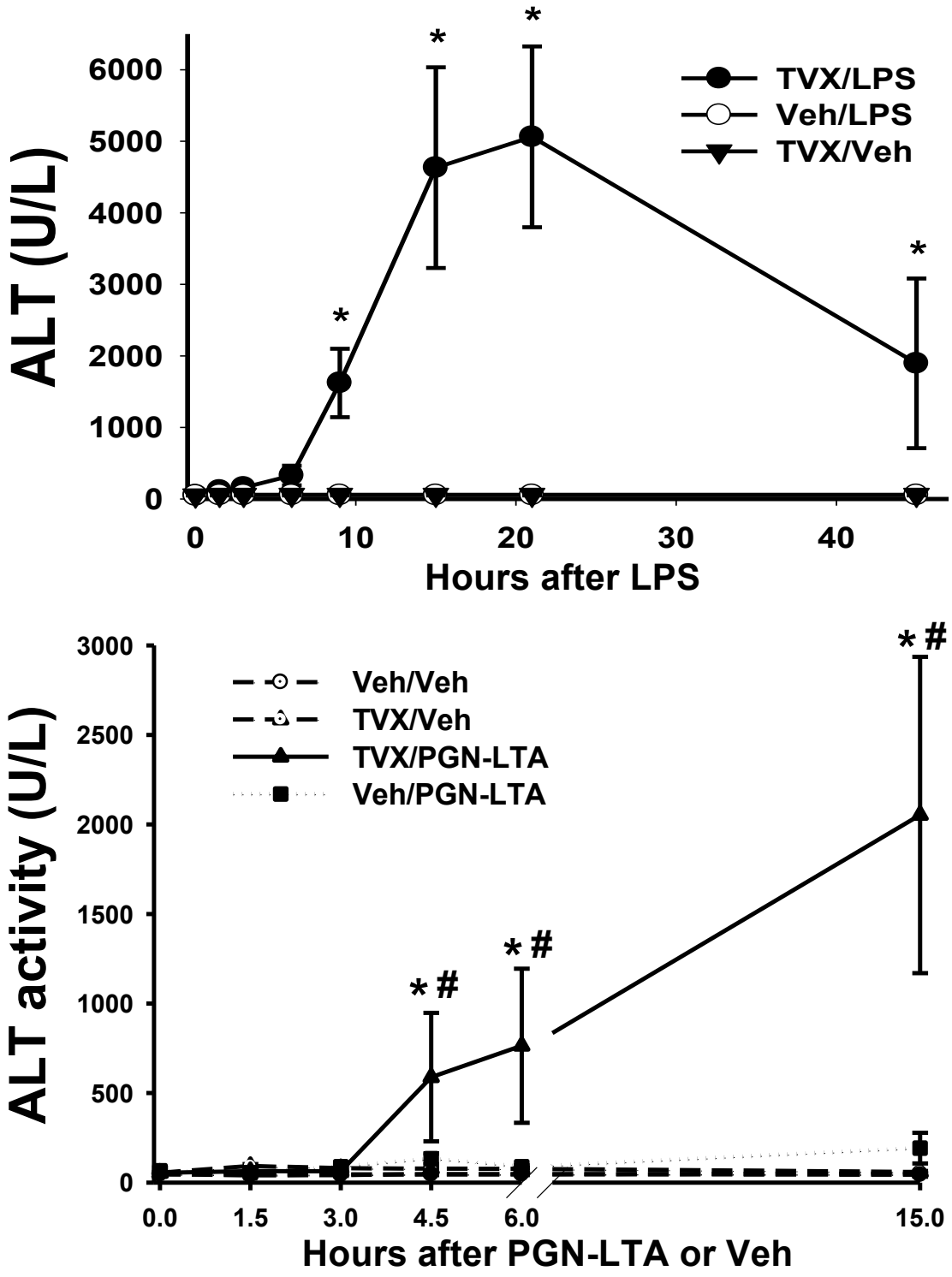


Figure 5: TVX synergizes with an inflammatory stress to precipitate liver injury in mice. Treatment with TVX or either inflammagen alone did not cause liver injury in

Figure 5 (cont'd)

mice. However, an inflammatory stress induced by inflammagens associated with either Gram-negative (LPS) or Gram-positive (PGN-LTA) bacteria both resulted in liver injury in the presence of TVX (From Shaw, et al., 2007 and 2009d).

2 and 1 alpha (MIP-2 and MIP-1a), each of which has chemotactic activity for neutrophils. Histopathology revealed that neutrophils were present in the livers of mice treated with an inflammagen alone, and that cotreatment with TVX did not change the numbers of neutrophils recruited. This suggests that TVX is somehow involved in neutrophil activation. Neutrophils are a common mediator of parenchymal cell injury resulting from inflammation (Chosay, et al., 1997). These cells cause hepatocellular injury by releasing numbers of mediators, including proteases such as neutrophil elastase (NE) (Ho, et al., 1996). Genetic knock out of NE in mice protected against both TVX/LPS-and TVX/PGN-LTN-induced liver injury, indicating a critical role for neutrophils in causing liver injury in this model (Shaw, et al., 2009d).

TVX/LPS administration in mice resulted in activation of the hemostatic system, which involves a complex network of tissue factor, platelet aggregation, coagulation and fibrinolytic factors. Loss of the homeostatic balance of the hemostatic system can contribute to excessive bleeding or pathologic coagulation and thrombi formation (Stassen, et al., 2004). Fibrin deposition was observed in the hepatic sinusoids of cotreated mice, and this was prevented by treatment with the anticoagulant heparin (Shaw, et al., 2009c). Heparin also significantly decreased the ALT activity in plasma of cotreated mice, indicating that the coagulation induced by TVX/LPS-cotreatment was contributing to hepatotoxicity. One manner in which fibrin deposition promotes liver injury is through the formation of occlusive thrombi in the sinusoids of the liver, which results in localized tissue hypoxia that can contribute to liver injury (Ganey, et al., 2007, Hewett and Roth, 1995, Luyendyk, et al., 2005).

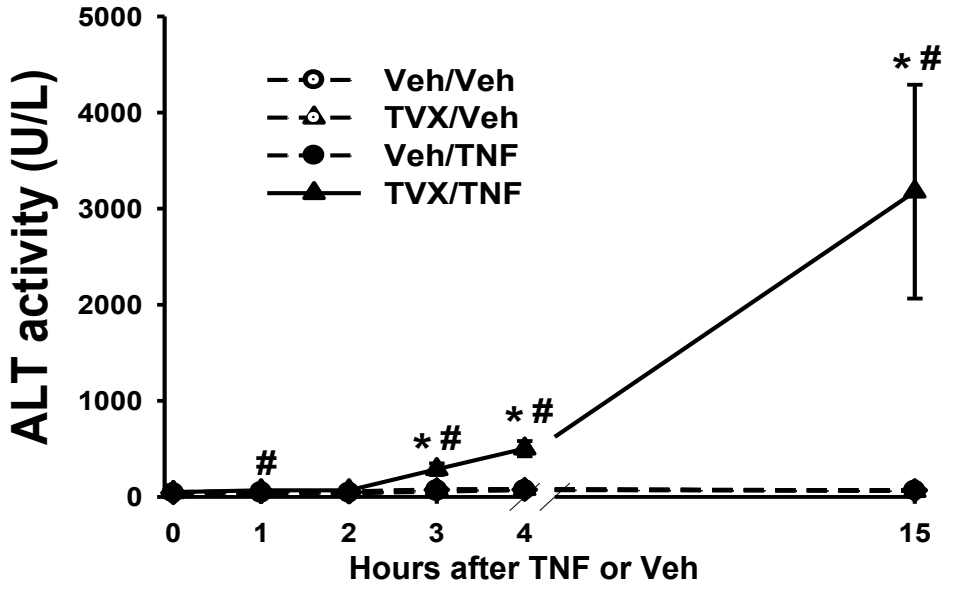
Another study examined changes in hepatic gene expression in mice treated with TVX and LPS. Gene array analysis revealed that a variety of interferon signaling genes were selectively expressed in cotreated mice at the onset of liver injury, compared to mice treated with either TVX or LPS alone that did not undergo liver injury. Genetic knock out of interferon-gamma (IFN) resulted in complete protection from TVX/LPS-induced liver injury, which indicates a significant role for this cytokine in promoting liver injury. In these studies, LVX did not cause a significant change in interferon-related genes. This suggests that drugs with the propensity to cause idiosyncratic liver injury in humans might induce unique gene expression profiles in liver, compared to negative comparator compounds (Shaw, et al., 2009b).

One factor that neutrophils, the hemostatic system, and IFN all have in common is that they can be affected by the proinflammatory cytokine, TNF. This cytokine is able to induce adhesion molecule expression on endothelial cells to promote neutrophil recruitment to the site of injury (Ohira, et al., 2003, Schlayer, et al., 1987) as well as activate neutrophils and stimulate respiratory burst, which can be damaging to neighboring tissue (Menegazzi, et al., 1994, Moore, et al., 1991, Segel, et al., 2011). TNF also increases tissue factor expression on endothelial cells and stimulates the production of interleukin 6 (IL-6), which can promote coagulation system activation and prothrombotic activity (Esmon, 1999, Friedl, et al., 2002). Lastly, both TNF and IFN are able to induce expression of one another, creating a positive feedback loop producing more cytokines (Hanlon, et al., 2002). TNF also is able to synergize with IFN to cause liver injury (Gantner, et al., 1995, Kusters, et al., 1997, Sass, et al., 2001).

TNF is an inducible cytokine in the liver. It is induced in response to Gram-negative stimuli such as LPS (Freudenberg and Galanos, 1990) and, to a lesser extent, Gram-positive stimuli such as PGN or LTA (Sriskandan and Cohen, 1999, Wang, et al., 2003). Kupffer cells are macrophages that reside in the liver, and they express both TLR4 and TLR2, which can be activated by LPS and PGN/LTA respectively. Upon TLR activation, an increase in the expression of several inflammatory cytokines, including TNF, occurs that could potentially play a significant role in promoting hepatotoxicity (Roberts, et al., 2007, Takeda and Akira, 2004, Usui and Kawarada, 1998, Visvanathan, et al., 2007). In mice treated with LPS, TNF protein in the plasma peaked 1.5 hours after treatment. The kinetic profile of TNF concentration in the plasma revealed that the presence of TVX prolonged the circulating TNF in response to LPS. At 4.5 hours after TVX/LPS, circulating TNF was significantly greater than in animals treated with either TVX or LPS alone. This time coincided with the onset of liver injury (Shaw, et al., 2007). Therefore, it was hypothesized that TNF contributes to liver injury in cotreated mice.

The role of TNF in TVX/LPS-induced murine liver injury was investigated in multiple studies (Figure 6). Administration of TNF directly, as a substitute for LPS, caused liver injury in combination with TVX similar to that seen in TVX/LPS-treated mice (Shaw, et al., 2009a). Also, the use of the nonspecific phosphodiesterase inhibitor pentoxifylline (PTX) to inhibit LPS-induced TNF production in macrophages (Witkamp and Monshouwer, 2000) significantly decreased plasma protein concentration of TNF after LPS treatment. PTX also significantly decreased the TVX/LPS-induced liver injury. Administration of a soluble TNF receptor, etanercept, to neutralize the activity of TNF

A



B

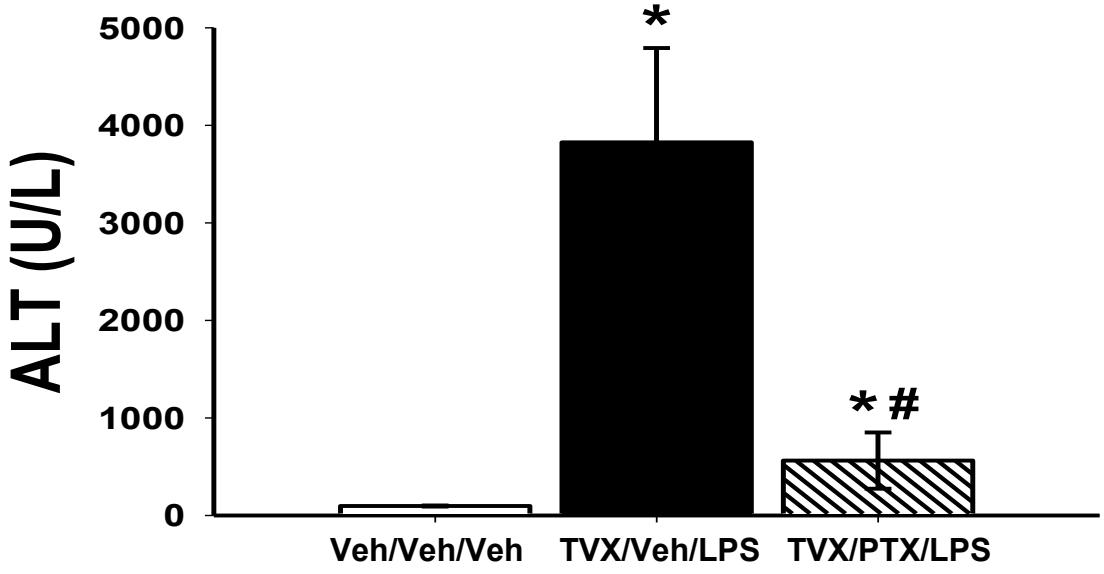
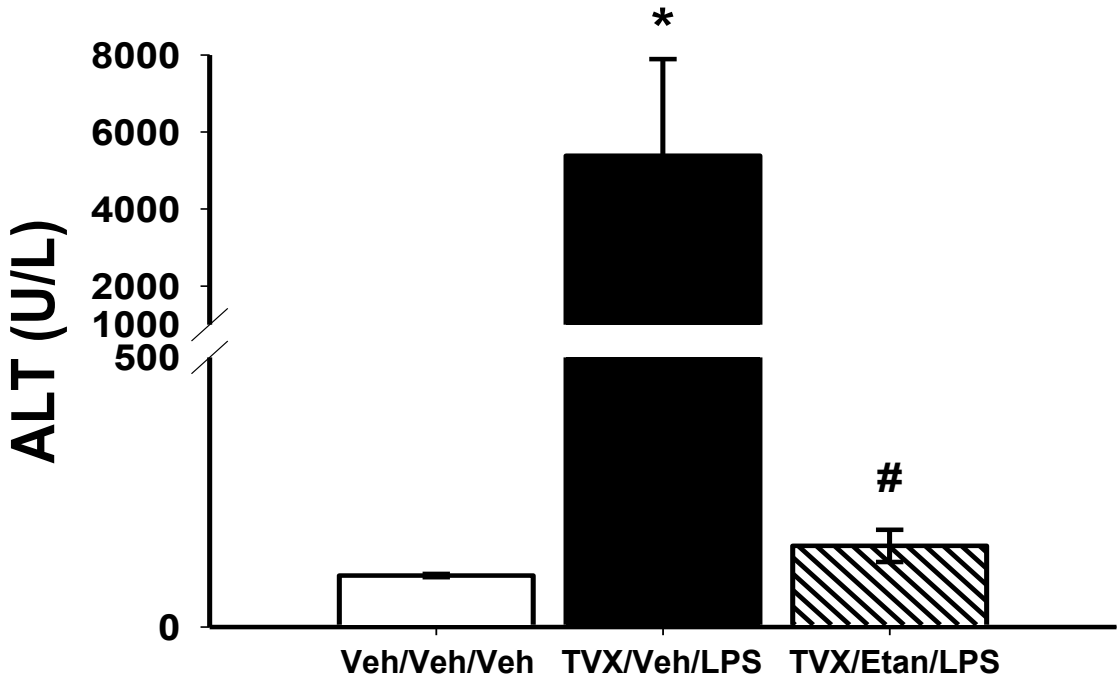


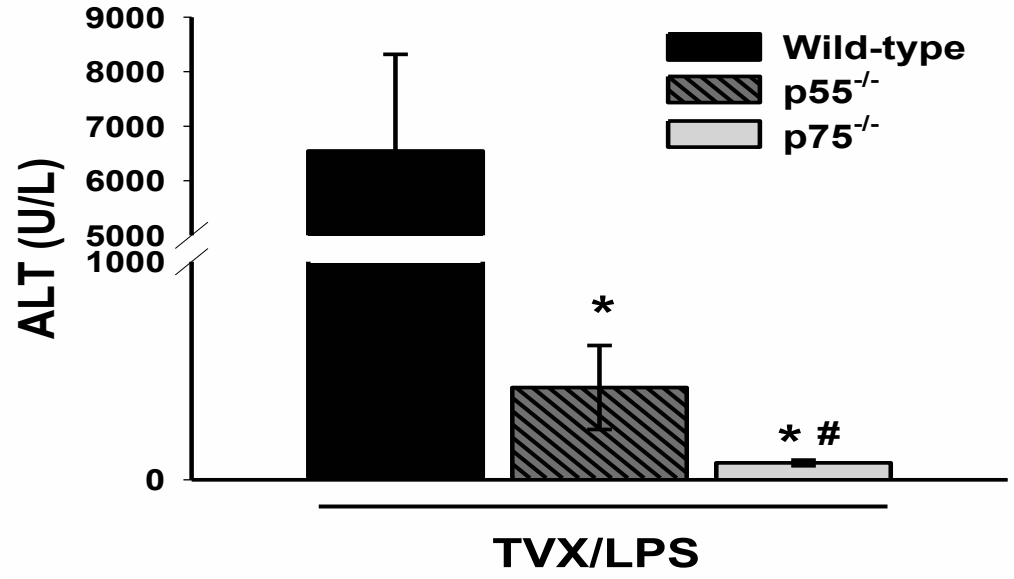
Figure 6: The role of TNF in TVX/LPS-induced liver injury. TNF is critical to the pathogenesis of liver injury induced by TVX/LPS. Substitution of TNF for LPS resulted in liver injury in the presence of TVX similar to the injury seen in TVX/LPS treatment (A) (From Shaw, et al., 2009e). Inhibition of LPS-induced TNF transcription with PTX attenuated liver injury in cotreated mice (B) (From Shaw, et al., 2007). Neutralization of

Figure 6 (cont'd)

C



D



TNF activity using etanercept protected against TVX/LPS-induced liver injury (C) (From Shaw, et al., 2007). Genetic knock out of either TNFR1 (p55) or TNFR2 prevented liver injury in response to TVX/LPS cotreatment (D) (From Shaw, et al., 2009e).

(Breedveld, 1998, Suffredini, et al., 1995) also decreased liver injury (Shaw, et al., 2007). Finally, the involvement of TNF receptors was investigated. TNF exerts its biological activity upon ligation to two cell surface receptors, TNF receptor 1 (TNFR1) and TNFR2 (Locksley, et al., 2001). Genetic knockout of either of these receptors resulted in protection against liver injury from TVX and LPS administration (Shaw, et al., 2009e). Clearly, these results demonstrate the importance of TNF in the pathogenesis of liver injury induced by TVX/LPS cotreatment. Further investigation into the molecular mechanisms associated with this liver injury should therefore focus on the signaling pathways associated with TNFR ligation.

1.3 Overview of tumor necrosis factor alpha

1.3.1 Significance of tumor necrosis factor in the inflammatory stress hypothesis

The role of TNF in promoting liver injury in rodents during concurrent inflammation has been demonstrated for a number of other drugs with idiosyncratic liability. These include ranitidine, sulindac and amiodarone, all of which interact with LPS to cause liver injury (Lu, et al., 2012, Tukov, et al., 2007, Zou, et al., 2009a). In each of these studies, administration of drug in combination with LPS resulted in an increase in the circulating TNF protein over animals treated with LPS alone. Neutralization of TNF with etanercept also decreased the liver injury induced by cotreatment of drug and LPS in each model. As described above, similar observations also were observed in mice treated with both TVX and LPS.

More recently, the ability of drugs with idiosyncratic liability to interact with TNF to cause cytotoxicity in hepatocytes has been demonstrated *in vitro*. Treatment of hepatocytes in culture with both TNF and drugs associated with IDILI caused cytotoxicity that was not induced by either agent alone. In the presence of TNF, cytotoxicity was observed in hepatocytes treated with ranitidine, nefazadone, nimesulide, diclofenac, sulindac, amiodarone, and trovafloxacin, all of which cause IDILI (Cosgrove, et al., 2009, Fredriksson, et al., 2011, Lu, et al., 2012, Shaw, et al., 2009a, Zou, et al., 2009a). Similar to the animal models using LPS, exposure of cells to negative comparator drugs including cimetidine, buspirone, aspirin, and levofloxacin, none of which are associated with IDILI, did not cause cell death in the presence of TNF (Cosgrove, et al., 2009).

Although these results offer insight into the potential mechanisms underlying IDILI, the interaction of a drug with idiosyncratic liability and TNF to cause cytotoxicity is not fully characterized. Further investigation of the signaling pathways involved in these models of *in vitro* cytotoxicity of hepatocytes could offer insight into the ultimate mechanism of action responsible for causing liver injury in humans. With this knowledge, pharmaceutical companies and regulatory agencies could implement high-throughput screening assays to detect drug candidates with the potential to cause idiosyncratic hepatotoxicity.

1.3.2 Tumor necrosis factor signaling in hepatocytes

TNF is a key mediator released in the liver by resident macrophages (Kupffer cells) upon LPS stimulation (Roberts, et al., 2007). Although TNF is produced mainly by Kupffer cells in response to immunological challenges such as LPS, PGN, or LTA, it also can be produced by T lymphocytes, sinusoidal endothelial cells (SECs) and natural killer (NK) cells, all of which are present within the liver (Bemelmans, et al., 1996). TNF is a pleiotropic cytokine that plays a dichotomous role in the liver. It is required for normal hepatocyte proliferation during regeneration after tissue injury; however, it also can induce hepatocellular apoptosis (Aggarwal, 2003, Bradham, et al., 1998, Micheau and Tschopp, 2003). These actions of TNF are accomplished through binding to its two distinct receptors located in the plasma membrane of cells, TNFR1 and TNFR2. Although TNFR1 is constitutively expressed in many different cell types and appears to be the key mediator of TNF signaling, expression of TNFR2 is highly regulated and typically located on cells of the immune system (Aggarwal, 2003, Wajant, et al., 2003).

In the liver, binding of TNF to TNFR1 causes receptor trimerization that initiates the recruitment of the TNF receptor-associated death domain (TRADD) to the receptor. Both the intracellular region of the TNFR1 and TRADD contain a death domain (DD) that allows for protein-protein interaction (Tartaglia, et al., 1993). Next, TRADD serves as a platform to recruit TNF receptor-associated factor 2 (TRAF2) through the interaction of a carboxy-terminal TRAF domain with an amino-terminal DD on TRADD (Hsu, et al., 1995). This is followed by recruitment of receptor-interacting protein 1 (RIP1), which also contains a DD (Hsu, et al., 1996). Together the interaction of these proteins forms what is known as complex I. Following the interaction of all these proteins to form this complex, the ubiquitin ligase domain of TRAF2 polyubiquitinates both itself and RIP1 on lysine 63 (Figure 7). These ubiquitin chains are recognized by TAK1 binding protein (TAB)2 and TAB3, which serve to recruit transforming growth factor beta (TGF-β) activated kinase 1 (TAK1) (Chen, et al., 2002). This active complex is able to phosphorylate downstream proteins through the kinase activity of TAK1, and this results in activation of both nuclear factor kappa B (NF-κB) and the mitogen-activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK) (Wajant, et al., 2003, Wullaert, et al., 2007).

NF-κB is a transcription factor that is sequestered in the cytoplasm by members of the inhibitor of κB (IκB) protein family. Upon exposure to TNF, hepatocellular TNFR1 activation results in TAK1-mediated phosphorylation and activation of the inhibitor of κB kinase (IKK) complex. Once activated, IKK phosphorylates IκB, which leads to ubiquitination of lysine-48 and subsequent proteosomal degradation by the 26S proteosome. Now free of the repressive IκB, NF-κB heterodimers such as the

prototypical p65/p50 complex are able to translocate into the nucleus and transcribe NF-κB responsive genes. Many of these genes are hepatoprotective and associated with liver survival (Andera, 2009, Wullaert, et al., 2007).

Also downstream of TNFR1 activation is the MAPK cascade. The classical MAPKs include p38, JNK and extracellular signal-related kinase (ERK), all of which are part of a kinase cascade that is activated in response to phosphorylation by upstream MAP kinase kinase proteins (MAP2Ks). These are regulated by another set of upstream kinases, the MAP kinase kinase kinase family members (MAP3Ks) (Cuschieri and Maier, 2005, Derijard, et al., 1995, Kyosseva, 2004). In hepatocytes, TNF is able to activate both p38 and JNK MAPKs (Ding and Yin, 2004, Hirano, et al., 2003, Lu and Cederbaum, 2010, Minero, et al., 2013, Win, et al., 2011, Wullaert, et al., 2007). Although TNF is able to activate ERK in some cell types (Gortz, et al., 2005, Yanagawa, et al., 2002), evidence of hepatocellular TNF-mediated ERK activation is lacking in the literature.

TAK1 is a member of the MAP3K family (MAP3K7) and is capable of activating downstream MAP2Ks (Shim, et al., 2005, Wang, et al., 2001). The MAP2Ks, MKK3 and MKK6 are responsible for phosphorylating and activating p38, and both MKK4 and MKK7 can activate downstream JNK (Derijard, et al., 1995). Upon their activation, p38 and JNK induce transcriptional activation of responsive genes, as well as cause posttranslational modifications of signaling proteins within hepatocytes (Wang, et al., 2004). However, JNK is the decidedly more involved kinase in hepatocellular responses to TNF (Schwabe, 2006, Seki, et al., 2012, Wullaert, et al., 2006). Similar to TNF itself, JNK is able to promote hepatocellular protection and proliferation (Brenner,

1998, Chen, et al., 1996, Krause, et al., 2001, Lamb, et al., 2003, Nishina, et al., 2004) as well as programmed cell death (Ding and Yin, 2004, Han, et al., 2009, Wullaert, et al., 2006). These effects depend on the duration of JNK activation. Ligation of TNF to TNFR1 causes a transient activation of JNK, often for duration of 15 minutes to 2 hours. However, during cellular stress, JNK can become activated for a prolonged period of time. This prolonged JNK activation is associated with cytotoxic signaling, and often can involve a deficiency in NF-κB mediated negative regulation of JNK (Javelaud and Besancon, 2001, Nakano, 2004, Tang, et al., 2002, Wicovsky, et al., 2007).

As mentioned before, TNF exerts a number of effects. The other component of TNFR1 signaling in hepatocytes involves the induction of apoptosis, mediated by a group of enzymes known as the cysteine-aspartate specific proteases (caspases) that play an integral role in this programmed cell death (Fan, et al., 2005, MacKenzie and Clark, 2012, Taylor, et al., 2008). After the formation of complex I and activation of MAPK and NF-κB signaling, TNFR1 undergoes a clathrin-dependent endocytosis. The internalization of TNFR1 results in the recruitment of caspase-associated ring protein-2 (CARP2), an ubiquitin E3 ligase that promotes the degradation of RIP1 and terminates NF-κB signaling. Fas-associated death domain (FADD) then interacts with TRADD, which leads to the recruitment of procaspase 8 and the formation of what is known as complex II in the cytosol of the cell (Micheau and Tschopp, 2003, Schutze, et al., 2008).

The recruited procaspase 8 autocatalytically cleaves itself to form active caspase 8, which targets and cleaves bcl2 interacting protein (Bid) to form truncated bid (tBid). Upon translocation to the mitochondria, tBid induces the release of cytochrome c into the cytosol through the opening of the permeability transition pore on the mitochondrial

outer membrane. Cytochrome c release prompts the formation of the apoptosome, consisting of apoptosis protease associated factor 1 (Apaf1) and pro-caspase 9. From this, active caspase 9 is formed and is able to cleave and activate downstream effector caspases 3, 6 and 7. These effector caspases can cleave a variety of substrates. Some of these substrates include caspase-activated DNAse (CAD), an endonuclease that cleaves DNA, as well as poly ADP-ribose polymerase (PARP) and DNA-dependent protein kinase (DNA-PK). Both PARP and DNA-PK are involved in DNA repair, and become inactivated upon cleavage. These events result in DNA degradation and chromatin fragmentation. Caspases also can cleave cellular cytoskeletal components, which results in cell shrinkage and the formation of apoptotic bodies. Ultimately, all of these events result in cellular apoptosis (Andera, 2009, Fan, et al., 2005, Wullaert, et al., 2007).

The dichotomous effects on cell function exemplify the complexity of TNF signaling in hepatocytes. TNF-induced NF-κB activation is responsible for the transcription of a number of cytoprotective genes that combat cellular stress. Induction of antioxidant genes such as manganese-dependent superoxide dismutase (mnSOD) and ferritin heavy chain (FHC) that neutralize reactive oxygen species (ROS), as well as growth arrest and DNA-damage-inducible 45 beta (GADD45β) that inhibits upstream MKK7, are largely responsible for maintaining as transient the JNK activation that occurs during TNFR1 activation. During oxidative stress, JNK activation becomes prolonged in response to activation of apoptosis signal-regulated kinase 1 (ASK1), an upstream MAP3K. ASK1 normally exists in an inactivated state due to its interaction with thioredoxin, but this interaction is interrupted upon oxidation of thioredoxin,

resulting in ASK1 activation (De Smaele, et al., 2001, Saitoh, et al., 1998, Wullaert, et al., 2006). Oxidative stress also can promote prolonged JNK activation by inactivating MAPK phosphatases, which are negative regulators of JNK phosphorylation (Kamata, et al., 2005).

TNF-induced activation of NF-κB also causes induction of antiapoptotic genes that can directly or indirectly prevent caspase activation. Proteins such as FADD-like interleukin converting enzyme (FLICE) inhibitory protein (FLIP), cellular inhibitor of apoptosis 1 (c-IAP1), c-IAP2, X-linked inhibitor of apoptosis (XIAP), and B-cell lymphoma-extra large (Bcl-xL), all of which are induced by NF-κB, can prevent the activation of caspase enzymes (Irmler, et al., 1997, Micheau and Tschopp, 2003, Wullaert, et al., 2007). These proteins interact with caspases and prevent their processing for activation. In the absence of any cellular stress that interferes with these events, the combined effects render hepatocytes resistant to TNF-induced apoptosis. However, if a cellular stress results in preventing transcription of these genes, hepatocytes will undergo TNF-induced apoptosis dependent on caspase activation. This scenario has been demonstrated with the use of transcriptional inhibitors such as actinomycin D, α-amanitin, and galactosamine (Leist, et al., 1994, Leist, et al., 1997a).

The other putative receptor for TNF, TNFR2, has some notable differences from TNFR1. Whereas membrane bound TNF is able to bind to and activate both TNF receptors, soluble TNF that has been released from the plasma membrane of cells only activates TNFR1 efficiently and has very limited signaling capacities on TNFR2. TNFR1 is a ubiquitous protein that is expressed in many tissues, but the expression of TNFR2 is limited and controlled. Ligation of TNFR2 results in direct recruitment of TRAF2

followed by TRAF1, which results in the activation of NF-κB and JNK, but not p38 or ERK. Unlike TNFR1, the intracellular domain of TNFR2 lacks a DD and is unable to process the activation of caspases and cell death signaling. However, there is evidence of functional crosstalk between the two receptors, and TNFR2 is able to enhance the cytotoxic effects of TNFR1 (Cabal-Hierro and Lazo, 2012, Jupp, et al., 2001, Wajant, et al., 2003, Wallach, et al., 1999). Together, this information implicates NF-κB, JNK, and caspase enzymes as critical mediators of TNF signaling in hepatocytes (Figure 7).

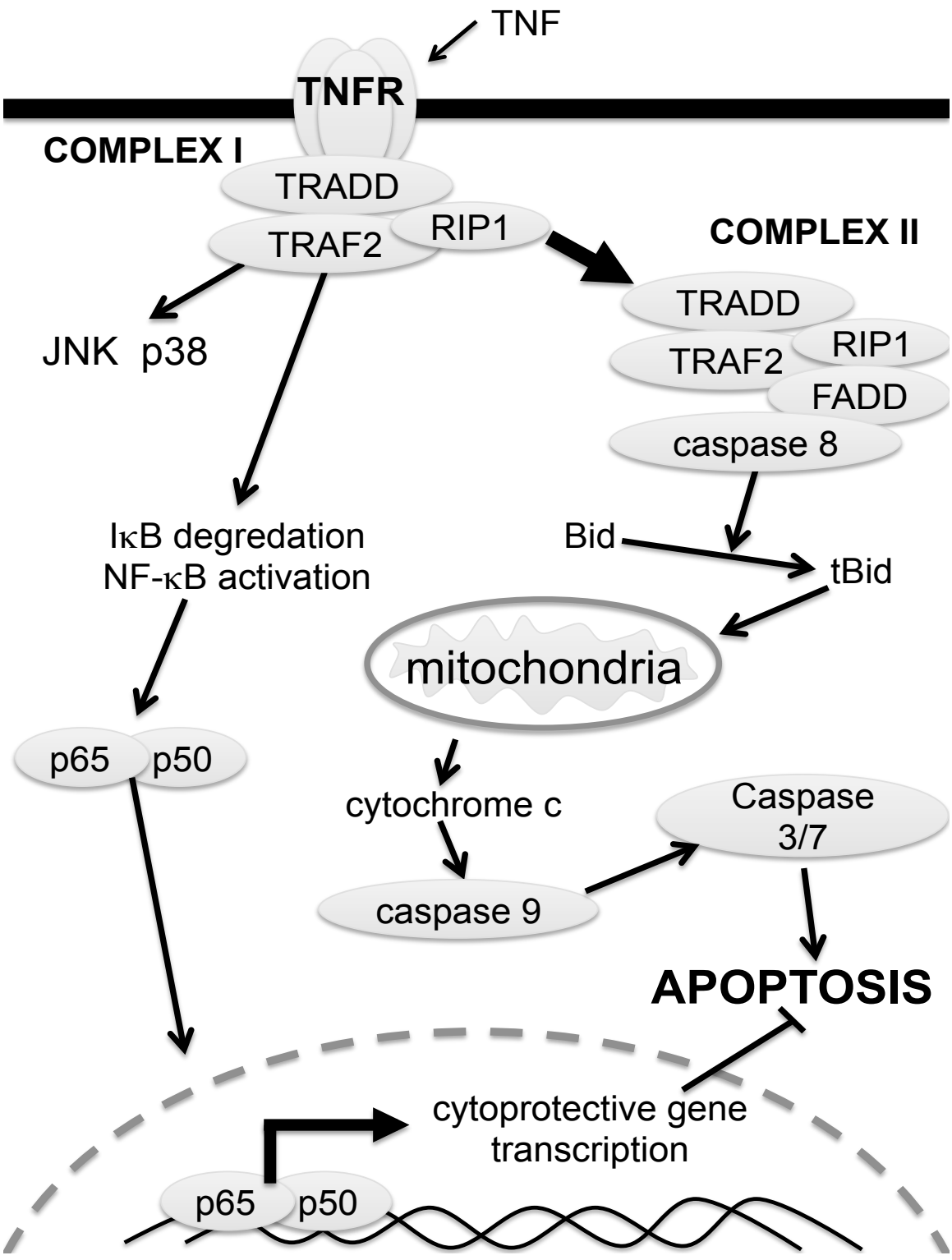


Figure 7: Hepatocellular responses to TNF. A schematic depicting the critical signaling events in hepatocytes induced by TNF (Adapted from Wullaert et al., 2006).

1.4 The DNA damage response

1.4.1 Idiosyncratic liability and cell cycle perturbations

Congruent with the finding that transcriptional interference sensitizes hepatocytes to TNF-induced apoptosis, similar results are observed in several cell types treated with a combination of eukaryotic topoisomerase inhibitors and TNF (Debernardis, et al., 1996, Hentze, et al., 2004, Utsugi, et al., 1990). This aligns with the observation that transcriptional arrest sensitizes cells to TNF, in the sense that topoisomerases are involved in transcriptional regulation (Mondal and Parvin, 2001). Of interest is the observation that some topoisomerase inhibitors, such as etoposide and doxorubicin, are associated with IDILI (Aviles, et al., 1984, Tran, et al., 1991). Altogether these findings suggest that topoisomerase enzymes might be involved in the hepatocellular responses to TNF.

Topoisomerases play an essential role in DNA replication, cell division, and transcriptional regulation. During cell division, these enzymes relieve topological constraints that occur ahead of DNA replication forks as the DNA becomes catenated and supercoiled. By introducing a transient double-strand break (DSB) in one section of DNA, then passing the other section through the cleavage site and religating, the DNA is decatenated in an ATP-dependent manner (Levine, et al., 1998, Vos, et al., 2011, Wang, 2002). The importance of these enzymes is demonstrated by their critical involvement in basic cellular functions. Their ability to introduce double strand breaks in DNA has made them a therapeutic target for the treatment of cancer and bacterial infection (Anderson, et al., 1998, Pommier, et al., 2010).

After topoisomerases cleave the phosphodiester bond of DNA, fluoroquinolones inhibit the religation action of the enzyme, forming a drug/enzyme/DNA ternary complex with stabilized double strand breaks (Drlica, 1999). These complexes and the DNA strand breaks are reversible, as the DNA can be religated (Howard, et al., 1994, Levine, et al., 1998, Wilstermann and Osheroff, 2003). The ternary complex also results in the inhibition of DNA synthesis by stalling the replication fork, as well as blocking transcription mediated by RNA polymerases (Hiasa, et al., 1996, Hong and Kreuzer, 2000, Willmott, et al., 1994). However, in the event that a DNA or RNA polymerase comes into contact with a drug-stabilized cleavage complex on the DNA, an irreversible DSB will form causing cellular genotoxic stress (Hiasa, et al., 1996, Howard, et al., 1994, Pohlhaus and Kreuzer, 2005, Stohr and Kreuzer, 2001).

Interestingly, some fluoroquinolone drugs have displayed activity against mammalian topoisomerases. These include moxifloxacin and ciprofloxacin. Although these antibiotics are designed to target prokaryotic type II topoisomerase enzymes, there appears to be some cross-reactivity towards the eukaryotic equivalents, albeit at higher drug concentrations than those experienced during antimicrobial therapy (Albertini, et al., 1995, Barrett, et al., 1989, Smart, 2008, Smart and Lynch, 2012). This presents the possibility that TVX might interact with eukaryotic topoisomerases, potentially sensitizing hepatocytes to TNF-mediated cytotoxicity. As a follow up to this idea, the ability of TVX to inhibit the activity of topoisomerase IIα, the human homolog of the bacterial target of TVX, was investigated in a cell-free system. Incubation of TVX with topoisomerase IIα inhibited the enzyme's activity towards its substrate, suggesting

TVX can inhibit mammalian topoisomerase IIα (Poulsen, el al., 2011), although whether or not this happens in hepatocytes remains uncertain.

In response to DNA damage, cellular checkpoint activation occurs to facilitate repair of damaged DNA, resulting in cell cycle arrest and slowing of proliferation (Houtgraaf, et al., 2006, Zhou and Elledge, 2000). Interestingly, a number of drugs with idiosyncratic liability, including TVX, are able to decrease proliferation *in vitro* (Ban, et al., 2011, Basta-Kaim, et al., 2006, Chennamaneni, et al., 2012, Francavilla, et al., 1989, Hamaguchi, et al., 2010, Holtom, et al., 2000, Jakubowska-Mucka, et al., 2012, Lee, et al., 2007, Parada-Turska, et al., 2006, Rajabalian, et al., 2009, Robles, et al., 1999, Supino, et al., 1977, Zakeri, et al., 2000) suggesting that these xenobiotics might initiate genotoxic stress in cells.

Collectively, these findings suggest that genotoxic stress might be a contributing factor involved in the development of IDILI. TVX inhibits mammalian topoisomerase activity, which can cause DNA strand breaks and sensitize cells to TNF-induced cytotoxicity. It also decreases cell proliferation. These observations suggest TVX is capable of inducing genotoxic stress. The interaction of the signaling events employed during genotoxic stress with those induced by TNF should be investigated.

1.4.2 Cellular response to genotoxic stress

Genotoxic stress occurs in response to a number of DNA damaging agents, including chemotherapeutic drugs, environmental toxicants, ionizing or ultraviolet radiation, and ROS. In response to this genotoxic stress, a complex signaling network known as the DNA damage response becomes activated. Minimal DNA damage will

activate repair mechanisms to remove the problematic sections of DNA and replace them with the proper nucleotide(s). In dividing cells, DNA damage results in cell cycle arrest through the activation of checkpoint pathways. These checkpoints halt the proliferating cell during its cell cycle progression to allow DNA repair machinery access to the site of genotoxicity. This cell cycle arrest is an attempt to restore the DNA to its proper form. However, if the DNA damage is too severe and exceeds the capability of the cell to repair, or if the DNA repair itself is ineffective, the DNA damage response will activate apoptotic-signaling mechanisms. These responses are an attempt to maintain genomic integrity and prevent mutations from occurring and being propagated to daughter cells after DNA replication and cell division (Houtgraaf, et al., 2006, Nowsheen and Yang, 2012, Roos and Kaina, 2006, Zhou and Elledge, 2000).

The DNA damage response is first initiated upon recognition of DNA damage. This is accomplished by the Mre11-Rad50-Nbs1 (MRN) complex and Rad9-Hus1-Rad1 (9-1-1) complex (Houtgraaf, et al., 2006, Jackson and Bartek, 2009, Parrilla-Castellar, et al., 2004, Polo and Jackson, 2011, Roos and Kaina, 2013). Upon recognition of the DNA damage, these complexes recruit and activate a family of phosphoinositide 3-kinase-like kinases (PIKK), Ataxia Telangiectasia Mutated (ATM), Ataxia Telangiectasia and Rad3 Related (ATR), and DNA-dependent protein kinase (DNA-PK). Although all three PIKKs share similarities, DNA-PK is unique compared to ATM and ATR, which share a number of signaling responses. Activation of DNA-PK occurs in response to radiation or V(D)J recombination in B and T lymphocytes. Activation of both ATM and ATR also occurs in response to radiation, as well as genotoxic compounds and DNA replication fork stalling (Yang, et al., 2003), and involves autophosphorylation events

(Bhatti, et al., 2011, Kozlov, et al., 2011, Lavin and Kozlov, 2007, Liu, et al., 2011, Nam, et al., 2011). Upon their activation, ATM and ATR phosphorylate and activate downstream targets to initiate checkpoint activation and halt proliferation. These targets include a couple of serine/threonine-protein kinases known as Chk1 and Chk2. Activation of either Chk1 or Chk2 results in phosphorylation of cell division cycle 25 (CDC25) phosphatases, targeting CDC25 for ubiquitination and proteosomal degradation (Smith, et al., 2010). With the phosphatase actions of CDC25 removed, inhibitory phosphate groups remain on downstream cyclin-dependent kinases (Cdks), conforming them to an inactive state and halting proliferation. When active, Cdks are critical proteins involved in promoting cell cycle progression and mitosis (Figure 9).

Chk1 and Chk2, as well as all three PIKKs (ATM, ATR and DNA-PK) are able to promote activation of the p53 transcription factor. The significance of p53 in the regulation of the cell cycle and response to DNA damage is noted in a study that observed that more than half of human tumors contained a mutated p53 gene (Greenblatt, et al., 1994). Much of the remaining instances of tumors are a result of compromised p53 activity (Vogelstein, et al., 2000). The phosphorylation of p53 promotes dissociation from its transcriptional repressor, mouse double minute 2 homolog (MDM2), which keeps p53 sequestered and inactive in the cytosol. MDM2 also contains an ubiquitin ligase domain that promotes the proteosomal degradation of p53, which has a half-life of about 5-30 minutes (Honda and Yasuda, 2000, Moll and Petrenko, 2003). Upon phosphorylation and dissociation from Mdm2, p53 undergoes tetramerization and becomes stabilized (Chene, 2001). This functional complex is capable of transcribing responsive genes that can promote cell cycle arrest or apoptosis

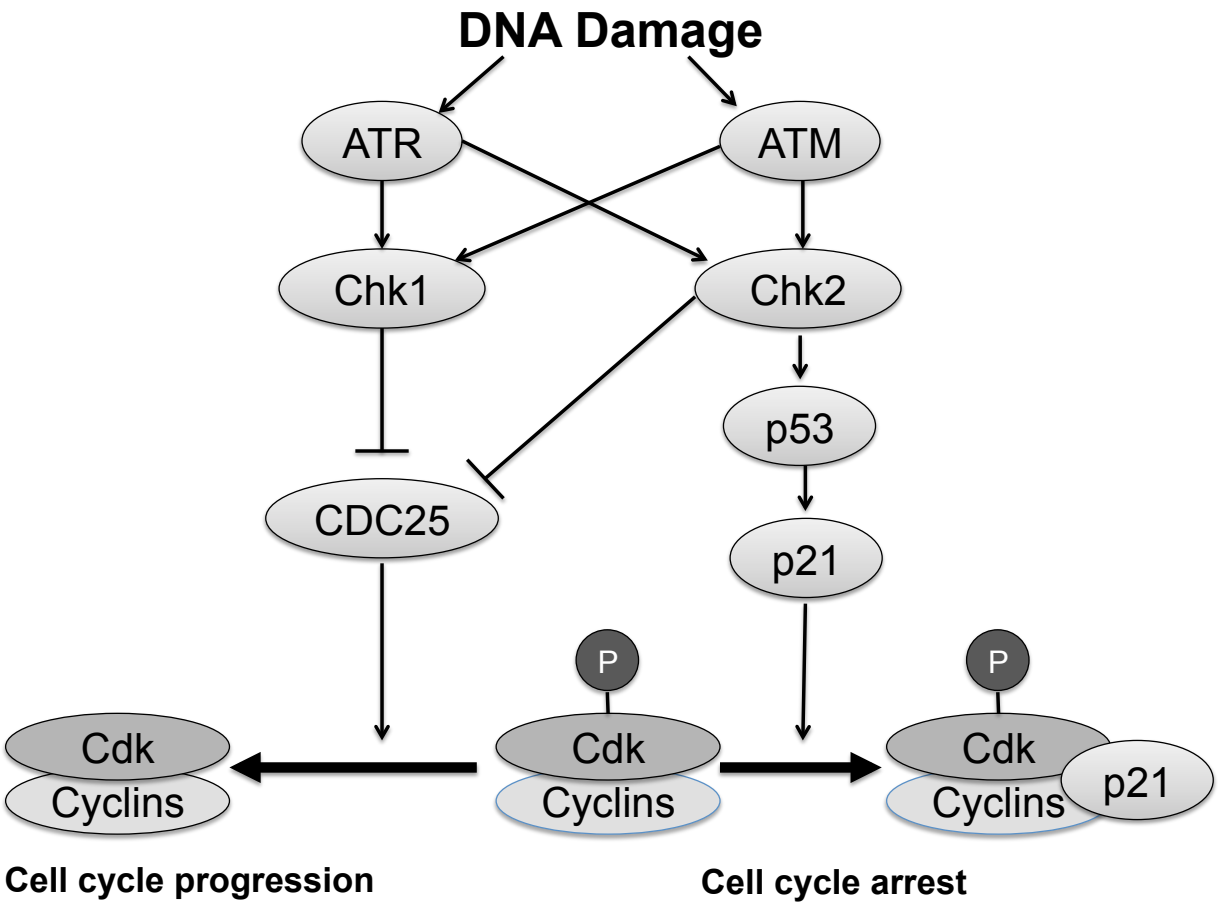


Figure 8. DNA damage-induced cell cycle arrest. In response to DNA damage, a signaling cascade occurs that prevents cell cycle progression by ultimately inhibiting the activity of cyclin-dependent kinases (Cdk) (adapted from De Veylder et al., 2007).

(Haupt, et al., 2003). In response to moderate genotoxic stress, p53 activation is associated with upregulation of the Cdk inhibitor p21, which promotes cell cycle arrest. However, if the genotoxic stress is severe, p53 can transcribe proapoptotic factors such as p53 upregulated modulator of apoptosis (PUMA) and Noxa, both of which promote cytochrome C release from the mitochondria and caspase 9 activation (Haupt, et al., 2003, Roos and Kaina, 2006). Genotoxic stess also activates MAPKs, which are another significant component of the DNA damage response.

DNA-damaging agents can activate all three of the major MAPKs, and can be either cytoprotective or apoptotic (Cagnol and Chambard, 2010, Chen, et al., 1996, Hayakawa, et al., 2003, Roos and Kaina, 2006, Tentner, et al., 2012, Wei, et al., 2011, Yoshida, et al., 2002). Although the direct signaling components involved between DNA damage and MAPK activation are not well characterized, it has been suggested that decreased global transcription in response to genotoxic stress (Heine, et al., 2008) yields less MKP expression, promoting the activation of MAPKs. This phenomenon seems particularly prominent for JNK and ERK (Cadalbert, et al., 2005, Hamdi, et al., 2005, Roos and Kaina, 2006).

In response to genotoxic stress, JNK activation is prolonged and capable of inducing apoptosis. The ability of JNK to promote cell death occurs by phosphorylating c-Jun, which forms a heterodimer with c-Fos to form the activator protein 1 (AP-1) transcription factor (Ventura, et al., 2003, Westwick, et al., 1994). AP-1 is responsible for the upregulation of proapoptotic genes such as Bax, as well as Fas ligand, which is a member of the TNF superfamily that can induce caspase activation upon binding to the Fas receptor on hepatocytes (Lauricella 2006, Liu Czaja 2002, Mandal 2001,

Schlosser 2000, Ni 1994, Mansouri 2003). JNK also postranslationally modifies proteins to promote caspase activation. This occurs prominently at the level of the mitochrondria. JNK phosphorylates Bax, which then localizes to the mitochondria to promote cytochrome c release, resulting in activation of caspase 9 (Lee, et al., 2005).

The involvement of ERK in DNA-damage induced apoptotic signaling is a relatively new finding. ERK signaling in cells is often associated as being cytoprotective (Pognonec, 2010, Rasola, et al., 2010, Stadheim, et al., 2001). However, recent investigation into the role of ERK during DNA damage revealed its potential to induce cytotoxicity. Intriguingly, the ability of ERK to promote apoptotic signaling coincides with JNK in several aspects. ERK is able to induce transcription of Bax, Bak and PUMA, all of which are proapoptotic. ERK also alters mitochondrial function, decreasing mitochondrial membrane potential and facilitating cytochrome c release. Also similar to JNK is the observation that the majority of studies focused on ERK-mediated cell death are associated with prolonged ERK activation, ranging from 6-72 hours (Cagnol, et al., 2006, Cagnol and Chambard, 2010, Tentner, et al., 2012).

1.4.3 Sensitivity of arrested cells to death receptor ligands

As mentioned previously, TVX is able to decrease cell proliferation *in vitro*. This is a common occurrence displayed by drugs with idiosyncratic liability. Other drugs that cause IDILI demonstrate the ability to alter proliferation *in vitro* including ranitidine, doxorubicin, diclofenac, sulindac, halothane, etoposide, chlorpromazine, nimesulide, troglitazone, valproic acid, and ketoconazole to name a few (Ban, et al., 2011, Basta-Kaim, et al., 2006, Chennamaneni, et al., 2012, Francavilla, et al., 1989, Hamaguchi, et

al., 2010, Jakubowska-Mucka, et al., 2012, Jensen, et al., 1999, Lee, et al., 2007, Parada-Turska, et al., 2006, Rajabalian, et al., 2009, Robles, et al., 1999, Sidana, et al., 2012, Supino, et al., 1977, Waxler, et al., 1994). This suggests that these xenobiotics which cause IDILI might be initiating genotoxic stress in cells.

Although DNA damage and cell cycle arrest do not always result in cell death, they can sensitize cells to the cytotoxic effects of members of the TNF superfamily, such as TNF, FasL and TNF-related apoptosis inducing ligand (TRAIL), all of which have similar receptor coupling that promotes apoptosis (Ehrhardt, et al., 2013, Gera, et al., 1993, Jiao, et al., 2013, Jin, et al., 2002, Kim, et al., 2009, Rodriguez, et al., 2007, Shih and Stutman, 1996). In these studies, induction of cell cycle arrest in dividing cells resulted in sensitizing the cells to apoptosis induced by the different death receptor ligands. Therefore, activation of the DNA damage response pathway by TVX, and perhaps other drugs with idiosyncratic liability, might be an underlying mechanism that sensitizes hepatocytes to TNF-induced apoptosis. This further implicates the involvement of the DNA damage response in cellular signaling events that might interact with TNF-mediated signaling to promote cell death.

1.5 Hypothesis and specific aims

The overall hypothesis of this thesis is that TVX interacts with TNF to promote hepatocellular cytotoxicity that is dependent on the interactive signaling pathways of NF-κB, JNK and caspase enzymes, as well as the DNA damage response. These factors were chosen based on the propensity of TNF to induce hepatocellular apoptosis involving alterations in NF-κB and JNK pathways that promote caspase activation. Investigation of DNA damage and its complex response network is based on the observation that drugs with idiosyncratic liability are able to promote characteristics of the DNA damage response in cells, such as cell cycle arrest, and also that TVX might interfere with eukaryotic topoisomerase enzymes, which can promote DNA damage. These signaling components will be investigated at the level of the hepatocyte, with a focus on a human cell line, and are represented in the following aims:

Aim 1 Hypothesis: TVX interacts with TNF to cause apoptotic cell death in HepG2 human hepatoblastoma cells and isolated primary murine hepatocytes in a concentration and time dependent manner (Chapter 2).

Aim 2 Hypothesis: The cytotoxic interaction of TVX and TNF involves changes in NF-κB and JNK signaling, as well as activation of caspase enzymes (Chapter 2 and 4).

Aim 3 Hypothesis: TVX activates the DNA damage response and causes cell cycle arrest, as well as induces the expression of p53 and p21 (Chapter 3).

Aim 4 Hypothesis: TVX activates ERK and the PIKK family kinases, which contribute to cell death in response to TVX/TNF (Chapter 3).

1.6 Overview and significance of dissertation

The studies in this dissertation provide mechanistic insight into the etiology of IDILI. Although incidents of IDILI are rare, the repercussions of even a few of these adverse events are widespread. These adverse events can result in restriction or removal of otherwise highly effective pharmaceuticals, liver failure, death, and litigations. As the underlying mechanisms responsible for IDILI remain unknown, these problems continue to persist. Identification of the critical signaling pathways involved in the cytotoxic events of the hepatocyte, in response to drugs with idiosyncratic liability in the presence of TNF, will facilitate the development of more reliable screening protocols capable of consistently identifying drug candidates with the potential to cause IDILI.

CHAPTER 2

Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor-alpha Interaction. Toxicol. Sci. (2013). Beggs, K.M., Fullerton, A.M., Miyakawa, K., Ganey, P.E., Roth, R.A.

2.1 Abstract

Idiosyncratic, drug-induced liver injury (IDILI) continues to be a significant human health problem. IDILI is characterized as occurring in a minority of individuals exposed to a drug, yet it accounts for upwards of 17% of all cases of acute liver failure. Despite these concerns, the mechanisms underlying IDILI remain unknown. Trovafloxacin (TVX), which causes IDILI in humans, also causes hepatocellular death *in vitro* when combined with tumor necrosis factor alpha (TNF) treatment. However, the molecular mechanisms involved in this toxicity are not fully characterized. The purpose of this study was to identify mechanisms by which TVX and TNF interact to cause hepatocellular death, with a focus on a human hepatocyte cell line. TVX and TNF interacted to cause cytotoxicity in HepG2 cells at drug concentrations similar to those in people undergoing TVX therapy. TVX/TNF treatment caused apoptosis and DNA damage in HepG2 cells that depended on caspase activation. Prolonged activation of c-Jun N-terminal kinase (JNK) occurred in TVX/TNF-induced cytotoxicity, and treatment with the JNK selective inhibitor SP600125 attenuated cytotoxicity. TVX/TNF cotreatment also caused cytotoxicity in isolated primary murine hepatocytes that was dependent on caspase activation. These results increase understanding of molecular signaling pathways involved in hepatocellular death caused by a drug with idiosyncratic liability in the presence of TNF.

2.2 Introduction

Drug-induced liver injury is the leading cause of acute liver failure in the United States (Ostapowicz, et al., 2002) as well as a common adverse effect preventing regulatory approval for new drugs. In addition, it is the primary cause of post-marketing actions resulting in warnings or withdrawal of drugs from the marketplace (Watkins, 2005). Idiosyncratic, drug-induced liver injury (IDILI) usually occurs in a small minority of individuals exposed to a drug, yet these reactions account for 13-17% of all acute liver failure cases (Hussaini and Farrington, 2007). Although current preclinical and clinical safety testing prevents many hepatotoxic drug candidates from entering the market, the ability to detect drugs capable of causing IDILI is still inadequate (Watkins, 2005).

Despite the prevalence of IDILI and the problems that it causes, mechanisms underlying the hepatotoxicity remain unknown. One hypothesis is that a modest inflammatory stress can render the liver sensitive to an otherwise nontoxic dose of a drug (Ganey, et al., 2004). It has been demonstrated that administration of lipopolysaccharide (LPS) to generate a modest inflammatory stress precipitates liver injury in animals co-exposed to drugs with IDILI liability. This phenomenon has been demonstrated in animal models using chlorpromazine, diclofenac, halothane, doxorubicin, amiodarone, trovafloxacin (TVX), and sulindac (Buchweitz, et al., 2002, Deng, et al., 2006, Dugan, et al., 2010, Hassan, et al., 2008, Lu, et al., 2012, Shaw, et al., 2007, Zou, et al., 2009b).

TVX is a fluoroquinolone antibiotic. Between February of 1998 and May of 1999, approximately 2.5 million prescriptions were written for TVX, and 140 adverse hepatic

events were reported (Dembry, et al., 1999), an incidence rate of 5.6 per 100,000 prescriptions written. These included 14 cases of acute liver failure, six of which were fatal (Nightingale, 1999). TVX is not hepatotoxic in mice but becomes so when combined with a modest inflammatory stress from LPS (Shaw, et al., 2007). Histological examination of TVX/LPS-induced liver injury identified both apoptotic as well as oncotic necrosis, and the pathogenesis of the TVX-LPS-induced liver injury in mice depended on the proinflammatory cytokine tumor necrosis factor-α (TNF). Interference with TNF activity by treatment with etanercept or genetic knockout of TNF receptors (TNFRs) protected mice from TVX-LPS-induced liver injury, signifying the importance of TNF signaling in the generation of hepatotoxicity (Shaw, et al., 2007, Shaw, et al., 2009e). Though TNF is able to induce apoptosis in many cell types, hepatocytes are largely resistant to its killing effects in the absence of a sensitizing event or factor (Leist, et al., 1994). Ligation of TNFR 1 on hepatocytes can activate several apoptotic factors, including caspase enzymes and c-Jun N-terminal kinase (Ding and Yin, 2004).

Exposure of cultured hepatocytes of human, rat, and murine lineage to cytokines in the presence of TVX and other drugs with IDILI potential caused cytotoxicity (Cosgrove, et al., 2009, Shaw, et al., 2009a). However, the intracellular signaling events in these models have not been elucidated fully. Accordingly, the aim of this study was to identify key signaling events in TVX-TNF-induced cytotoxicity. The hepatoblastoma HepG2 cell line was chosen for these studies because this cell is currently used in preclinical testing and is of human lineage. TVX also causes a number of similar changes in gene expression in both HepG2 cells and primary human

hepatocytes (Liguori, et al., 2008). Identification of critical signaling events could lead to development of more effective preclinical screening protocols to identify drugs with idiosyncratic liability.

2.3 Materials and Methods

2.3.1 Materials

Unless otherwise noted, all materials were purchased from Sigma-Aldrich (St. Louis, MO). TVX was synthesized by Cayman Chemical (Ann Arbor, MI). Recombinant truncated form of murine TNF, recombinant human TNF, z-VAD-FMK, z-IETD-FMK, z-LEHD-FMK, z-DEVD-FMK, and caspase fluorometric assay kits were purchased from R&D Systems (Minneapolis, MN). Antibiotic-antimycotic (ABAM), 0.25% trypsin-EDTA, phosphate-buffered saline (PBS), L-glutamine, Dulbecco's Modified Eagles Medium (DMEM) and Williams' Medium E were purchased from Life Technologies (Carlsbad, CA). SP-600125 and lactate dehydrogenase were purchased from Calbiochem (San Diego, CA). Cell Staining Buffer, Annexin V Binding Buffer, Alexa Fluor 647 conjugated Annexin V (AnnV), and propidium iodide (PI) were all purchased from Biolegend (San Diego, CA). Alanine Aminotransferase (ALT) reagent was purchased from Thermo Scientific (Pittsburgh, PA).

2.3.2 Animals

Mice received humane care, and the Michigan State University Committee on Animal Use and Care approved all procedures. Nine-week-old, male C57Bl/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Prior to use, mice were allowed 1 week to acclimate in a 12-hour light/dark cycle. They had continual access to bottled spring water and were fed a standard chow (8640 Teklad 22/5 Rodent Diet, Harlan Laboratories, Madison, WI) *ad libitum*.

2.3.3 Cell Culture

HepG2 human hepatoblastoma cells (American Type Culture Collection,

Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% ABAM in 25-cm^2 tissue culture flasks. Primary murine hepatocytes were isolated as described by Klaunig et al. and modified by Bajt et al. (Bajt, et al., 2004, Klaunig, et al., 1981). Briefly, hepatocytes were isolated using a two-step collagenase perfusion method. Isolated hepatocytes with a viability of 85% or greater, as determined by trypan blue exclusion, were plated and supplemented with Williams' medium E containing 10% FBS, 1% ABAM, 2 mM L-glutamine, and 100 nM insulin and allowed 3 hours to adhere before treatment. Both cell types were cultured at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2. Confluent HepG2 cell cultures were detached from the flask with 0.25% trypsin-EDTA and allowed to adhere for at least 7 hours before treatment.

2.3.4 HepG2 Cytotoxicity Assessment

HepG2 cells were plated at 4 x 10^4 cells per well in white-walled, 96-well tissue culture plates. For treatment, TVX and levofloxacin (LVX) were reconstituted to a stock solution of 200 mM and 500 mM respectively in dimethyl sulfoxide (DMSO) which resulted in a maximal final concentration of 0.01% DMSO in treated wells. Vehicle controls for TVX or LVX are represented as “Veh” throughout. LVX was used as a negative comparator drug for TVX in this model because it is in the same pharmacological class as TVX but has far less propensity to cause IDILI in humans or liver injury in mice cotreated with LPS (Shaw, et al., 2007). TNF was reconstituted to a stock solution of 100 μg/mL in PBS. Cytotoxicity was measured using the CytoTox-Glo Cytotoxicity Assay from Promega (Madison, WI). After initial concentration-response

studies, a combination treatment of 20 μM TVX and 4 ng/mL TNF was chosen for subsequent experiments. For the caspase inhibitor studies, the peptide-based caspase inhibitors were reconstituted in DMSO, and cells were exposed to 40 μM of each inhibitor or 0.2% DMSO Vehicle. For studies using the JNK inhibitor SP600125, cytotoxicity was assessed by trypan blue exclusion. This was done because SP600125 interfered with the CytoTox-Glo cytotoxicity assay.

2.3.5 Cytotoxicity Timecourse Study

LDH activity was measured as described (Vanderlinde, 1985). Briefly, HepG2 cells were plated at 2.5 x 10^5 cells per well in 12-well tissue culture plates. Cells were treated, and 40 μL of culture medium was collected every 4 hours for 24 hours starting at the time of treatment and kept at 4°C until LDH activity was measured. LDH enzyme (Calbiochem) was used to create a standard curve of enzyme activity, and LDH activity was measured in the collected supernatants at each time spectrophotometrically.

2.3.6 Flow Cytometry

Cells were plated at 5 x 10^5 cells per well in 12 well tissue culture plates. After 24 hours of exposure, cells and supernatant were collected in 12mm x 75mm round bottomed tubes (BD Biosciences, San Jose, CA) on ice. Cells were pelleted by centrifugation at 4°C for 5 minutes at 70 x g. Culture medium was aspirated before cells were washed with cold Cell Staining Buffer. After the cells were pelleted again and Cell Staining Buffer was removed, they were resuspended in 200 μL Annexin V Binding Buffer with 10 μL each of AnnV and PI. They were incubated for 15 minutes at

room temperature before an additional 300 μL Annexin V Binding Buffer was added to each sample. Cells were then analyzed using a BD FACS Canto II flow cytometer. All data were analyzed using Kaluza software (Beckman Coulter, Brea, CA). Gating parameters were determined using Veh/PBS-treated cells as a negative control. Unstained cells were run to account for auto fluorescence.

2.3.7 TUNEL Assay

TUNEL labeling was conducted using the *In Situ* Cell Death Detection Kit from Roche Diagnostics Corporation (Indianapolis, IN). Cells were plated in 8-chamber culture slides (BD Falcon) at 9 x 10^4 cells per chamber. After 24 hours of treatment, the cells were processed following the manufacturer's instructions. An anti-fade mounting medium containing DAPI was applied (Vector Laboratories, Burlingame, CA). Slides were imaged using an Olympus IX71 inverted fluorescence microscope and appropriate filters. Images were taken with an Olympus F-View II digital monochrome camera and were processed using Image J software.

2.3.8 Caspase Activity Assay

Caspase activity was determined using fluorometric caspase activity assay kits (R&D Systems). Briefly, cells were plated at 1.2 x 10^6 cells per well in 6 well tissue culture plates. Cells were treated for 8, 16, or 24 hours before collection. They were lysed, and lysates were centrifuged. 50 μL of supernatant from each sample was added to black walled 96-well plates, along with reaction buffer and fluorogenic substrate and incubated at 37°C for 1 hour. After incubation, the plate was read on a fluorescent microplate reader with filters for excitation at 400 nm and emission at 505

nm.

2.3.9 Protein Isolation

HepG2 cells were plated at 1.2 x 10^6 cells per well in 6 well tissue culture plates. They were treated for 1 hour or 8 hours before being washed with ice-cold PBS and treated with radio-immunoprecipitation assay (RIPA) buffer containing HALT protease and phosphatase inhibitors (Thermo Scientific, Pittsburgh, PA). Cells were scraped, collected in tubes and kept on ice. After incubating in RIPA buffer for 10 minutes, each sample was sonicated with one 5-second pulse. Lysates were centrifuged at 20,000 x g for 20 minutes, and supernatants containing whole cell extract were collected. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo Scientific).

2.3.10 Western Blot Analysis

For phospho-JNK (p-JNK) and lamin B1 (Lamin) detection, 15 μg of protein was loaded and separated on NuPAGE 12% Bis-Tris gels (Life Technologies) by electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), which were then blocked for 1 hour with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST). Membranes were then probed with p-JNK or Lamin primary antibodies (Cell Signaling Technology, Beverly, MA). Antibodies were diluted in 5% BSA in TBST to 1:1000 for p-JNK and 1:10,000 for Lamin. Membranes were incubated with primary antibodies at 4°C for at least 18 hours. PVDF Membranes were then washed with TBST and probed with goat anti-rabbit HRP-conjugated secondary antibody (Sana Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were diluted in 5% BSA in TBST to 1:2500 for p-JNK

and 1:10,000 for Lamin. HRP was visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and developed on HyBlot CL Film (Denville Scientific, Metuchen, NJ). Densitometry was performed on the developed films using Image J software.

2.3.11 Primary Murine Hepatocyte Studies

After isolation, hepatocytes were plated at 1.25×10^5 cells per well in 24-well, collagen-coated tissue culture plates. After attachment, the cells were washed twice with warm PBS and treated with serum-free Williams' Medium E containing 2 mM glutamine and 1% ABAM. At 24 hours, medium and cell lysates were collected and analyzed for ALT activity as described previously (Luyendyk, et al., 2005).

2.3.12 Statistical Analysis

Results are expressed as mean $\pm$ S.E.M. Percentile data were subjected to arcsine transformation. Analysis of data was performed using one-way or two-way analysis of variance (ANOVA) followed by pairwise multiple comparisons using the Holm Sidak or Tukey's method where appropriate. Nonparametric data were analyzed using Kruskal-Wallis test followed by pairwise multiple comparisons using Tukey's or Dunn's method where appropriate. The criterion for statistical significance was $p<0.05$.

2.4 Results

2.4.1 Concentration response and time course of TVX/TNF-induced cytotoxicity

HepG2 cells were treated with TVX (1.25-20 μM), TNF (0.015-16 ng/mL), or their vehicles simultaneously for 24 hours before cytotoxicity was measured. TVX alone (0 ng/mL TNF) did not cause significant cytotoxicity at any concentration. Similarly, TNF alone (0 μM TVX) was not cytotoxic. In TVX/TNF-treated cells, synergistic cytotoxicity was observed (Figure 9). Cytotoxic interactions occurred with concentrations as small as 5 μM TVX and 62.5 pg/mL TNF. LVX, another fluoroquinolone antibiotic with a much lower propensity for causing IDILI in humans, was used as a negative comparator drug for TVX in this model. Clinically, LVX is prescribed at a dose that is 2.5-fold greater than the dose of TVX to achieve a similar therapeutic effect (Lubasch, et al., 2000). Accordingly, HepG2 cells were treated with a larger concentration of LVX (50 μM). LVX treatment alone or in combination with TNF did not cause cytotoxicity in HepG2 cells (Figure 10).

The activity of LDH released into medium was not increased in HepG2 cells treated with Veh/PBS, TVX/PBS, or Veh/TNF at any time up to 24 hours (Figure 11). TVX/TNF cytotoxicity appeared to begin at 8 hours and became statistically significant by 20 hours.

2.4.2 Flow cytometric analysis of cell viability

To determine if the cytotoxicity induced by TVX/TNF cotreatment was oncotic or apoptotic, cells were stained with cell impermeable PI to identify compromised plasma membranes characterized by oncosis and with Annexin V that binds to exposed phosphatidylserine residues on the outer leaflet of the plasma membrane during

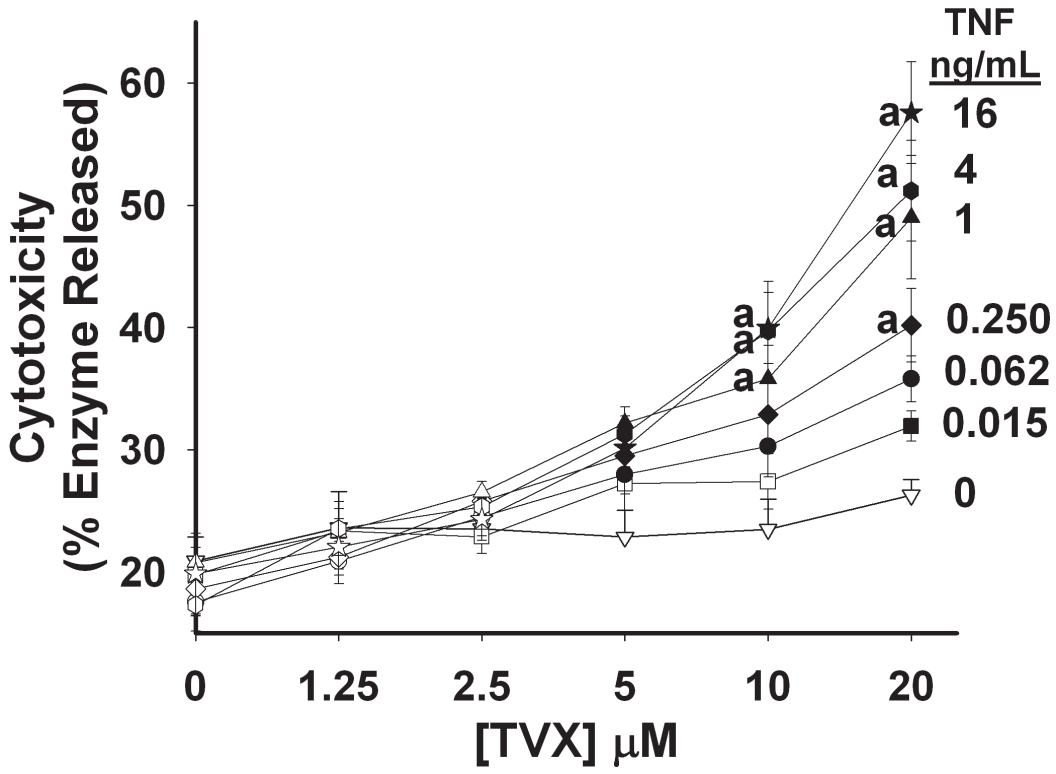


Figure 9. Concentration-response for TVX/TNF-induced cytotoxicity.

HepG2 cells were treated simultaneously with Veh or TVX (1.25-20 μM) and with PBS or TNF (0.015-16 ng/mL). Cytotoxicity was measured 24 hours after treatment. Data represent the mean ± SEM of 4 separate experiments performed in duplicate. Closed symbols are significantly different from 0 μM TVX at the same concentration of TNF. **a** significantly different from 0 ng/mL TNF at the same concentration of TVX

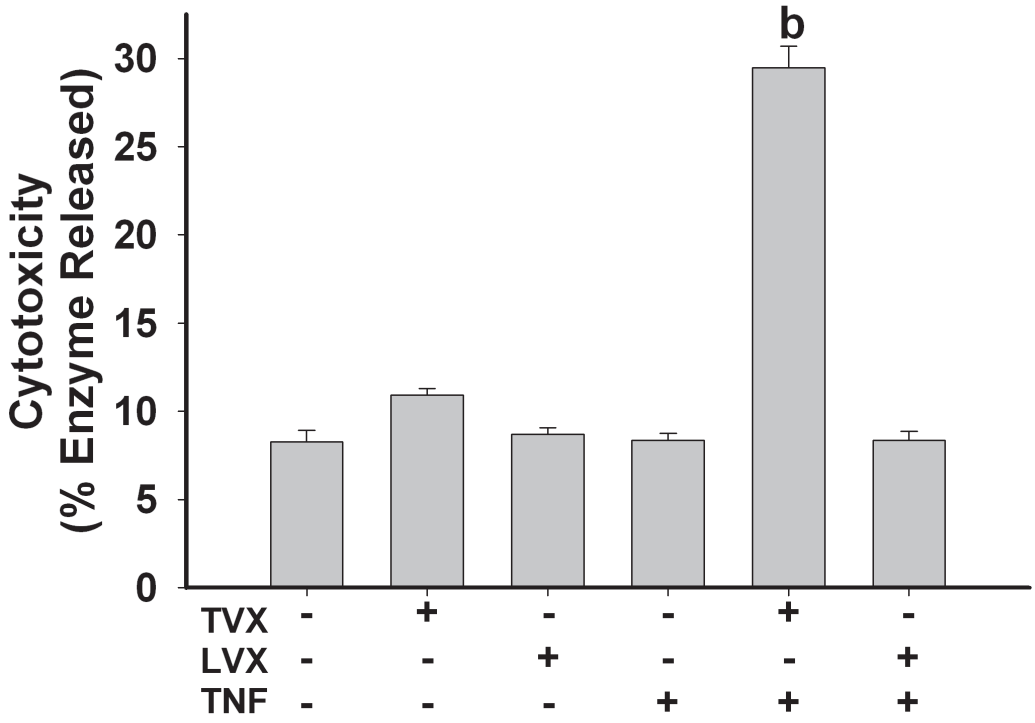


Figure 10. LVX does not interact with TNF to cause cytotoxicity. HepG2 cells were treated with Vehicle, 20μM TVX or 50μM LVX in the presence of 4 ng/mL TNF or PBS. Cytotoxicity was measured 24 hours after treatment. Data represent the mean ± SEM of 3 separate experiments performed in quadruplicate. **b** Significantly different from all other treatment groups.

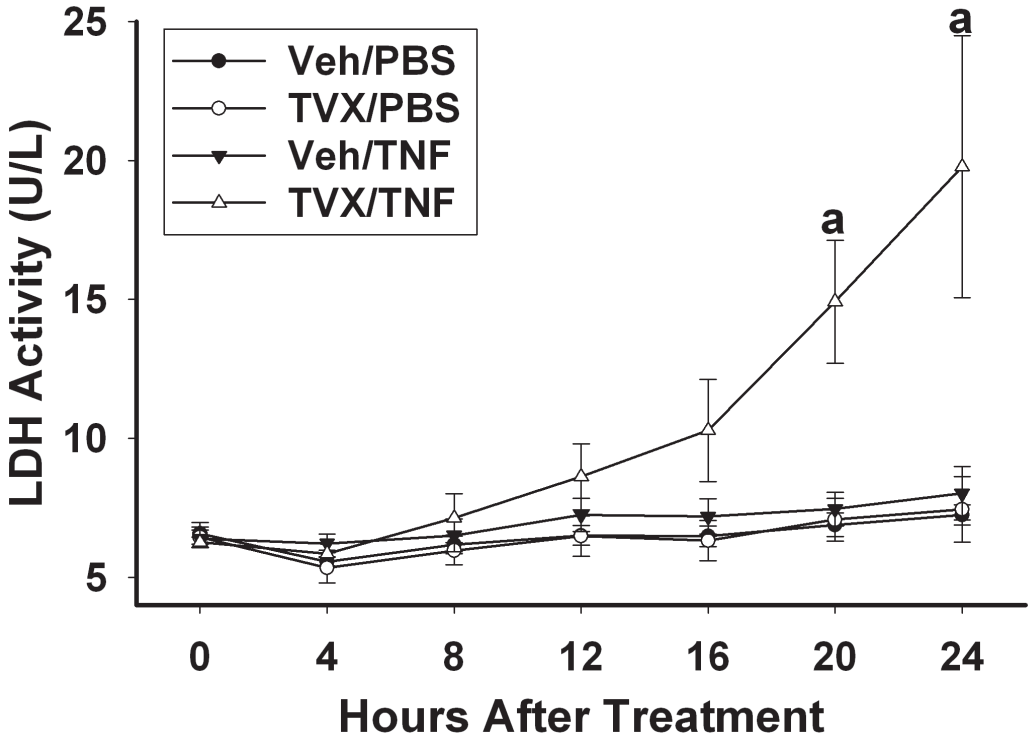


Figure 11. Time course of TVX/TNF-induced cell death.

HepG2 cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS. Cell culture supernatant was collected every 4 hours after treatment, and LDH activity was measured as described in Materials and Methods. **a** Significantly different from other treatment groups at same time point. Data represent the mean ± SEM of 3 separate experiments in performed triplicate.

apoptosis (Vermes, et al., 1995). Cells were analyzed using flow cytometry after 24 hours of exposure (Figure 12A). TVX/PBS and Veh/TNF treatments each caused a small increase in the percentage of early apoptotic (AnnV+/PI-) cells compared to Veh/PBS-treated group (Figure 12B). TVX/TNF treatment caused a much greater increase in the percentage of early apoptotic cells. Cotreatment with ZVAD resulted in a decrease in the percentage of early apoptotic cells treated with TVX/PBS or TVX/TNF. Treatment of cells with TVX/PBS resulted in an increase in the percentage of late apoptotic (AnnV+/PI+) cells (Figure 12C), and TVX/TNF treatment caused a more pronounced increase. Treatment with ZVAD decreased the percentage of late apoptotic cells in TVX/TNF-treated groups. Cytotoxicity, measured by enzyme release, was also increased in cells treated with TVX/TNF, an effect that was prevented in the presence of ZVAD (Figure 13). A small percentage (less than 2%) of cells appeared to have undergone oncotic necrosis (AnnV-/PI+) and this percentage was similar in all treatment groups (data not shown).

2.4.3 TUNEL Staining

DNA strand breakage was examined using the TUNEL assay. Compared to Veh/PBS-treated cells, treatment with TVX/PBS or Veh/TNF did not increase TUNEL staining (Figure 14A and 14B). TVX/TNF treatment increased TUNEL staining compared to all other treatment groups.

2.4.4 Caspase activity timecourse and inhibition

The activities of caspases 8, 9, and 3 were measured 8, 16, and 24 hours after treatment. Treatment with TVX/PBS or Veh/TNF did not cause significant changes in the activities of any of the caspases at any time examined (Figure 15). In cells treated

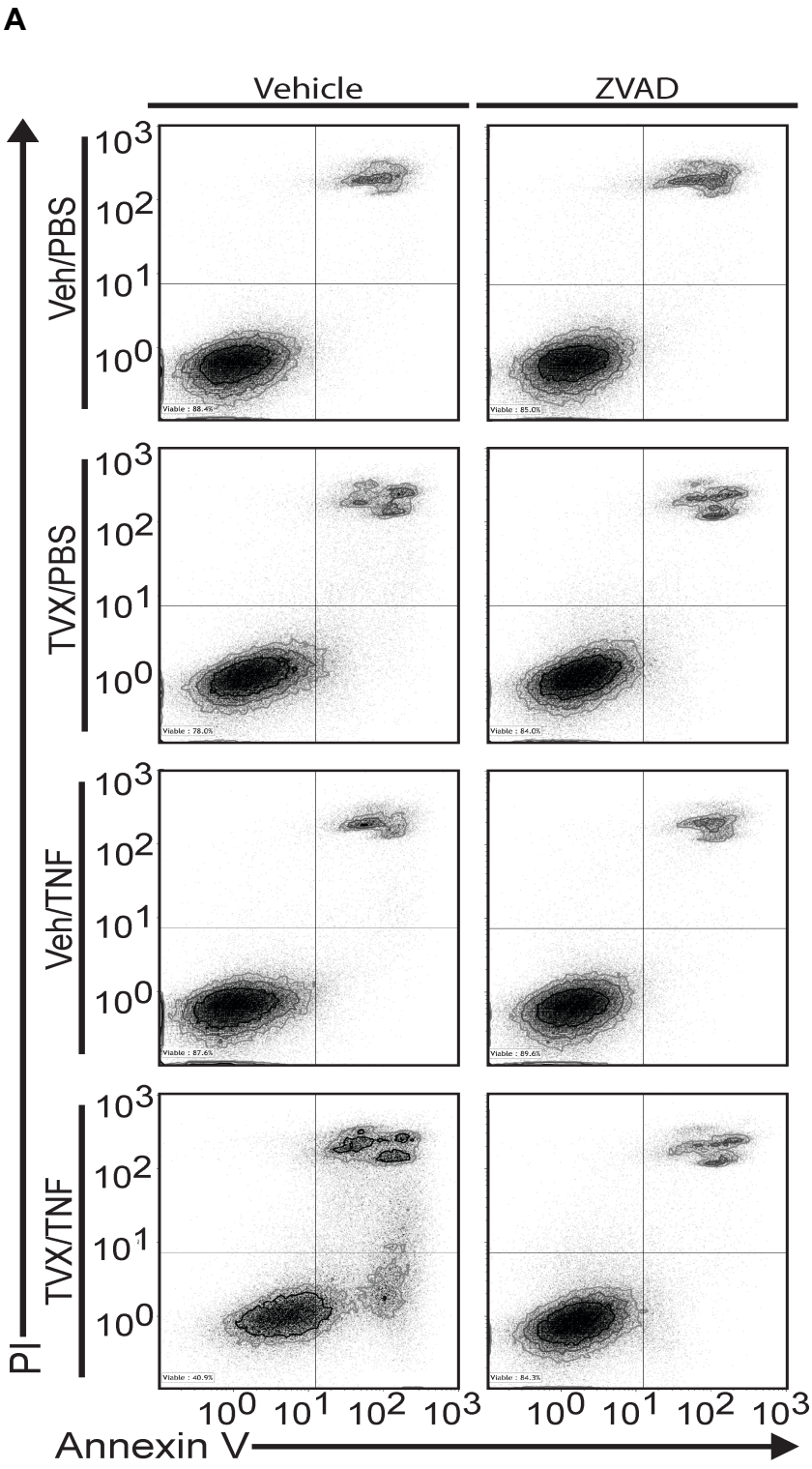
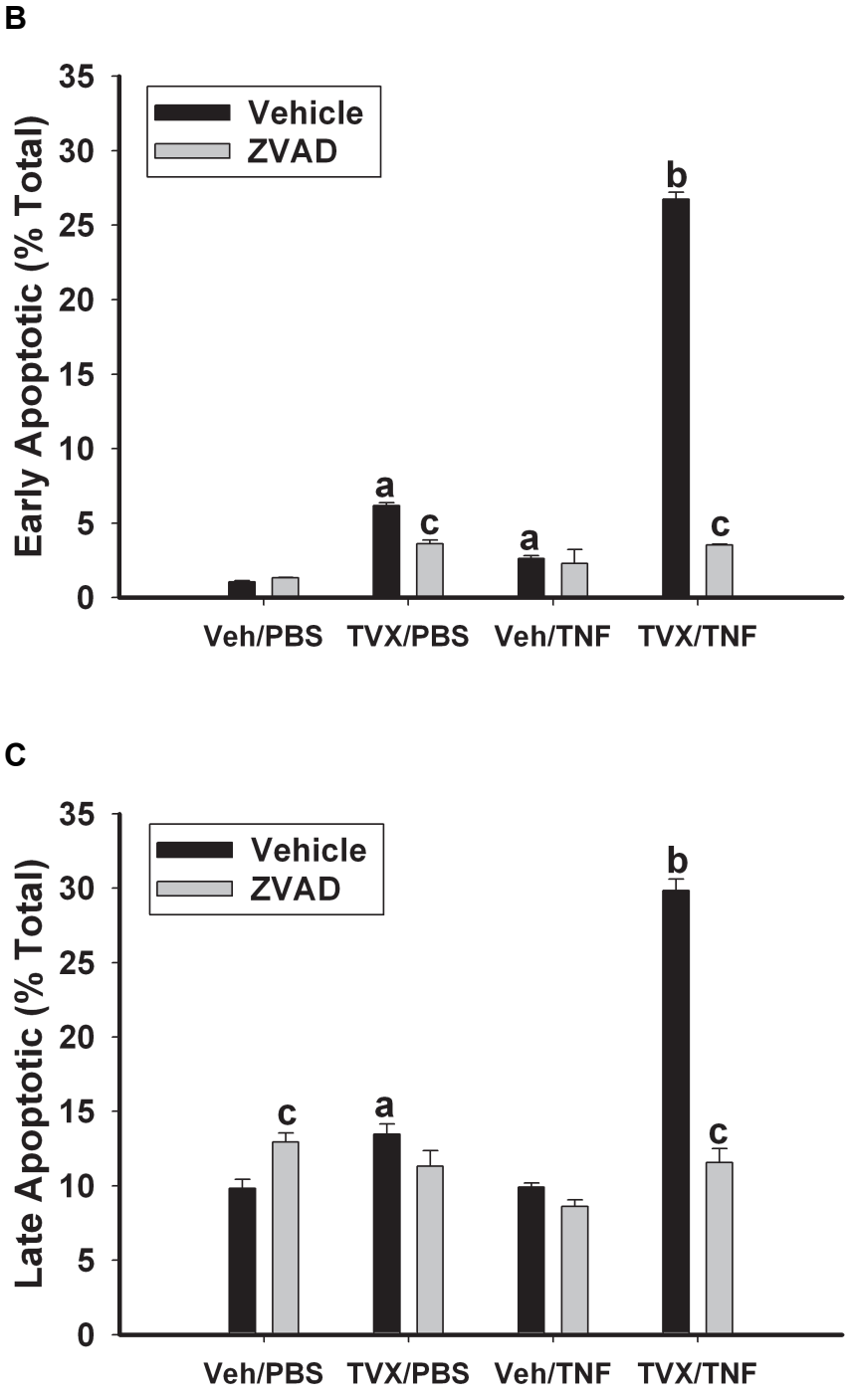


Figure 12. TVX/TNF treatment causes caspase-dependent apoptosis. HepG2 cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS, as well as 40 μM

Figure 12 (cont'd)



ZVAD or its vehicle control. After 24 hours, viability was assessed using flow cytometry. (A) Representative quadrant plots for each treatment. (B) Percentage of the total cells

Figure 12 (Cont'd)

gated undergoing early apoptosis (AnnV+, PI-). (C) Percentage of the total cells gated undergoing late apoptosis (AnnV+, PI+). **a** Significantly different from Veh/PBS-treated group without ZVAD. **b** Significantly different from Veh/PBS-, TVX/PBS-, and Veh/TNF-treated groups without ZVAD. **c** Significantly different from same treatment without ZVAD. Data represent the mean ± SEM of 3 separate experiments. 100,000 cells were sampled from each experiment. Oncotic necrosis (AnnV-, PI+) represents less than 2% of the total cells gated in any experiment.

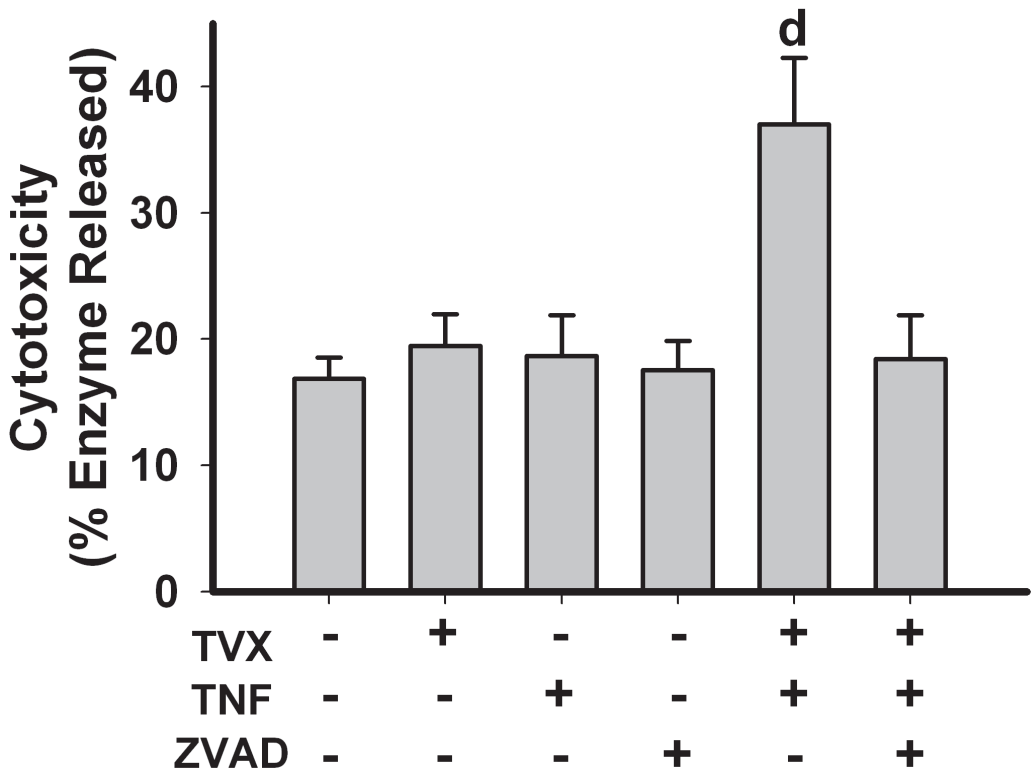


Figure 13. Cells were treated with TVX, TNF, or ZVAD alone or altogether. After 24 hours cytotoxicity was assessed by Cytotox-Glo membrane leakage assay. **d** Significantly different from all other treatment groups. Data represent the mean ± SEM of 4 separate experiments in quadruplicate.

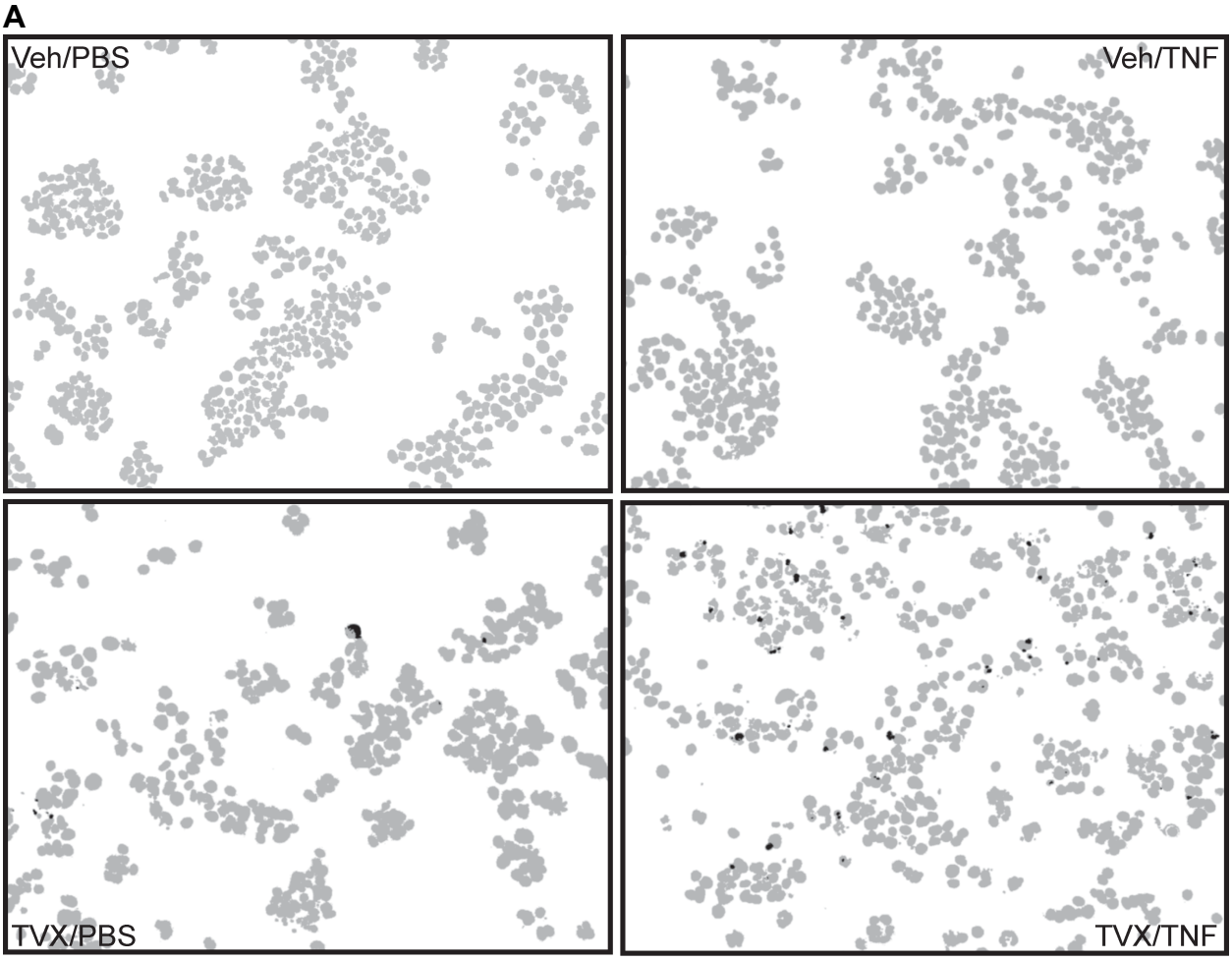
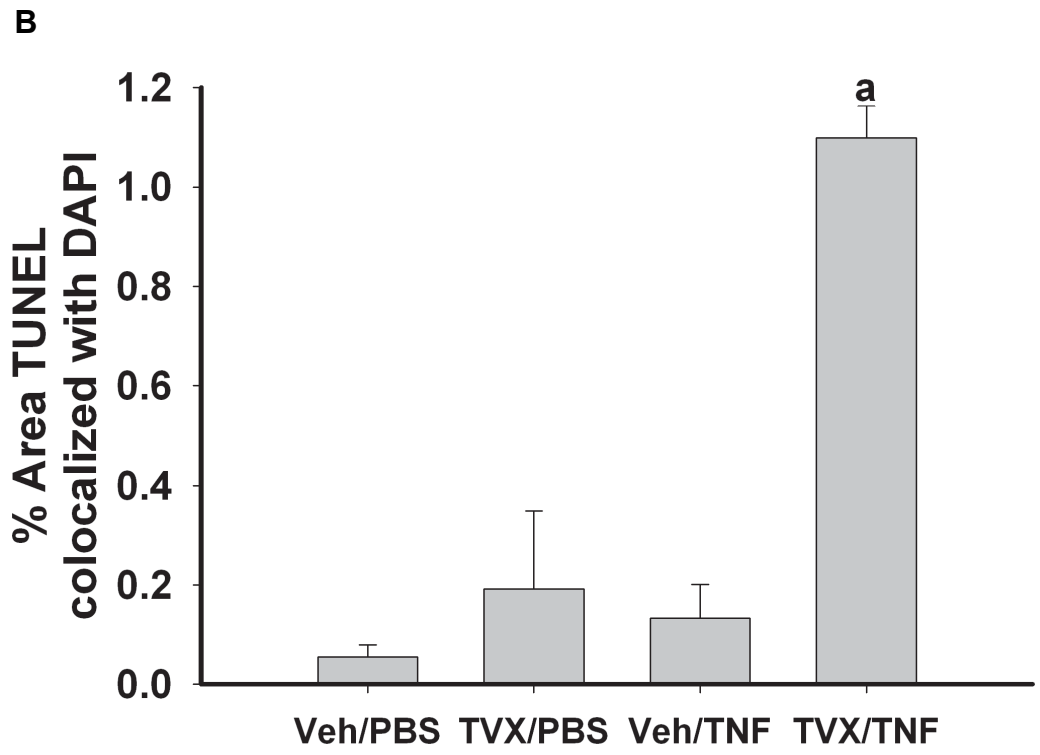


Figure 14. TVX/TNF-induced DNA damage.

HepG2 cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS. 24 hours after treatment cells were subjected to TUNEL labeling as described in Materials and Methods and imaged using fluorescence microscopy. (A) Representative images from each treatment. Gray areas represent DAPI signal. Black areas represent colocalized FITC and DAPI signal indicating TUNEL positive-signal located in nuclei.

Figure 14 (Cont'd)



(B) Quantification of the area of TUNEL signal colocalizing with DAPI in response to treatment. **a** Significantly different from all other treatment groups. Data represent the mean ± SEM of 3 separate experiments and 3-4 images from each treatment group.

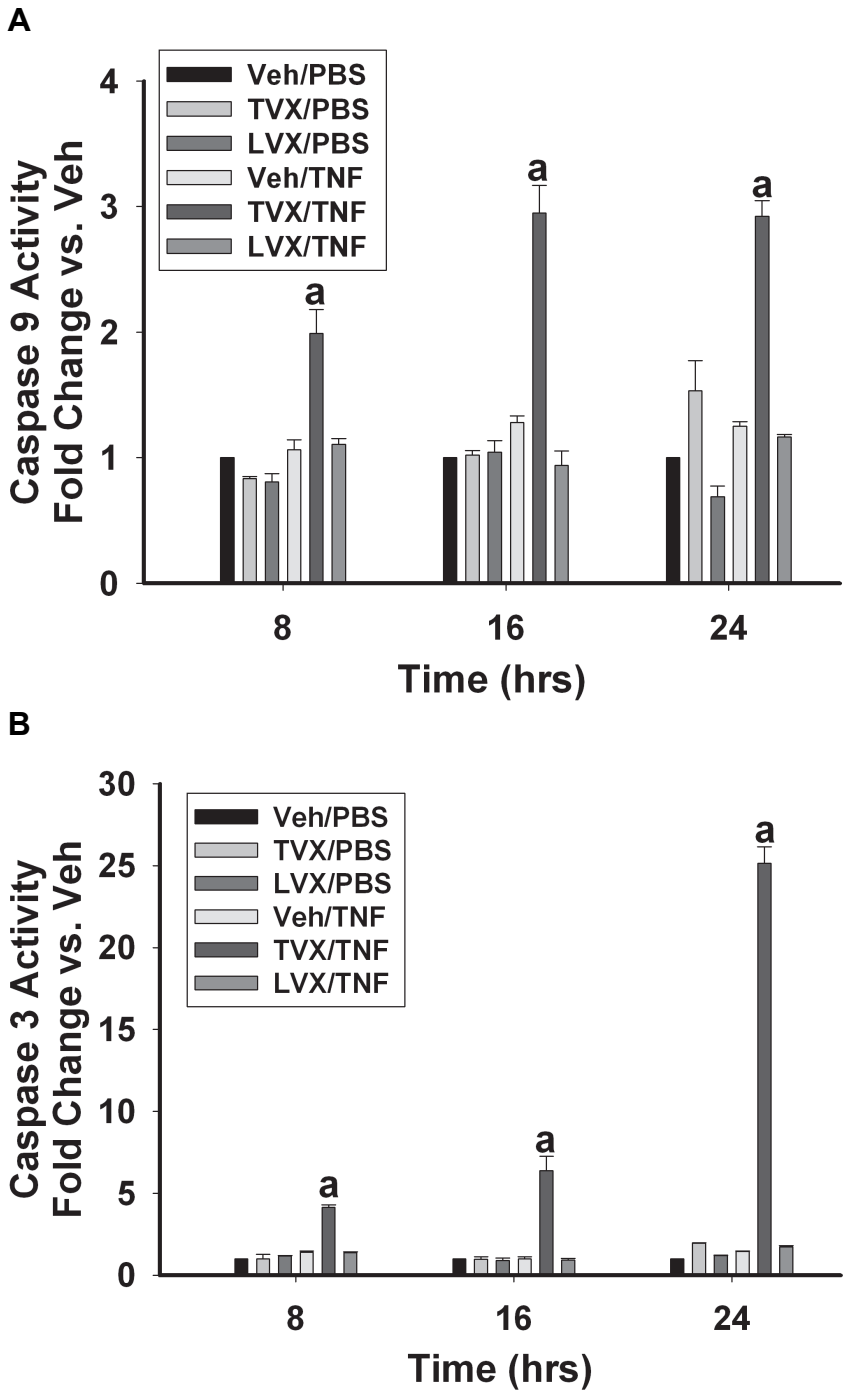
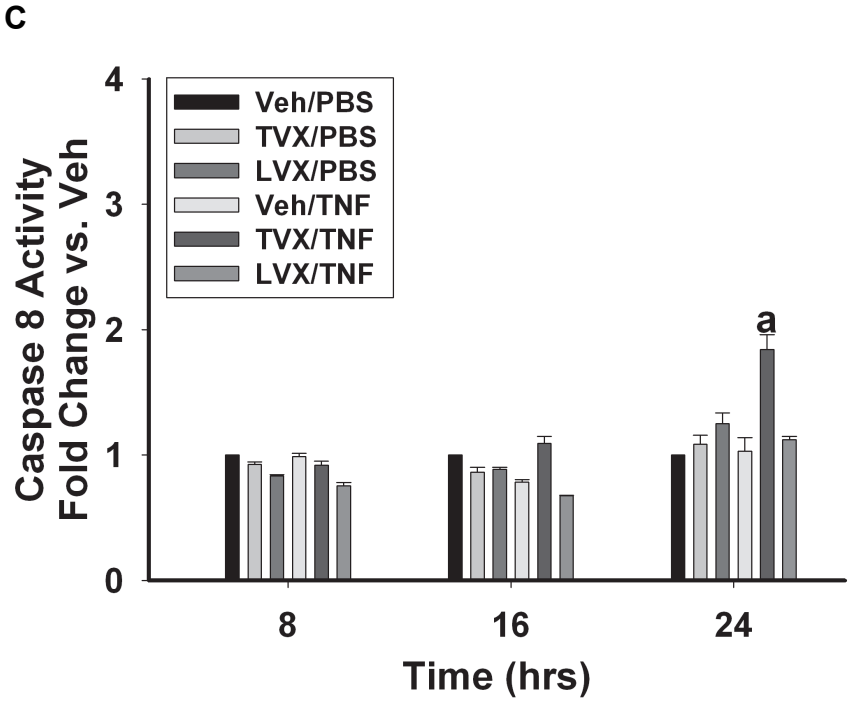


Figure 15. Timecourse of caspase activation. HepG2 cells were treated with 20 μM TVX, 50 μM LVX, or their vehicle controls in the presence of 4 ng/mL TNF or PBS. Activities of caspase 9 (A), caspase 3 (B), and caspase 8 (C) are represented relative to

Figure 15 (cont'd)



activities measured in Veh/PBS-treated groups. Caspase activities were measured 8, 16, and 24 hours after treatment as described in Materials and Methods. **a** Significantly different from all other treatment groups within the same time. Data represent the mean ± SEM of 3 separate experiments run in triplicate.

with TVX/TNF, the activity of caspase 9 was increased twofold 8 hours after treatment (Figure 15A) and threefold by 16 hours after treatment. Caspase 9 activity remained increased 24 hours after TVX/TNF treatment. Caspase 3 activity was increased fourfold compared to Veh/PBS-treated cells 8 hours after treatment with TVX/TNF and increased with time, reaching twenty-five fold after 24 hours (Figure 15B). TVX/TNF treatment caused a significant increase (1.8-fold) in activity of caspase 8 at 24 hours (Figure 15C). At no time did LVX/PBS or LVX/TNF increase activity of any of the caspases.

The effects of selective caspase inhibitors on TVX/TNF-induced cytotoxicity were evaluated (Figure 16). In all studies, TVX/TNF treatment significantly increased cytotoxicity. Treatment with the caspase inhibitors themselves did not cause any cytotoxicity. Treatment with either LEHD (caspase 9 inhibitor) or IETD (caspase 8 inhibitor) abolished TVX/TNF-induced cytotoxicity (Figure 16A and 16C), whereas DEVD (caspase 3/7 inhibitor) attenuated TVX/TNF-induced cytotoxicity (Figure 16B).

2.4.5 JNK activation and SP600125 treatment

TVX/PBS treatment did not affect JNK phosphorylation after 1 hour (Figure 17A). Treatment with Veh/TNF led to JNK activation after 1 hour. A greater increase in this early JNK activation was observed in TVX/TNF-treated cells. After 8 hours of treatment, Veh/PBS and Veh/TNF had similar levels of phosphorylated JNK (Figure 17B). Both TVX/PBS and TVX/TNF treatments were associated with increased JNK phosphorylation at this time. Total JNK expression was not affected by any treatment (data not shown).

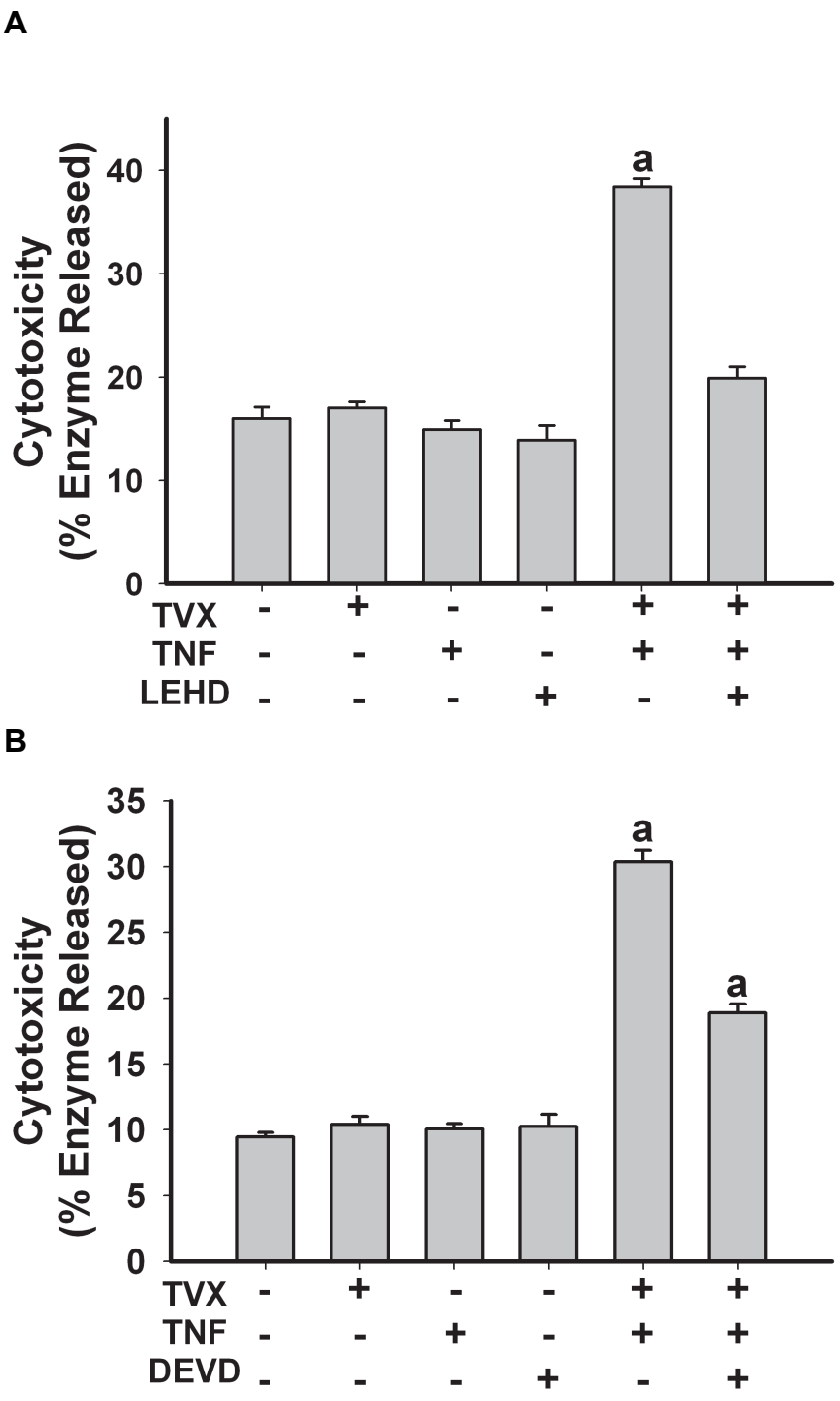
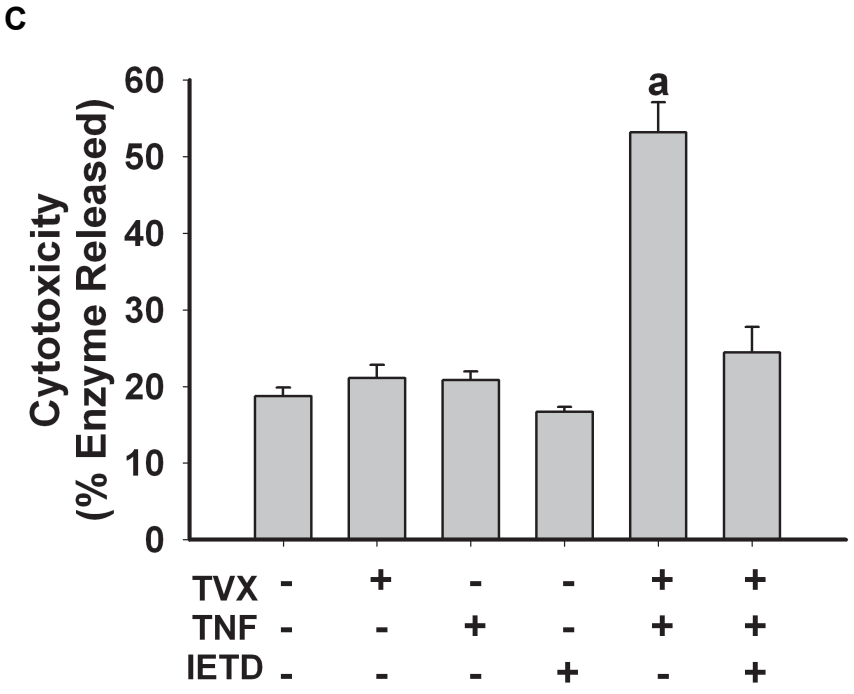


Figure 16. Caspase inhibition protects against TVX/TNF-induced cytotoxicity.

HepG2 cells were treated simultaneously with 20 μM TVX or vehicle and with 4 ng/mL

Figure 16 (cont'd)



TNF or PBS. At the same time some cells also received 40 μM of z-LEHD-fmk to inhibit caspase 9 (A), z-DEVD-fmk to inhibit caspase 3 (B), or z-IETD-fmk to inhibit caspase 8 (C). Cytotoxicity was measured 24 hours after treatment. **a** Significantly different from all other treatment groups. In panel B, bars labeled **a** are also different from one another. Data represent the mean ± SEM of 4-5 separate experiments.

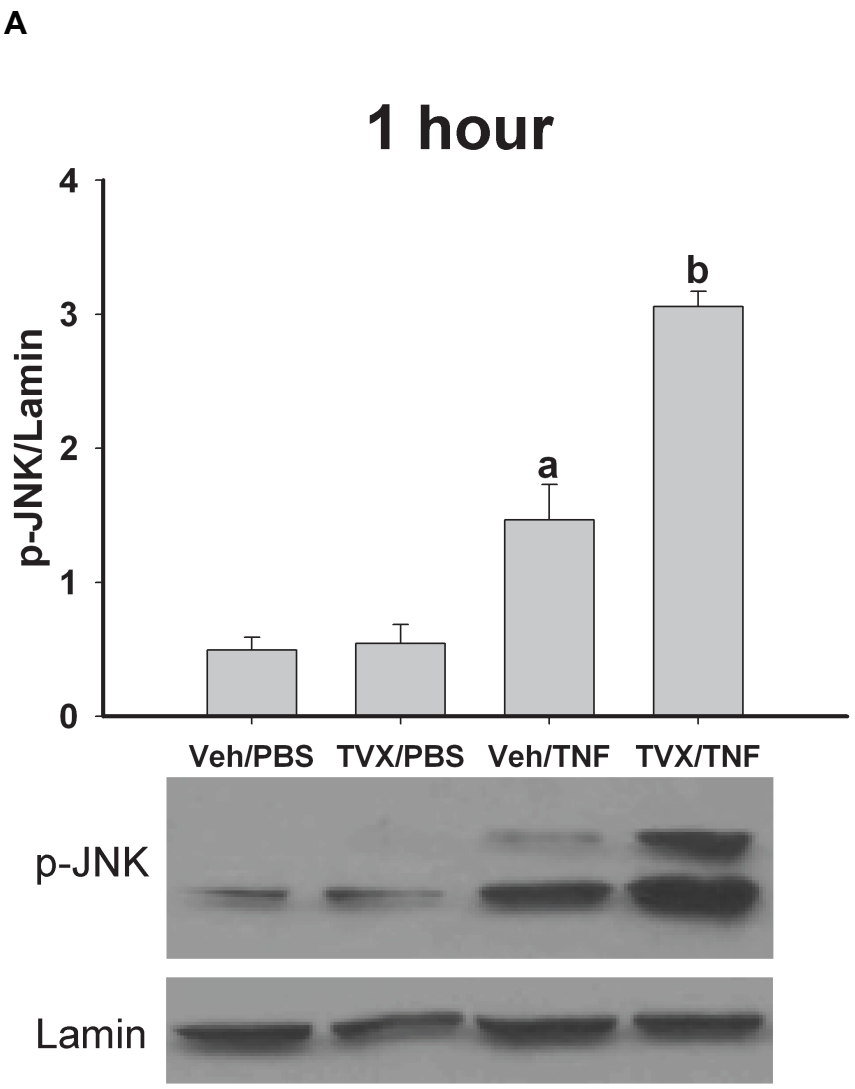
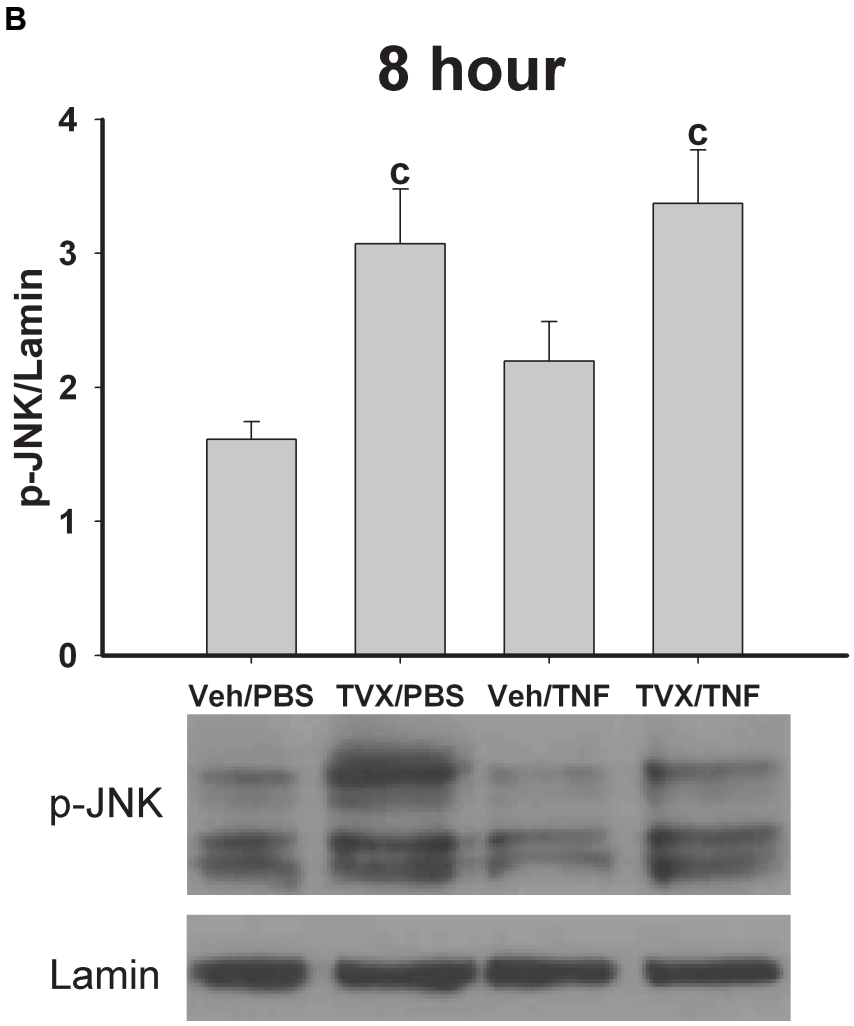


Figure 17. TVX treatment enhances JNK activation. HepG2 cells were treated simultaneously with 20 μM TVX or vehicle and with 4 ng/mL TNF or PBS for 1 or 8 hours. Phosphorylation of JNK was determined by western analysis. Representative blots are shown. Densitometry was performed on phospho-JNK (p-JNK) and Lamin bands, and the ratio of p-JNK to Lamin is represented. (A) p-JNK 1 hour after

Figure 17 (cont'd)



treatment. (B) p-JNK 8 hours after treatment. **a** Significantly different from Veh/PBS treated group. **b** Significantly different from all other treatment groups. **c** Significantly different from respective group in the absence of TVX. Data represent the mean ± SEM of 4 separate experiments.

HepG2 cells were treated with SP600125, a selective inhibitor of JNK (Bennett, et al., 2001), and cytotoxicity was assessed. Treatment with TVX, TNF or SP600125 alone did not increase cytotoxicity (Figure 18). Cytotoxicity was increased by treatment with TVX/TNF, and addition of SP600125 significantly reduced this effect.

2.4.6 Studies using isolated primary murine hepatocytes

Primary murine hepatocytes were treated simultaneously with TVX (0-20 μM) and/or TNF (10 ng/mL). ALT activity released into the medium was measured 24 hours after treatment. Neither TNF nor TVX alone at any concentration tested caused an increase in ALT release (Figure 19A). Treatment with 10 or 20 μM TVX in combination with TNF caused a significant increase in release of ALT compared to all other treatment groups. Treatment of cells with ZVAD decreased the TVX/TNF-induced ALT release. Treatment of primary murine hepatocytes with concentrations of LVX up to 50 μM caused no significant increase in release of ALT, regardless of the inclusion of TNF (Figure 19B).

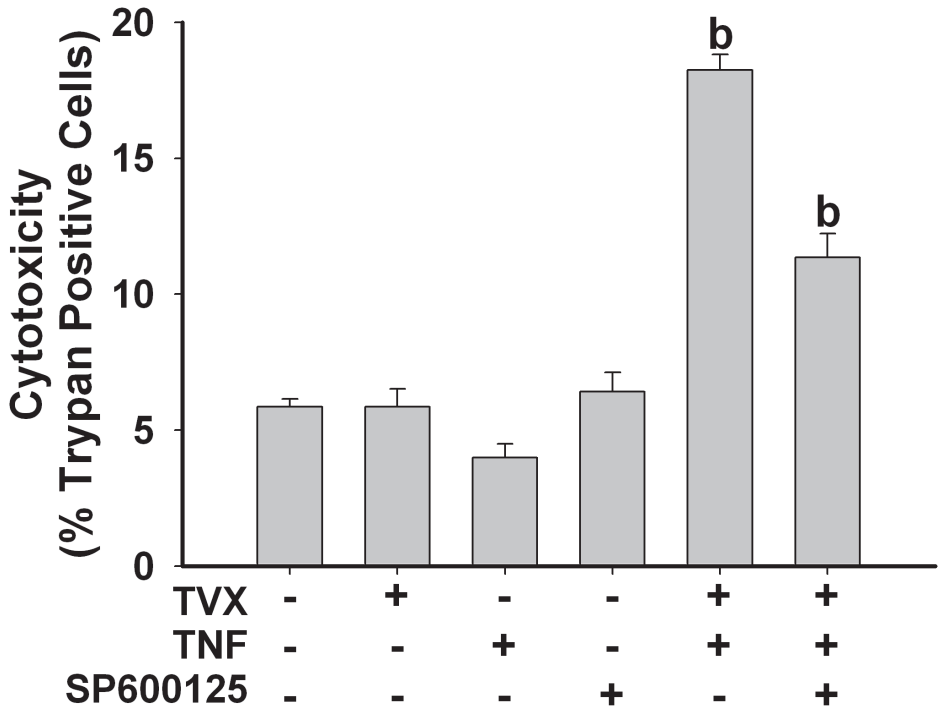


Figure 18. Inhibition of JNK attenuates TVX/TNF-induced cytotoxicity. Cells were also treated with 10 μM SP600125. Cytotoxicity was assessed 24 hours after treatment by trypan blue exclusion: a minimum of 300 cells was counted for each treatment group. **b** Significantly different from all other treatment groups. Groups labeled **b** are different from each other. Data represent the mean ± SEM of 4 separate experiments.

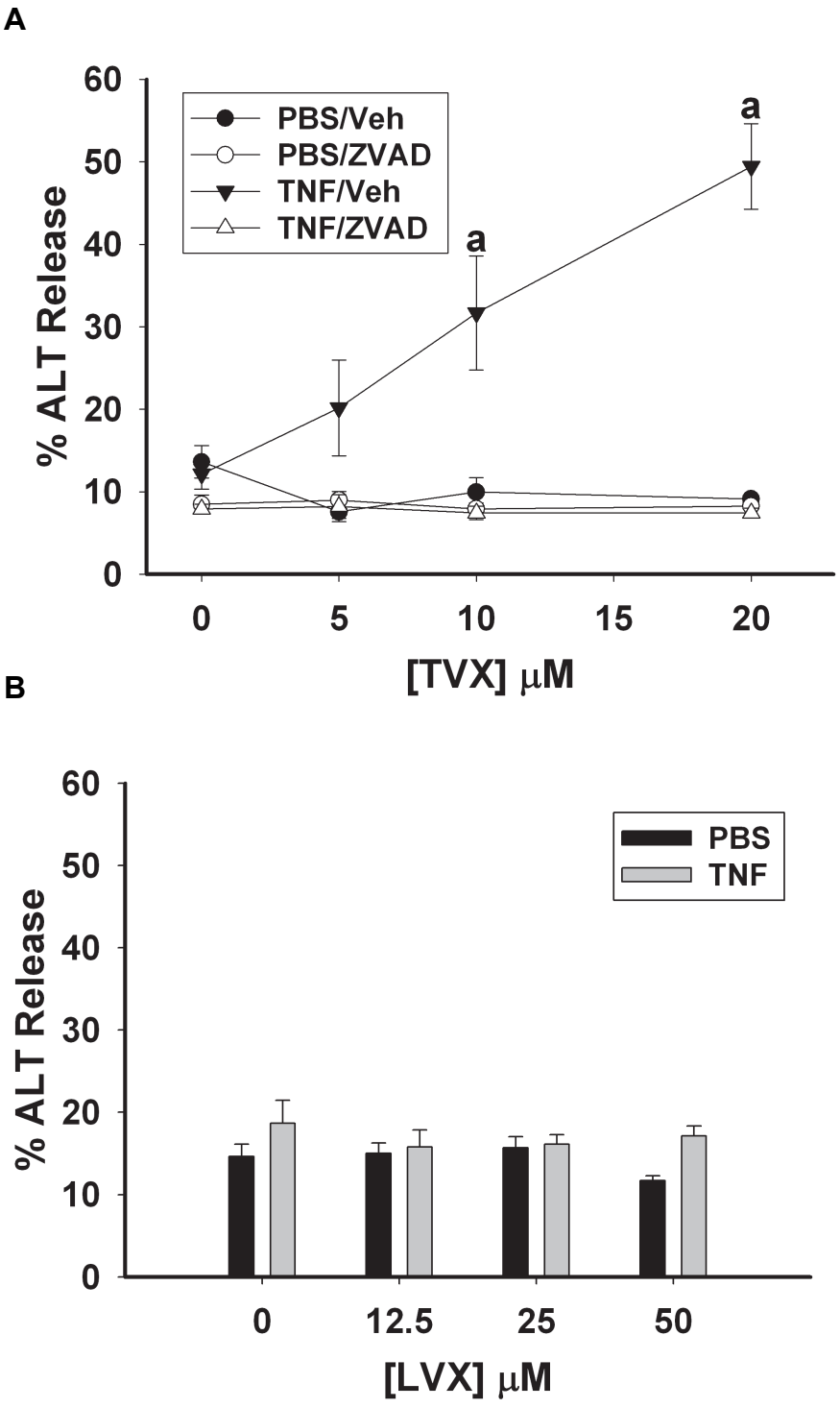


Figure 19. TVX/TNF-induced cytotoxicity of murine hepatocytes is caspase-dependent. (A) Primary murine hepatocytes were treated with Vehicle or TVX (5-20 μM) and with 10 ng/mL TNF or PBS, as well as 10 μM ZVAD or its vehicle. ALT release

Figure 19 (cont'd)

was measured 24 hours after treatment. (B) Cells were treated with LVX or Veh at various concentrations and with 10 ng/mL TNF or PBS. **a** Significantly different from all other treatment groups at same TVX concentration. Data represent the mean ± SEM of hepatocytes isolated from four mice run in duplicate.

2.5 Discussion

For the initial experiments, the range of TVX concentrations was based on the plasma concentrations of TVX observed in patients treated with the drug, which ranged from 2-10 μM (Melnik, et al., 1998, Teng, et al., 1996, Vincent, et al., 1997). These concentrations should reflect those to which the human liver might be exposed during TVX therapy. Similarly, the concentrations of TNF (0-16 ng/mL) were chosen to mimic what is experienced physiologically in humans during inflammatory stress (Copeland, et al., 2005, Taudorf, et al., 2007), as well as what was observed in the mouse model of TVX/LPS-induced liver injury (Shaw, et al., 2007). Treatment with either TVX or TNF alone at any of the concentrations tested did not cause cytotoxicity in HepG2 cells. Also, treatment with TVX at concentrations smaller than 5 μM in combination with TNF at any concentration did not cause statistically significant cytotoxicity (Figure 9). However, a combination of TVX at 5 or 10 μM interacted with TNF at concentrations as small as 62.5 pg/mL to cause cytotoxicity. Previous studies have demonstrated a cytotoxic interaction between TVX and TNF on HepG2 cells, but those studies used a combined treatment of 450 μM TVX and 100 ng/mL TNF (Cosgrove, et al., 2009). A treatment combination of 20 μM TVX and 4 ng/mL TNF was chosen for additional study because it: (1) caused a robust and reproducible increase in cytotoxicity and (2) represents concentrations of TVX and TNF that are similar to those occurring *in vivo*. The cytotoxic interaction of TVX and TNF was not limited to the HepG2 transformed human cell line; TVX also interacted with TNF to kill primary murine hepatocytes in a concentration-dependent manner. Unlike TVX, LVX did not interact with TNF to cause cytotoxicity in either cell type; thus the cytotoxic interaction of these two drugs with TNF

matched their propensity to cause IDILI in humans. The cell death that occurred from TVX/TNF in HepG2 cells appeared to start about 8 to 12 hours after treatment and became significant at 20 hours.

The livers of mice treated with TVX/LPS displayed histological signs of both apoptotic and oncotic necrosis, and this injury depended on TNF (Shaw, et al., 2009c). In a previous study, effector caspase activation occurred in liver after treatment of mice with TVX/TNF and in hepatocytes treated with TVX/TNF *in vitro*, suggesting that cells died at least partially by apoptosis (Shaw, et al., 2009a). Flow cytometric analysis corroborated that observation (Figure 12). TVX/TNF-treated cells with compromised plasma membranes registered as positive for PI uptake largely costained with Annexin V, suggesting an apoptotic cell death. TVX/TNF treatment also caused an increase in the fraction of cells in the early apoptotic (AnnV+/PI-) population. Interestingly, TVX treatment alone caused a small but significant increase in both the early and late apoptotic population. These findings suggest that TVX stressed cells in a way that did not result in overt cell death after 24 hours of treatment.

Apoptosis is associated with DNA damage, which can be detected using the TUNEL method (Heatwole, 1999). In this study, significant DNA damage was observed only in cells treated with TVX/TNF. Though not statistically significant, there was a trend toward an increase in DNA damage caused by TVX treatment alone. This suggests that TVX might have caused some modest genomic stress by itself. Taken together, these results demonstrate that TVX/TNF treatment causes HepG2 cell apoptosis.

Hepatocellular apoptosis is largely regulated by activation of caspase enzymes and can be either extrinsic or intrinsic (Malhi and Gores, 2008). Treatment with the pancaspase inhibitor ZVAD resulted in fewer early apoptotic cells after both TVX and TVX/TNF treatment, and it also decreased late apoptotic cells as well as cytotoxicity after TVX/TNF-treatment. Similarly, the cytotoxic interaction of TVX and TNF to kill primary murine hepatocytes was completely abrogated when caspases were inhibited with ZVAD. These results suggest that caspase activation was critically involved in the TVX/TNF-induced apoptosis. Further investigation revealed that caspase 9 and 3 were significantly activated starting 8 hours after TVX/TNF treatment, a time corresponding to the onset of cell death. Caspase 8 was also significantly activated, but not until 24 hours after treatment and to a lesser extent than caspases 9 and 3. The latency in caspase 8 activation suggests that it might not play a role in the initiation of TVX/TNF-induced cell death. Taken together these results suggest that TVX/TNF treatment initiated an intrinsic pathway to apoptosis.

To demonstrate the role that these activated caspases play in TVX/TNF-induced apoptosis, selective inhibitors of individual capases were used. Treatment with IETD or LEHD, inhibitors of caspases 8 and 9 respectively, resulted in complete protection. The effectiveness of IETD was surprising, given that caspase 8 activation was detected only after the onset of cell death (Figure 15C). However, recent findings indicate that caspase inhibitors are less selective for individual caspases than previously reported (Pereira and Song, 2008). Treatment with DEVD to inhibit caspase 3 and 7 attenuated the cytotoxicity caused by TVX/TNF, but did not fully prevent it. Attempts to inhibit cytotoxicity further by increasing the concentration of DEVD resulted in DEVD-induced

cytotoxicity. It is possible that the degree of caspase 3 activation, which was much greater than the activation of initiator caspases 8 or 9, was too extensive to be fully inhibited by DEVD. Nevertheless, these findings identify caspase activation as being critical to TVX/TNF-induced apoptosis, and the latency in caspase 8 activation suggests that apoptosis was intrinsically mediated.

Another signaling pathway involved in hepatocellular apoptosis involves JNK (Ding and Yin, 2004). JNK can be activated in the liver by many cellular stresses, including cytokines such as TNF (Seki, et al., 2012). If cellular stress is short-lived, JNK activation is typically transient and does not result in cell death. Prolonged activation of JNK is associated with cell death signaling in hepatocytes and can occur during oxidative or genotoxic stress (Kobayashi and Tsukamoto, 2001, Seok, et al., 2008, Win, et al., 2011, Wullaert, et al., 2006). TVX by itself did not cause early (1 hour) JNK activation, but the early activation of JNK by TNF was enhanced by TVX coexposure. At a later time when the degree of activated JNK was similar in TNF-treated cells and vehicle controls (8 hour), TVX caused more JNK activation by itself, independent of TNF. Thus, because of the early enhancement of JNK activation by TVX, cells exposed to both TVX and TNF experienced JNK activation that was prolonged. Treatment of cells with SP600125 to inhibit JNK attenuated TVX/TNF-induced cytotoxicity. These results indicate that prolonged JNK activation is important in promoting cytotoxicity from TVX/TNF coexposure.

In summary, TVX and TNF interact to cause hepatocellular toxicity in a concentration-dependent manner in both HepG2 human hepatoblastoma cells and cultured primary murine hepatocytes at drug concentrations that are near what is

measured in humans undergoing TVX therapy. Cotreated hepatocytes undergo an apoptotic mode of cell death that requires active caspases. LVX is not associated with causing IDILI in humans, and treatment of hepatocytes with LVX, in the presence or absence of TNF, does not cause hepatocellular toxicity. TVX alone significantly increases the percentage of early apoptotic cells, and in the presence of TNF it prolongs JNK activation that is required for cytotoxicity. Apoptotic cells were identified *in vivo* in the mouse model of TVX/LPS- or TVX/TNF-induced liver injury. However, oncotic necrosis was also observed (Shaw, et al., 2007, Shaw, et al., 2009a). Identifying the signaling mechanisms involved in hepatocellular toxicity due to TVX/TNF could help the understanding of IDILI pathogenesis and the development of more effective preclinical screening tools to prevent drugs with idiosyncratic liability from reaching the market.

CHAPTER 3

Trovafloxacin Activates the DNA Damage Response and Sensitizes HepG2 Cells to Tumor Necrosis Factor-alpha-Induced Cytotoxicity Involving ERK and ATR. Submitted for review. Beggs, K.M., Fullerton, A.M., Poulsen, K.L., Ganey, P.E., Roth, R.A.

3.1 Abstract

Use of the fluoroquinolone antibiotic trovafloxacin (TVX) was restricted due to idiosyncratic hepatotoxicity in people taking the drug. Previous studies demonstrated that at a clinically relevant concentration of TVX and of tumor necrosis factor alpha (TNF), these two agents interact to cause hepatocellular death through the intrinsic pathway of apoptosis. The cell death involved prolonged activation of c-Jun N-terminal kinase (JNK), activation of caspases 9 and 3, and DNA damage. One cause of intrinsic apoptosis and prolonged JNK activation is DNA damage, suggesting that genotoxicity might be an initiating event in TVX/TNF-induced cytotoxicity. Other reports have indicated that cells arrested in the cell cycle, a common result of DNA damage, can become sensitive to the cytotoxic effects of TNF. The purpose of this study was to determine 1) whether TVX activates the DNA damage response, and 2) how that response might interact with TNF to cause cytotoxicity. TVX treatment caused DNA damage, cell cycle arrest, decreased proliferation and increased expression of p21 in HepG2 cells but was not cytotoxic by itself. In the presence of TNF, DNA damage observed from TVX treatment was exacerbated, and cytotoxicity occurred that involved signaling from extracellular signal-related kinase (ERK) and from one of the key elements of the DNA damage response, ataxia telangiectasia and Rad3 related protein (ATR). These results increase understanding of molecular signaling pathways involved in hepatocellular death caused by a drug with idiosyncratic liability and suggest a role for the interaction of the DNA damage response with cytokines such as TNF.

3.2 Introduction

Idiosyncratic, drug-induced liver injury (IDILI) is a typically rare and currently unpredictable adverse response that accounts for as much as 17% of all cases of acute liver failure (Hussaini and Farrington, 2007). Due to a lack of understanding of mechanisms of toxicity, host susceptibility, and outcome factors, IDILI is presently not preventable. One hypothesis to explain IDILI etiology is that an otherwise nontoxic dose of a drug interacts with a concurrent inflammatory stress to precipitate liver injury (Roth, et al., 2003). Several animal models have been developed based on this hypothesis (Roth and Ganey, 2011). Exploration of these models has allowed the identification of some common factors, including the importance of the proinflammatory cytokine, tumor necrosis factor-alpha (TNF), in promoting liver injury from drugs with idiosyncratic liability (Shaw, et al., 2007, Tukov, et al., 2007, Zou, et al., 2009a). Consistent with this finding in vivo, cotreatment of primary hepatocytes and hepatocyte cell lines with TNF and drugs with IDILI liability caused a synergistic cytotoxicity (Beggs, et al., 2013, Cosgrove, et al., 2009, Fredriksson, et al., 2011, Shaw, et al., 2009a, Zou, et al., 2009a). These results suggest that such drugs sensitize hepatocytes to the cytotoxic effects of TNF.

Trovafloxacin (TVX) is a fluoroquinolone antibiotic that received a black box warning in 1999 after it was associated with life-threatening IDILI in people (Nightingale, 1999). In vitro, TVX and TNF synergized to cause cytotoxicity in hepatocytes (Beggs, et al., 2013, Cosgrove, et al., 2009, Shaw, et al., 2009a). The cell death was apoptotic and depended on prolonged activation of c-Jun N-terminal kinase (JNK) as well as upon caspase activation, and was associated with DNA damage (Beggs, et al., 2013).

Prolonged activation of JNK can occur from genotoxic stress arising from DNA damage (Roos and Kaina, 2006, Seok, et al., 2008), which can also activate a multitude of other signaling events. These events include induction of the cyclin-dependent kinase inhibitor p21, cell cycle arrest, decreased cell proliferation and activation of extracellular signal-regulated kinase (ERK). Other events associated with DNA damage include activation of the DNA damage sensor kinases ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK). Together, these signaling events can arrest cells to allow for repair of DNA damage or, in cases of severe genotoxic stress, promote apoptotic signaling leading to cell elimination (Cagnol and Chambard, 2010, Houtgraaf, et al., 2006, Roos and Kaina, 2006, Yang, et al., 2003).

The purpose of this study was to determine 1) whether TVX activates the DNA damage response, and 2) how that response might interact with TNF to cause cytotoxicity. Identification of critical, drug-induced signaling events that render hepatocytes sensitive to cell death from cytokines could facilitate the development of predictive preclinical screening assays to identify drug candidates with idiosyncratic liability.

3.3 Materials and Methods

3.3.1 Materials

Unless otherwise noted, all materials were purchased from Sigma-Aldrich (St. Louis, MO). Cayman Chemical (Ann Arbor, MI) synthesized the TVX. Recombinant human TNF was purchased from R&D Systems (Minneapolis, MN). Phosphate-buffered saline (PBS), high glucose Dulbecco's Modified Eagles Medium (DMEM) Antibiotic-Antimycotic (ABAM), L-glutamine, and 0.25% trypsin-EDTA were purchased from Life Technologies (Carlsbad, CA). For flow cytometry experiments, Cell Staining Buffer was purchased from Biolegend (San Diego, CA), Perm/Wash Buffer from BD Biosciences (San Jose, CA), and Propidium Iodide/RNase Staining Solution from Cell Signaling Technology (Beverly, MA). U0126 was purchased from Calbiochem (San Diego, CA). KU55933 was purchased from Tocris Bioscience (Minneapolis, MN).

3.3.2 Cell Culture

HepG2 human hepatoblastoma cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% ABAM in 25-cm^2 tissue culture treated flasks. Cells were cultured in a humidified atmosphere composed of 95% air and 5% CO_2 and a temperature of 37°C. Cells were passaged twice each week. 0.25% trypsin-EDTA was used to detach confluent HepG2 cells from the flask. After plating, cells were allowed 7 hours to adhere before treatment. TVX was reconstituted to a stock solution of 200 mM in dimethyl sulfoxide (DMSO): when added to culture wells the maximal final concentration of DMSO was 0.01%. Vehicle controls for TVX are represented as "Veh" throughout. TNF was reconstituted to a stock solution of 100 μg/mL in PBS.

3.3.3 Protein Isolation

HepG2 cells were plated at 1.2 x 10^6 cells per well in 6-well tissue culture plates. Cells were treated for various times before being washed with ice-cold PBS. After washing, they were treated with radio-immunoprecipitation assay (RIPA) buffer containing HALT protease and phosphatase inhibitors (Thermo Scientific, Pittsburgh, PA). Cells were scraped, collected in tubes and kept on ice. They were incubated in RIPA buffer for 10 minutes before each sample was sonicated with one 5-second pulse. Lysates were centrifuged at 20,000 x g for 20 minutes, and the supernatant was collected for analysis. The bicinchoninic acid (BCA) assay (Thermo Scientific) was used to determine protein concentration.

3.3.4 Western Blot Analysis

Phospho-H2AX (γH2AX), p53, p21, phospho-ERK (p-ERK) and Lamin B1 (Lamin) were detected by loading 15 μg of protein on NuPAGE 10% Bis-Tris gels (Life Technologies). The proteins were then separated by electrophoresis. Proteins were transferred from gels onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After transfer, the membranes were blocked for 1 hour in a solution of 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST). Membranes were then probed with primary antibodies (Cell Signaling Technology, Beverly, MA). Antibodies were diluted in 5% BSA in TBST to 1:2000 for γH2AX and p21, 1:1000 for p53, 1:10,000 for p-ERK and 1:10,000 for Lamin. Membranes were incubated with primary antibodies at 4°C for at least 18 hours. PVDF membranes were then washed with TBST and probed with goat anti-rabbit HRP-conjugated secondary antibody for one hour at room temperature (Santa Cruz Biotechnology,

Santa Cruz, CA). Secondary antibodies were diluted in 5% BSA in TBST to 1:2500 for γH2AX, 1:5000 for p53 and p21, and 1:10,000 for p-ERK and Lamin. HRP was visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA). Membranes were developed on HyBlot CL Film (Denville Scientific, Metuchen, NJ), and densitometry was performed on the developed films using Image J software.

3.3.5 Flow Cytometry

Cells were plated at 5 x 10^5 cells per well in 12-well tissue culture plates. After 12 hours of exposure, cells and supernatant were collected in 12mm x 75mm round-bottomed tubes (BD Biosciences) on ice. Cells were pelleted by centrifugation at 4°C for 5 minutes at 70 x g. Culture medium was aspirated before cells were washed with cold Cell Staining Buffer. This cell suspension was added drop wise to an ice-cold solution of %70 ethanol. The suspension was kept at 4°C for at least 12 hours to allow for fixation. After the cells were fixed, they were spun down, and the ethanol solution was aspirated. Cells were washed once with Cell Staining Buffer and pelleted, and the buffer was aspirated. The cells were resuspended in Perm/Wash Solution and kept on ice for 5 minutes. After centrifugation and aspiration of the Perm/Wash Solution, cells were resuspended in PI/RNase Staining Solution at room temperature for 30 minutes. After incubation, cell cycle analysis was performed using a BD FACS Canto II flow cytometer. Data were analyzed using Kaluza software (Beckman Coulter, Brea, CA). Unstained cells were also analyzed to account for auto fluorescence.

3.3.6 HepG2 Proliferation Studies

For the manual cell count study, cells were plated at 1 x 10^6 cells per well in 6-

well tissue culture plates. After treatment time (0, 24, or 48 hours), culture medium supernatant was collected, and cells were detached with trypsin. The collected supernatant was returned to detached cells to neutralize trypsin, and cell concentration was determined using a hemocytometer. Each sample was counted 6 times (technical replicates), and the average cell concentration was recorded as n=1 when calculating the group mean. For the fluorescent probe study, cells were plated at 1×10^4 cells per well in black-walled, 96-well tissue culture plates. For each experiment, 4 wells were plated for each treatment group. Relative DNA content was determined using the CyQUANT NF Cell Proliferation Assay (Life Technologies) following the manufacturer's instructions. Briefly, at time of measurement culture medium was gently aspirated and replaced with an equal volume of dye solution. Cells were incubated at 37°C for 1 hour to allow for intercalation of the proprietary fluorescent dye into DNA. After incubation, the plates were read on a fluorescent microplate reader with filters for excitation at 485 nm and emission at 530 nm.

3.3.7 RNA Isolation and RT-PCR

Cells were plated at 1.2×10^6 cells per well in 6-well tissue culture plates. After 6 hours of treatment, RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A NanoDrop 2000 spectrophotometer (Thermo Scientific) was used to assess the quantity and quality of the collected RNA. Complementary DNA (cDNA) was prepared from 1 µg of RNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). The expression of TP53, CDKN1A and ACTB genes were determined using a StepOne Real-Time PCR system

using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Copy number was determined by comparison with standard curves of the respective genes generated from pooled cDNA of all treatment groups. TP53 and CDKN1A expression was normalized to the expression of ACTB (β-Actin). PCR primers were used as follows: human TP53, 5'- gagcgtgctttccacgac-3' (forward) and 5'- tgtttcctgactcagagggg -3' (reverse), human CDKN1A, 5′- accgaggcactcagaggag -3′ (forward) and 5′- gccattagcgcatcacagt -3′ (reverse), human ACTB, 5′- gcacagagcctcgcctt -3′ (forward) and 5′- gttgtcgacgacgagcg -3′ (reverse).

3.3.8 HepG2 Cytotoxicity Assessment

HepG2 cells were plated at 4 x 10^4 cells per well in white-walled, 96-well tissue culture plates. Cytotoxicity was measured after 24 hours of treatment using the CytoTox-Glo Cytotoxicity Assay (Promega, Madison, WI) following the manufacturer's instructions. All inhibitors were reconstituted in DMSO and represented as "Vehicle" in each study. Cells were exposed to 25 μM PFT or 0.05% DMSO, 10 μM U0126 or 0.05% DMSO, 12.5 μM NU6027 or 0.0625% DMSO, 20 μM KU55933 or 0.1% DMSO, or 10 μM wortmannin or 0.1% DMSO. The concentrations chosen have been shown to inhibit their respective target proteins (Hickson, et al., 2004, Komarov, et al., 1999, Okayasu, et al., 1998, Peasland, et al., 2011).

3.3.9 ATR Measurement

Labeling for phospho-ATR (p-ATR) was conducted using a polyclonal antibody against phosphorylated threonine 1989 on ATR (Genetex, Irvine, CA). Cells were

plated in 8-chamber culture slides (BD Biosciences) at 9 x 10^4 cells per chamber. After 6 hours of treatment, culture medium was gently aspirated. Cells were air-dried and submerged in acetone chilled to -20°C for fixation and permeabilization. Cells were then rinsed with PBS and incubated with a blocking buffer composed of 10% goat serum in PBS. Primary p-ATR antibody was diluted to 1:100 in blocking buffer, and cells were incubated with primary antibody solution in a humidified chamber overnight at 4°C. Cells were rinsed with cold PBS and incubated with a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (Life Technologies) diluted 1:500 in blocking buffer for 2 hours at room temperature. They were then washed, and an anti-fade mounting medium containing DAPI was applied (Vector Laboratories, Burlingame, CA). Slides were imaged using an Olympus IX71 inverted fluorescence microscope and appropriate filters. Images were taken with an Olympus F-View II digital monochrome camera and were processed using Image J software. 3 to 6 images were taken for each chamber. Cells exposed to only the secondary antibody were used as a negative control, and no significant signal was detected in these groups.

3.3.10 Statistical Analysis

Results are expressed as mean ± S.E.M. Percentile data were subjected to arcsine transformation. Analysis of data was performed using one-way or two-way analysis of variance (ANOVA) followed by pairwise multiple comparisons using the Holm Sidak or Tukey's method. Nonparametric data were analyzed using Kruskal-

Wallis test followed by pairwise multiple comparisons using Tukey's or Dunn's method as appropriate. The criterion for statistical significance was $p<0.05$.

3.4 Results

3.4.1 DNA Damage Timecourse

Phosphorylated histone H2AX (γH2AX), a marker of DNA damage (Rogakou, et al., 1998), was measured 6, 12, and 24 hours after treatment. Cells were treated with 20 μM TVX and 4 ng/mL TNF, or their vehicles simultaneously. This TVX/TNF combination was shown previously to result in cytotoxicity within 20 hours (Beggs, et al., 2013). After 6 hours, none of the treatments caused a change in the γH2AX signal (Figure 20). Treatment with vehicles or TNF did not alter γH2AX levels at 12 or 24 hours either. In contrast, TVX/TNF treatment caused a nine-fold increase in γH2AX compared to Veh/PBS-treated cells 12 hours after treatment, and γH2AX was further increased to thirteen-fold at 24 hours. TVX alone also caused an increase in γH2AX (3.8-fold) at 24 hours.

3.4.2 HepG2 Cell Cycle Analysis and Proliferation Time Course

In response to DNA damage, many cell types undergo cell cycle arrest (Houtgraaf, et al., 2006). Distribution of cells in the various stages of the cell cycle was analyzed by flow cytometry after 12 hours of treatment. The distribution of cells in G0/G1, S, and G2/M after vehicle treatment was 49%, 27%, and 19%, respectively (Figure 21). TVX treatment caused an increase in the percentage of G0/G1 cells (71%) and a decrease in the percentage of cells in S phase (6%) but did not change the percentage of cells in G2/M (21%). Cells treated with TNF also had an increase in the percentage of G0/G1 cells (59%) as well as a decrease in the percentage of cells in S phase (18%) without changing the percentage of cells in G2/M (20%) relative to vehicle controls. Treatment of cells with TVX/TNF resulted in a cell cycle distribution almost

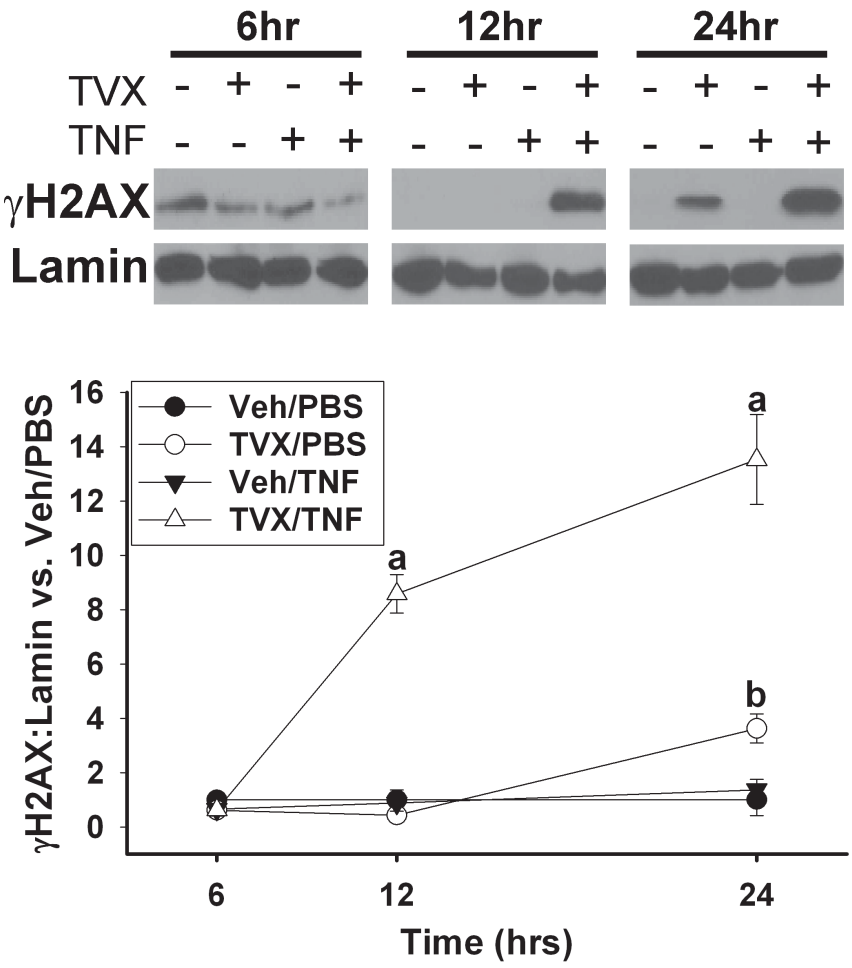


Figure 20. TVX-induced DNA damage is exacerbated by TNF. Cells were treated simultaneously with 20 μM TVX or vehicle and with 4 ng/mL TNF or PBS for 6, 12 or 24 hours. Phosphorylation of H2AX was determined by western analysis. Representative blots are shown. Densitometry was performed on phospho-H2AX (γH2AX) and Lamin bands, and the ratio of γH2AX to Lamin for each treatment group relative to the ratio for Veh/PBS treatment is shown for each time. **a** Significantly different from all other treatment groups at the same time. **b** Significantly different from Veh/PBS treatment group at the same time. Data represent the mean ± SEM of 4-6 separate experiments.

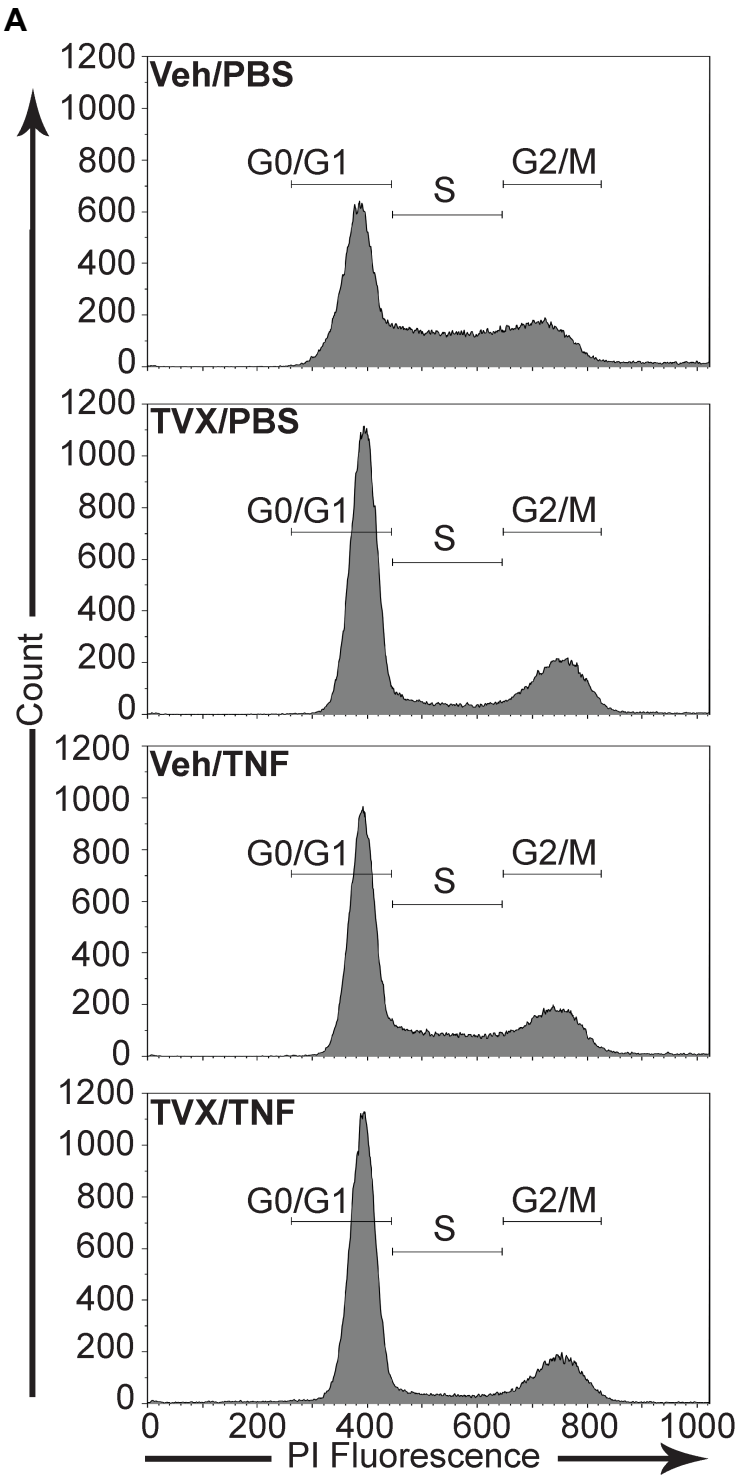
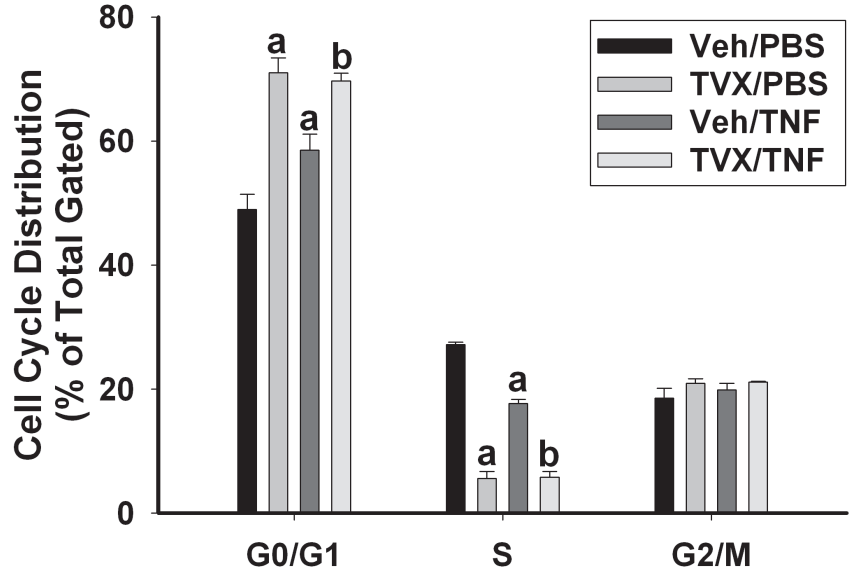


Figure 21. TVX causes cell cycle arrest. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS. After 12 hours, cell cycle distribution was assessed using flow cytometry. (A) Representative histograms for each treatment.

Figure 21 (cont'd)

B



(B) Percentage of the total cells gated in the various stages of the cell cycle. **a** Significantly different from Veh/PBS-treated group within same phase of cell cycle. **b** Significantly different from Veh/TNF-treated group within same phase of cell cycle. Data represent the mean ± SEM of 5 separate experiments. 100,000 cells were sampled from each experiment.

identical to treatment with TVX alone; that is, TVX/TNF caused an increase in the percentage of cells in G0/G1 as well as a decrease in the percentage in S phase.

Effects on HepG2 cell proliferation were investigated by manual counting using a hemocytometer as well as by measuring DNA content with an intercalating fluorescent probe. Measurements were made at the time of treatment (0 hour), as well as 24 and 48 hours after treatment. Proliferation was apparent in cells treated with vehicle but was halted completely by addition of TVX (Figure 22A). Similarly, DNA content relative to vehicle controls was progressively less as result of TVX treatment (Figure 22B). TNF was without significant effect. By 48 hours cotreatment with TVX/TNF caused a more pronounced decrease in DNA content than treatment with either TNF or TVX alone.

3.4.3 p53 Expression and Contribution to Cytotoxicity

DNA damage can lead to upregulation of the transcription factor and tumor suppressor protein, p53 (Lakin and Jackson, 1999). Though many transformed cell lines have a mutated p53 gene, HepG2 cells express a functional, wild type p53 (Hosono, et al., 1991). Expression of TP53 mRNA was not altered by treatment with TVX, TNF or the combination after 4 or 6 hours of exposure (Figure 23). Similarly, expression of p53 protein was not altered after 6 or 12 hours (Figure 24). The contribution of p53 to cytotoxicity was examined using an inhibitor of p53-mediated transcription, pifithrin-α (PFT) (Komarov, et al., 1999). Simultaneous treatment of cells with 25 μM PFT did not alter the cytotoxicity caused by TVX/TNF after 24 hours (Figure 25). Similar results were observed after treatment with 50 or 100 μM PFT (data not shown).

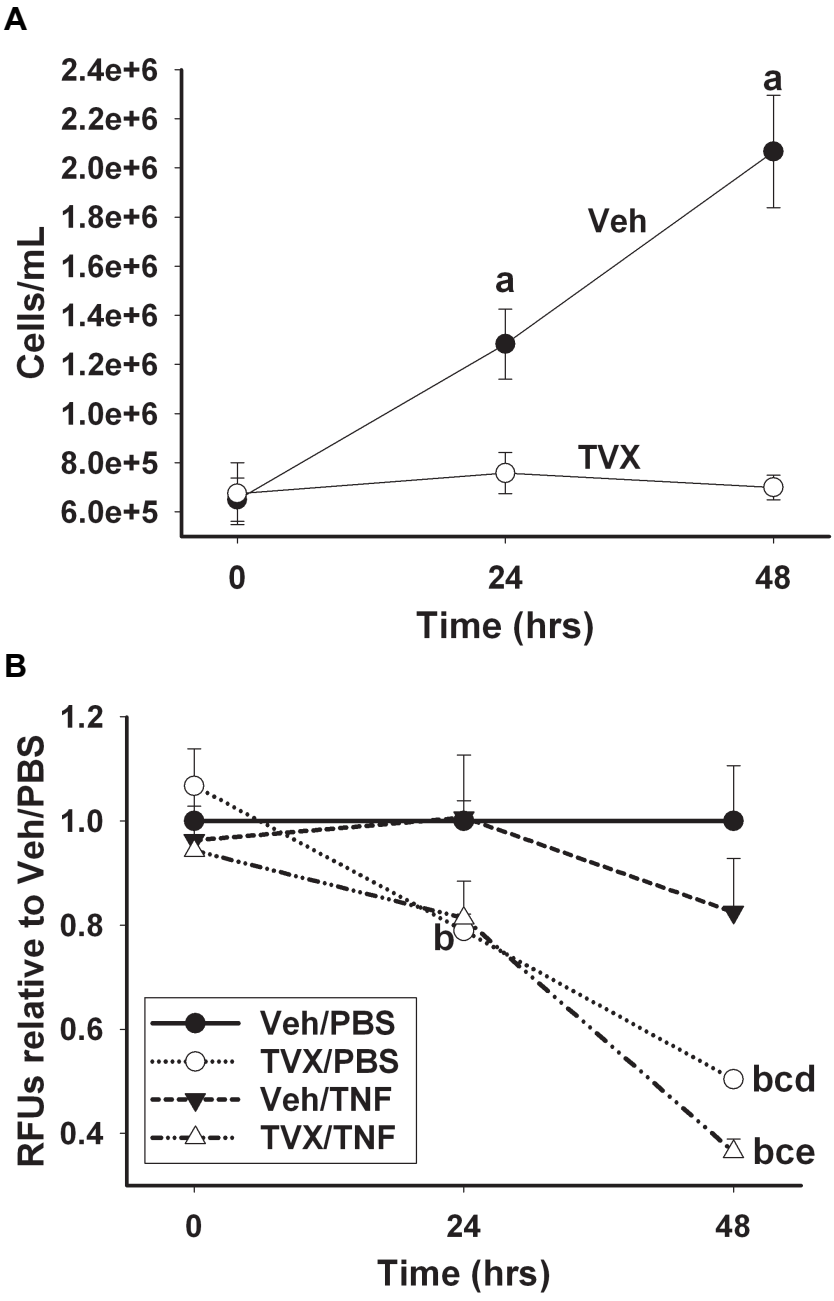


Figure 22. TVX decreases HepG2 cell proliferation. (A) Cells were treated with 20 μM TVX or its vehicle and counted 0, 24 and 48 hours later as described in Methods. Data represent the mean ± SEM of 3 separate experiments and are expressed as number of cells per mL of culture medium. **a** Significantly different from TVX-treated

Figure 22 (cont'd)

group at same time. (B) Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS. Fluorescence of the DNA probe was measured 0, 24 and 48 hours after treatment as described in Methods. Data represent the mean ± SEM of 4 separate experiments and are expressed as fluorescence relative to fluorescence in the Veh/PBS group at each time. **b** Significantly different from same treatment at 0 hour. **c** Significantly different from same treatment at 24 hours. **d** Significantly different from Veh/PBS-treated group at the same time. **e** Significantly different from all other treatment groups at same time. Data represent the mean ± SEM of 4 separate experiments.

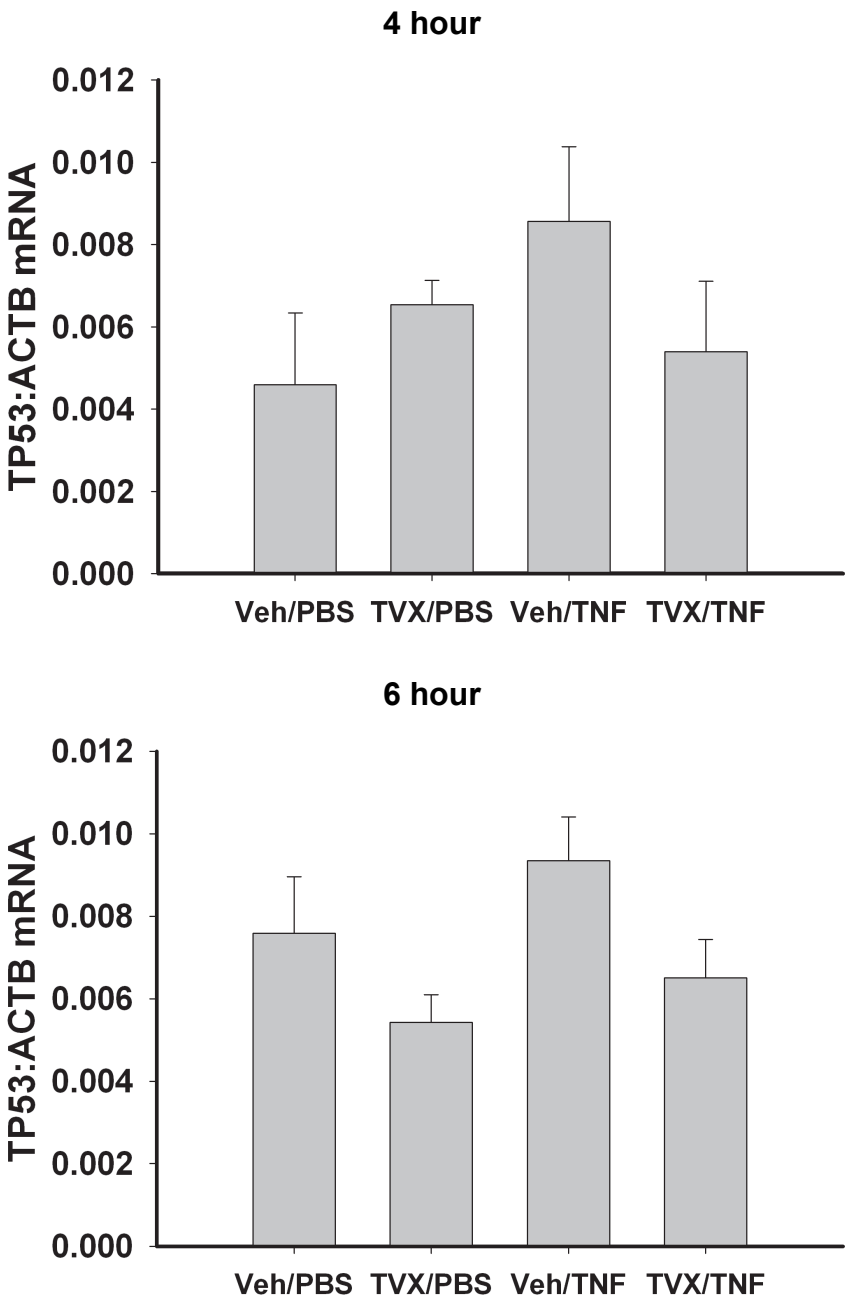


Figure 23. Transcription of TP53 gene is unaltered by treatment. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS. After 4 or 6 hours of exposure, mRNA was isolated as described in Materials and Methods. TP53 mRNA expression relative to ACTB mRNA is shown at 4 and 6 hours. Data represent the

Figure 23 (cont'd)

mean ± SEM of 4 separate experiments. No significant differences in TP53 mRNA were observed.

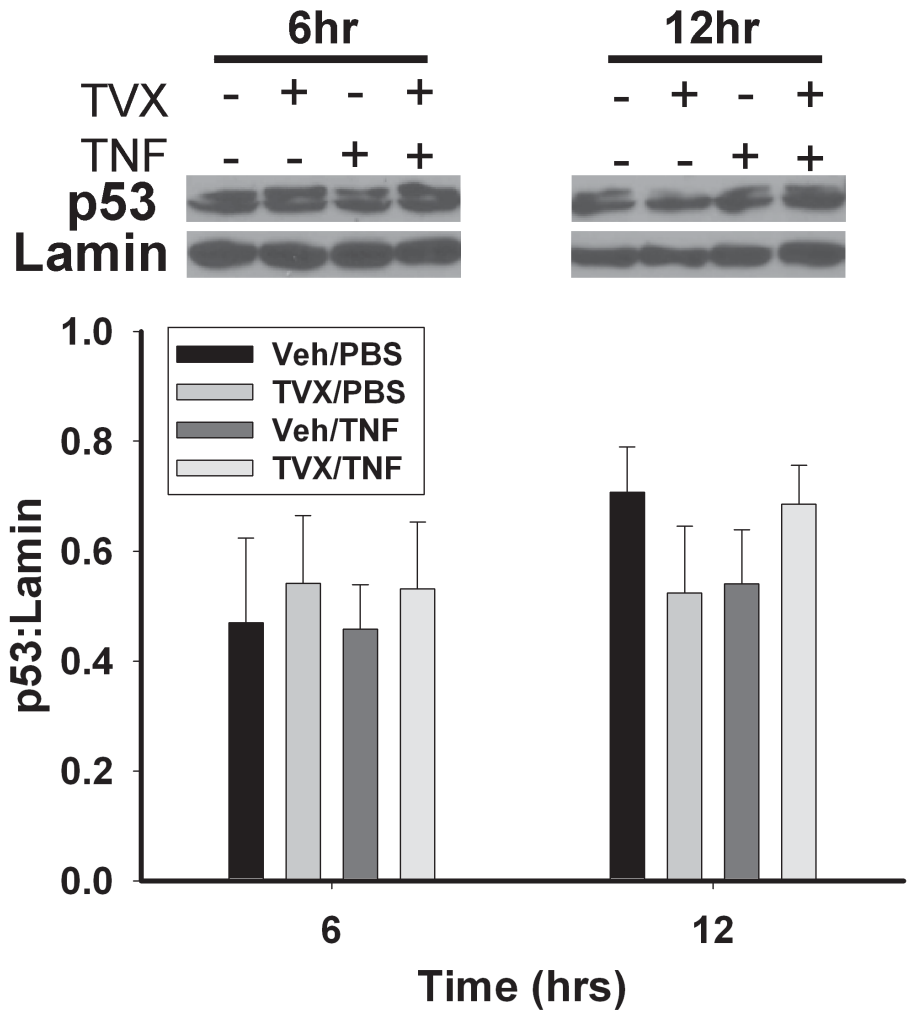


Figure 24. p53 protein expression is unchanged by treatment. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS. After 6 or 12 hours of treatment protein was isolated as described in Materials and Methods. Representative blots of p53 and Lamin 6 and 12 hours after treatment, and quantification of p53 protein expression. Data represent the mean ± SEM of 4-6 separate experiments. No significant differences in protein expression were observed.

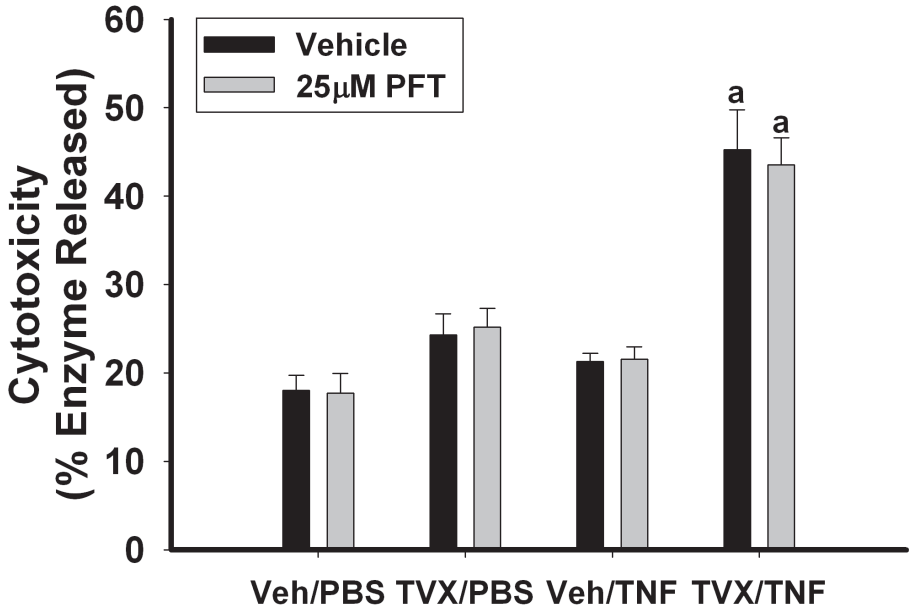


Figure 25. p53 is not involved in TVX/TNF-induced cytotoxicity. To inhibit p53 activity cells were treated with 25 μM PFT (a selective p53 inhibitor) or its vehicle. Cytotoxicity was measured after 24 hours. Data represent the mean ± SEM of 4 separate experiments. No significant differences between PFT- and Vehicle-treated groups were observed.

3.4.4 p21 Transcription and Protein Expression

Transcription of the CDKN1A gene and expression of its protein product p21 were examined. After 6 hours of treatment, both TVX alone and TVX/TNF caused an increase in CDKN1A mRNA expression compared to cells treated with vehicle or TNF (Figure 26A). None of the treatments changed p21 protein expression at 6 hours (data not shown). However, TVX caused an increase in p21 expression compared to vehicle controls 12 hours after treatment (Figure 26B).

3.4.5 ERK Activation and Its Role in Cell Cycle and Cytotoxicity

A common response to DNA damage is activation of the ERK MAP kinase. ERK signaling also can be involved in either progressing or halting the cell cycle (Cagnol and Chambard, 2010). For these reasons, the activation of ERK was investigated. Although none of the treatments affected ERK phosphorylation after 1 hour (data not shown), by 6 hours both TVX and TVX/TNF treatments led to an increase in ERK activation compared to cells treated with vehicles or TNF (Figure 27A). This increase in ERK activation was maintained through 24 hours (Figure 27B). Total ERK expression was not affected by any treatment (data not shown). Treatment with U0126, a selective inhibitor of the MEK1/2 kinases upstream of ERK (Favata, et al., 1998), completely prevented ERK phosphorylation in all treatment groups. Cell cycle analysis was performed on cells treated with U0126 or its vehicle. In cells treated with either vehicle or TNF, U0126 caused an increased percentage of cells in G0/G1 (Figure 28A), as well a decreased percentage of cells in S phase (Figure 28B). However, in cells treated with TVX alone or TVX/TNF, U0126 failed to cause such changes. The percentage of cells in G2/M was not affected by U0126 for any of the treatment groups.

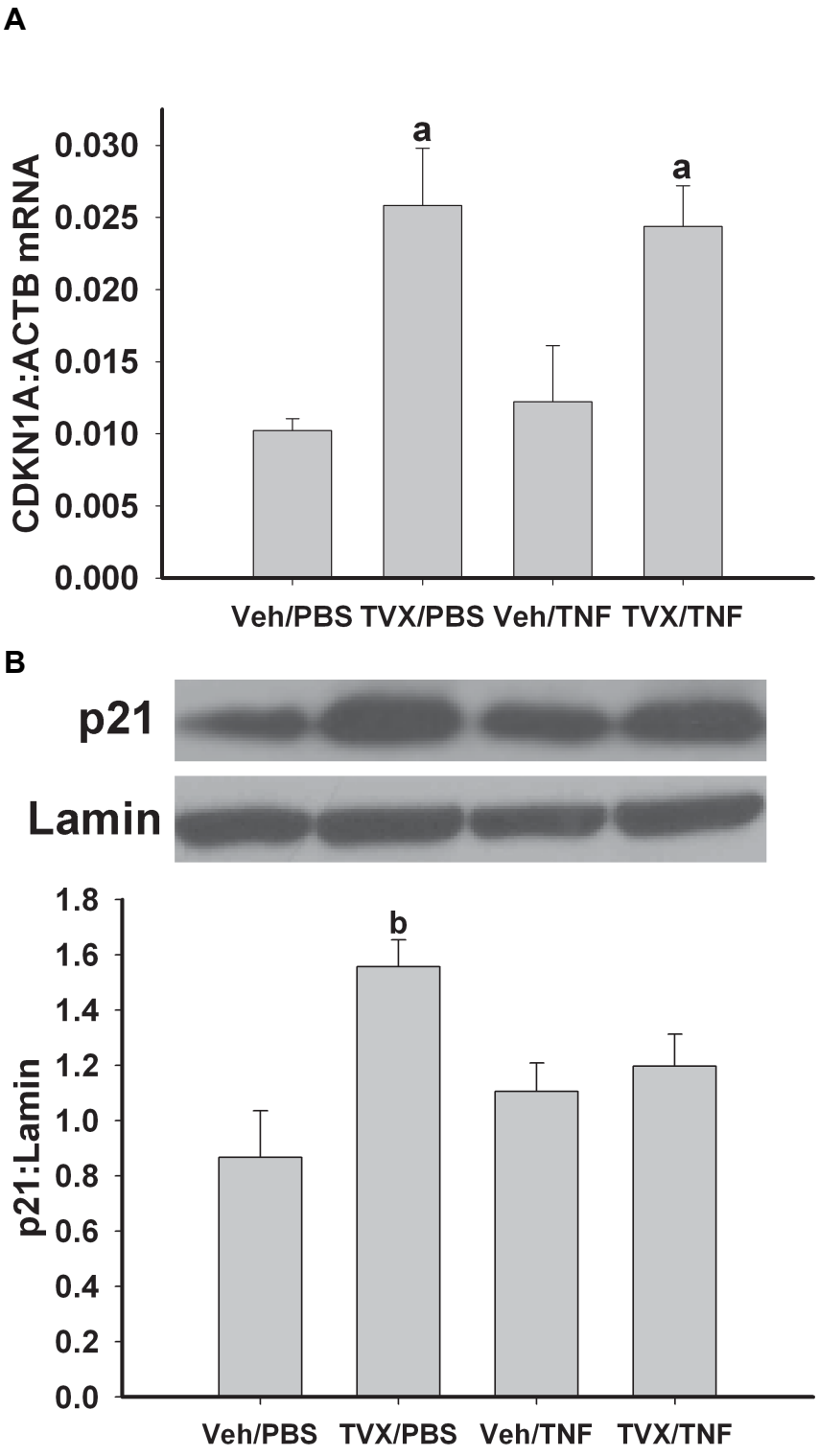


Figure 26. TVX-induced upregulation of p21. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS. After 6 hours of treatment mRNA was collected,

Figure 26 (cont'd)

and after 12 hours of treatment protein was isolated as described in Materials and Methods. CDKN1A mRNA expression was determined by qRT-PCR, and p21 protein expression by western analysis. For p21 protein, densitometry was performed on p21 and Lamin bands, and the ratio of p21 to Lamin is represented. (A) CDKN1A mRNA expression relative to ACTB mRNA. **a** Significantly different from respective group in the absence of TVX. Data represent the mean ± SEM of 4 separate experiments. (B) Representative blots of p21 and Lamin 12 hours after treatment, and quantification of p21 protein expression. **b** Significantly different from Veh/PBS-treated group at same time. Data represent the mean ± SEM of 3 separate experiments.

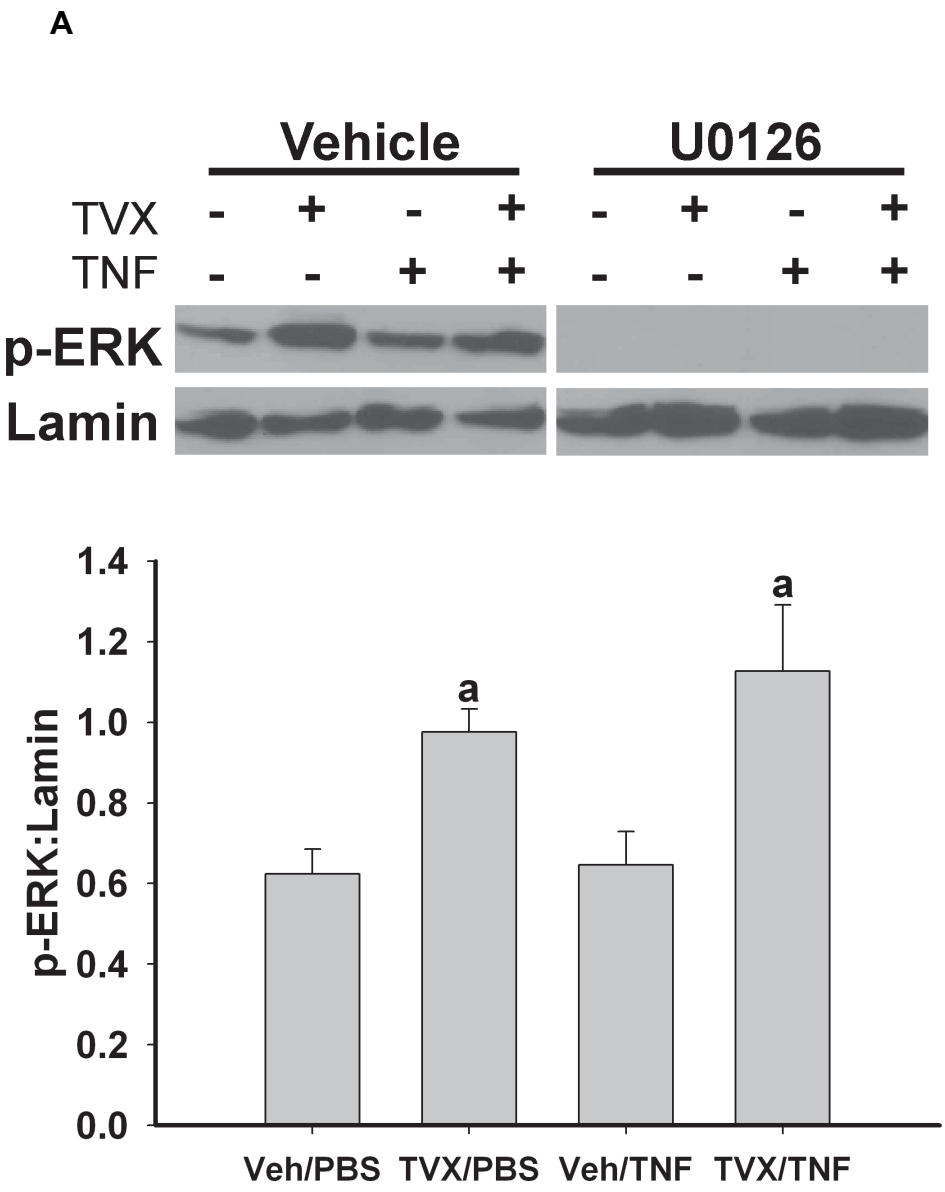
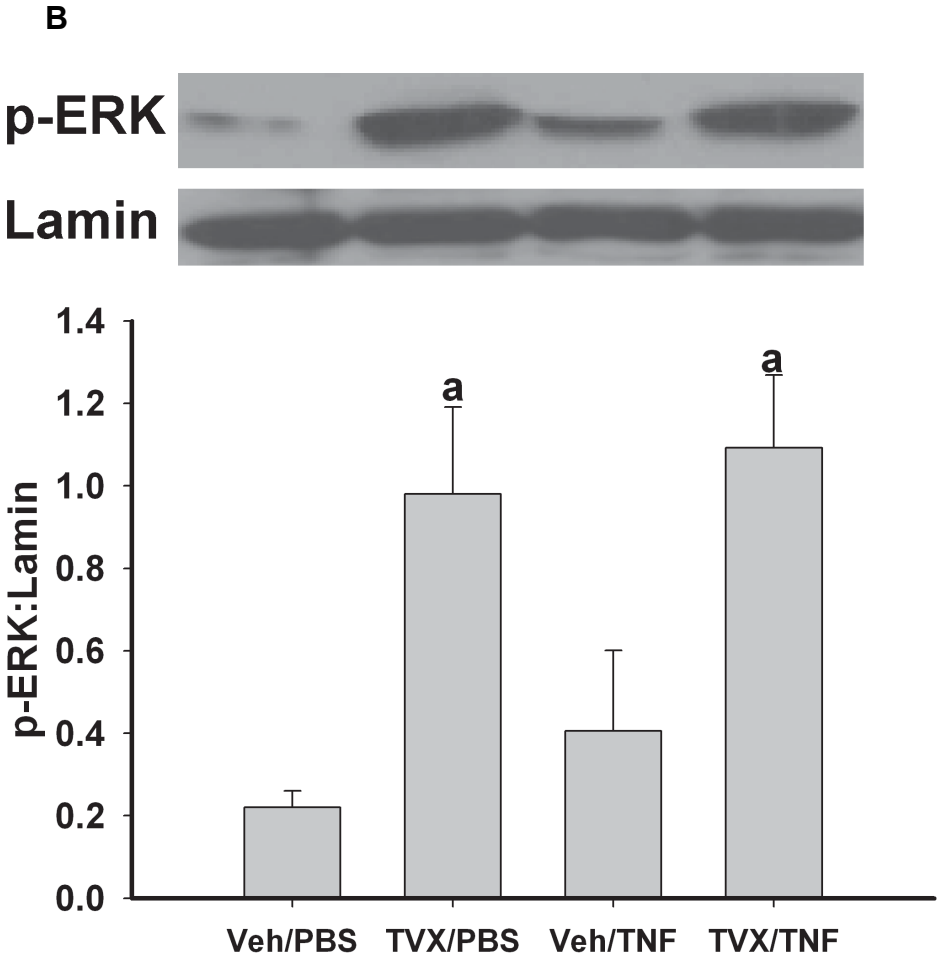


Figure 27. TVX exposure activates ERK. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS in the presence of 10 μM of the MEK-1/2 inhibitor U0126 or its vehicle. Phosphorylation of ERK was determined by western analysis. Representative blots of 6 hour (A) and 24 hour (B) treatment are shown. Densitometry was performed for cells not treated with U0126, and the ratio of p-ERK to Lamin is

Figure 27 (cont'd)



shown. **a** Significantly different from respective group in the absence of TVX. Data represent the mean ± SEM of 4 separate experiments.

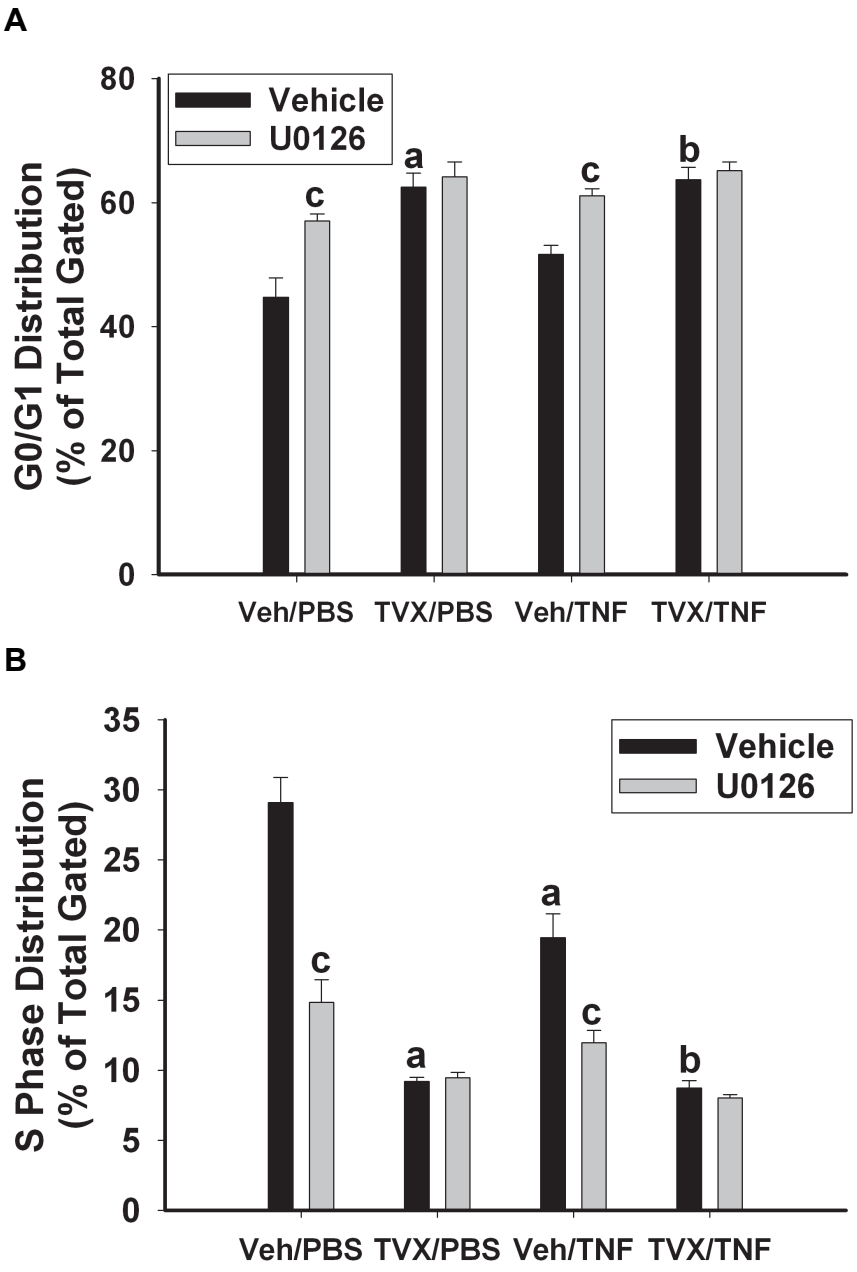


Figure 28. ERK does not mediate TVX-induced cell cycle arrest. Cells were treated simultaneously with 20 μM TVX or vehicle and with 4 ng/mL TNF or PBS in the presence of 10 μM U0126 or its vehicle. After 12 hours of treatment, cell cycle

Figure 28 (cont'd)

distribution was analyzed by flow cytometry. (A) Percent distribution of cells gated in the G0/G1 phase of the cell cycle. (B) Percent distribution of cells gated in the S phase of the cell cycle. **a** Significantly different from respective Veh/PBS-treated group. **b** Significantly different from respective Veh/TNF-treated group. **c** Significantly different from same treatment without U0126. Data represent the mean ± SEM of 3 separate experiments. 100,000 cells were counted for each sample.

To examine the role that ERK signaling plays in promoting cell death, HepG2 cells were treated with TVX and/or TNF in the presence and absence of U0126. Cytotoxicity was measured 24 hours after treatment. Neither TVX nor TNF alone caused cytotoxicity in the presence or absence of U0126 (Figure 29). As shown previously (Beggs, et al., 2013), the TVX/TNF combination was cytotoxic, and U0126 markedly reduced the cytotoxicity.

3.4.6 ATR Activation

ATR is a kinase involved in sensing DNA damage and promoting cell cycle arrest. It is activated by phosphorylation of threonine 1989 (Nam, et al., 2011). The activation of ATR was determined by immunofluorescent detection of phosphorylated ATR (p-ATR) in cell nuclei after 6 hours of treatment. Cells treated with vehicle or TNF displayed minimal p-ATR in the nuclei (Figure 30). Treatment with either TVX or TVX/TNF caused a significant increase in nuclear p-ATR. Treatment with NU6027, an inhibitor of ATR (Peasland, et al., 2011), significantly reduced the p-ATR detected in the nuclei of TVX- or TVX/TNF-treated cells.

3.4.7 Pharmacological Inhibition of DNA Damage-Sensing Kinases

Along with ATR, both ATM and DNA-PK can detect DNA damage. Together, these three make up a family of PI3K-like kinases involved in the DNA damage response (Houtgraaf, et al., 2006, Yang, et al., 2003). To investigate the role that these three kinases might play in TVX/TNF-induced cytotoxicity, a pharmacological inhibitor for each kinase was administered, and cytotoxicity was measured after 24 hours. In the absence of any inhibitors, treatment with TVX/TNF caused cytotoxicity. Treatment of

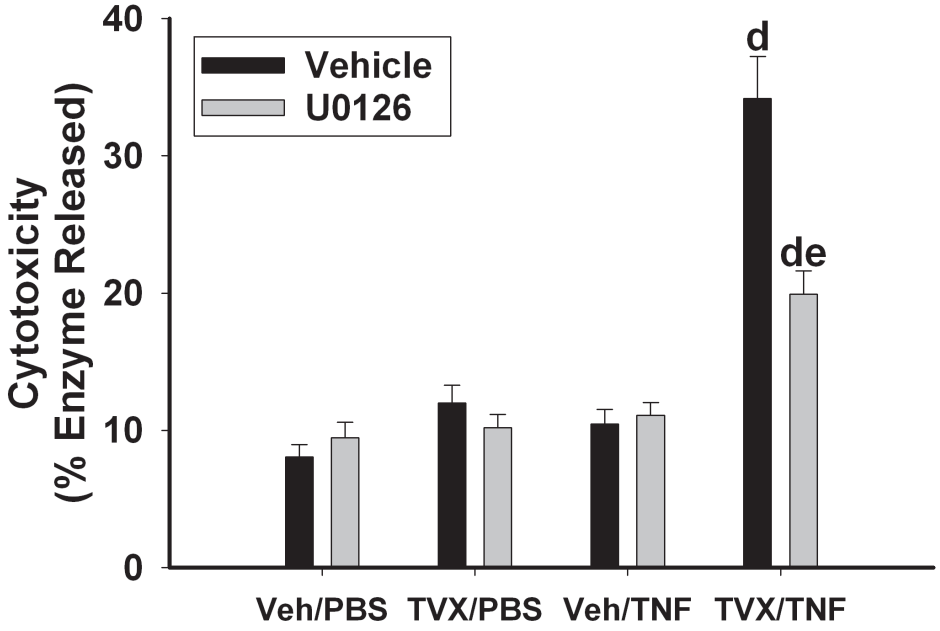


Figure 29. ERK is involved in TVX/TNF-induced cytotoxicity. Cells were treated simultaneously with 20 μM TVX or vehicle and with 4 ng/mL TNF or PBS in the presence of 10 μM U0126 or its vehicle. Cytotoxicity was assessed 24 hours after treatment. **d** Significantly different from respective TVX and TNF alone treated groups. **e** Significantly different from TVX/TNF-treated group in the absence of U0126. Data represent the mean ± SEM of 4 separate experiments.

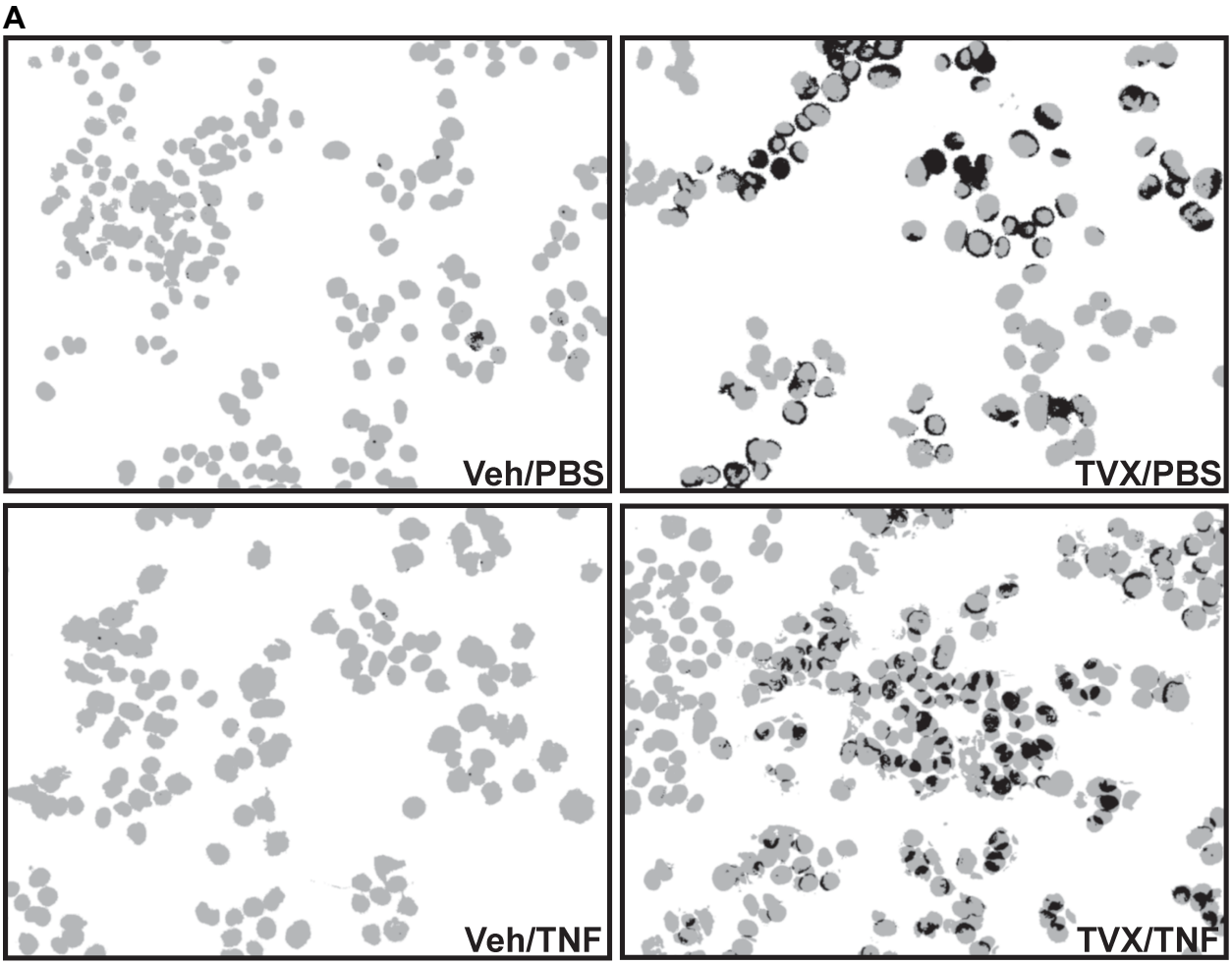
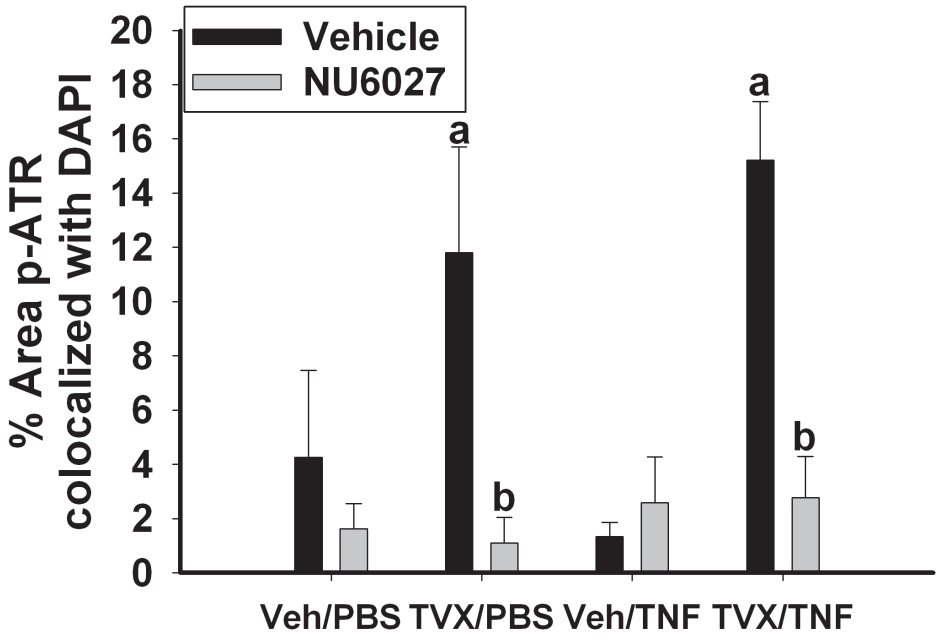


Figure 30. TVX treatment causes ATR activation. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS in the presence of 12.5 μM of the ATR inhibitor NU6027 or its vehicle. 6 hours after treatment, cells were subjected to immunolabeling for phospho-ATR (p-ATR) as described in Materials and Methods and imaged using fluorescence microscopy. (A) Representative images from each treatment without NU6027. Gray areas represent a positive DAPI signal. Black areas represent colocalized PI and DAPI signals, indicating p-ATR positive signal located in nuclei of cells. (B) Quantification of the area of p-ATR signal colocalizing with DAPI in

Figure 30 (cont'd)

B



response to treatment. **a** Significantly different from respective group without TVX. **b** Significantly different from same treatment without NU6027. Data represent the mean ± SEM of 3-4 separate experiments.

cells with NU6027 to inhibit ATR attenuated the TVX/TNF-induced cytotoxicity (Figure 31A). In contrast, treatment with either the ATM-selective inhibitor KU55933 (Hickson, et al., 2004) or wortmannin to inhibit DNA-PK (Okayasu, et al., 1998) failed to affect the cytotoxicity caused by TVX/TNF (Figure 31B and C).

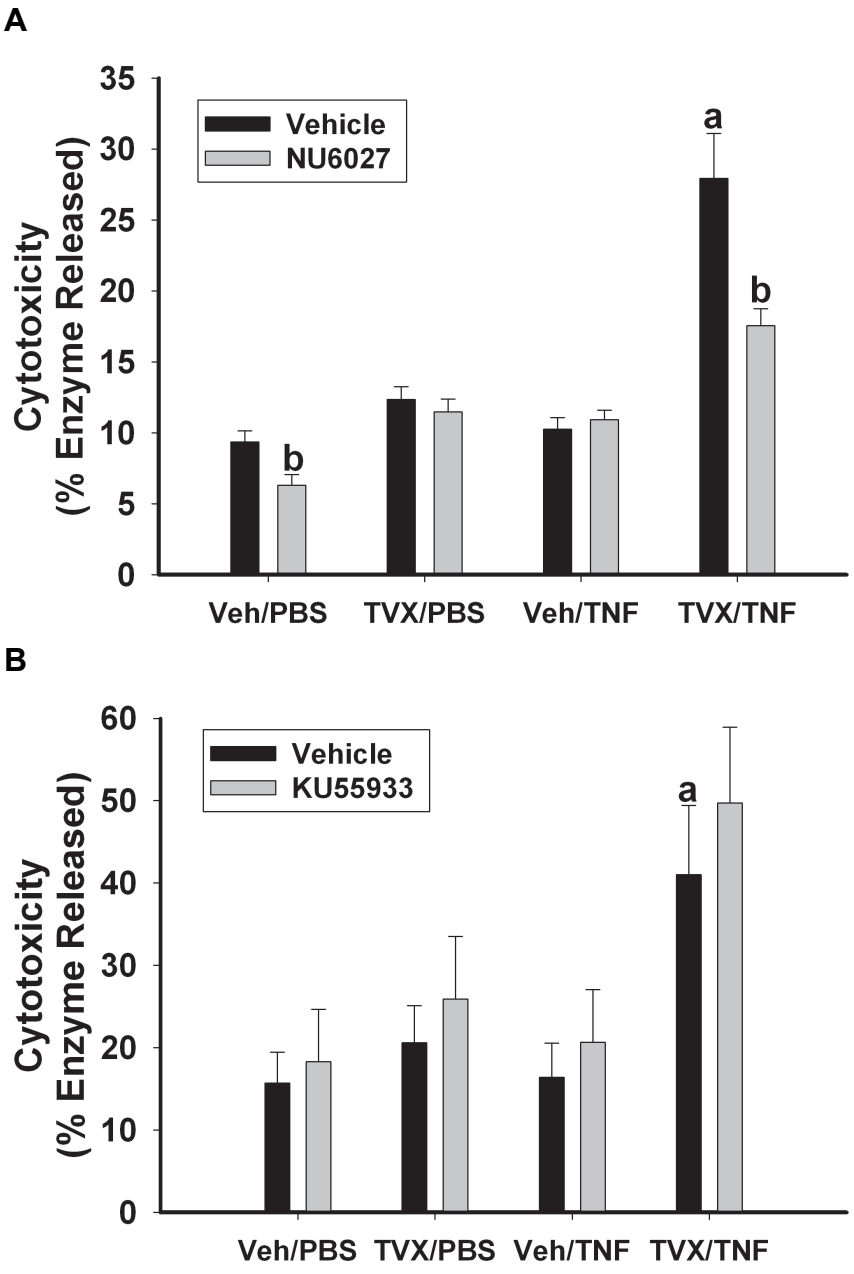
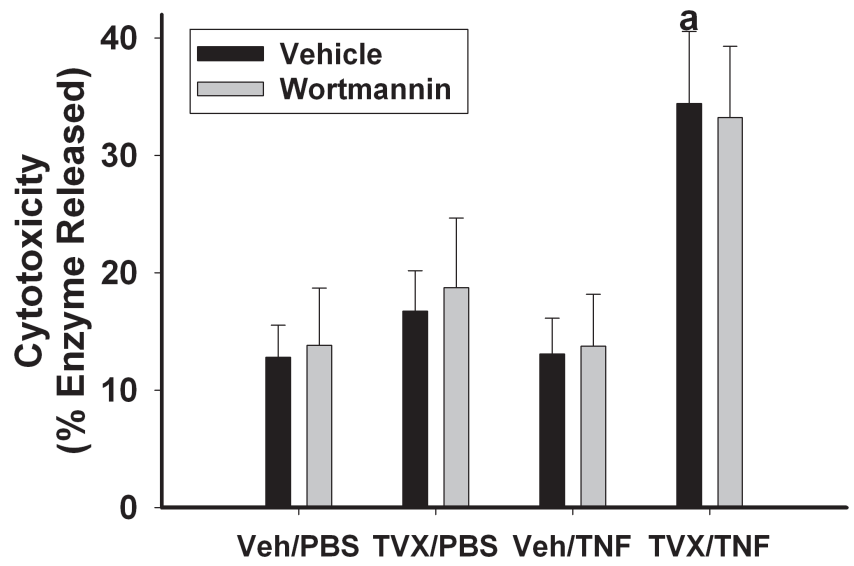


Figure 31. An inhibitor of ATR, but not of ATM or DNA-PK, attenuates TVX/TNF-induced cytotoxicity. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS in the presence of (A) 12.5 μM NU6027, (B) 20 μM KU55933, and (C) 10 μM wortmannin, or their respective vehicles. Cytotoxicity was assessed after 24 hours. **a** Significantly different from all other treatment groups in the absence of an inhibitor.

Figure 31 (cont'd)

c



b Significantly different from same treatment without inhibitor. Data represent the mean ± SEM of 3-7 separate experiments.

3.5 Discussion

We reported previously that treatment of hepatocytes with TVX plus TNF *in vitro* caused prolonged activation of JNK. Caspases 9 and 3 were also activated at the onset of cytotoxicity, and this was followed by DNA damage, suggesting an intrinsically mediated apoptotic cell death (Beggs, et al., 2013). One feature these events have in common is that each can be activated by genotoxic stress (Roos and Kaina, 2006), suggesting that the cell death signaling in response to TVX/TNF treatment might occur in response to DNA damage. In support of this, exposure of HepG2 cells to TVX/TNF led to an increase in γH2AX, a marker of DNA damage (Figure 20). This happened before the onset of cell death, which occurs between 16 and 20 hr (Beggs, et al., 2013).

Another common response to DNA damage is cell cycle arrest resulting in decreased cell proliferation (Houtgraaf, et al., 2006). Studies *in vitro* demonstrated that TVX causes a decrease in the rate of proliferation and cell cycle arrest in several cell types (Holtom, et al., 2000, Thadepalli, et al., 2005, Zakeri, et al., 2000). In a cell free system TVX inhibited eukaryotic topoisomerase-IIα, the human homolog to its target in bacteria (Poulsen, et al., 2011), which is involved in DNA replication and cell cycle regulation (Larsen, et al., 1996). Together, these findings suggest that TVX might cause cell cycle arrest through inhibition of human topoisomerase enzymes. Indeed, treatment of HepG2 cells with TVX resulted in cell cycle arrest and decreased proliferation (Figures 21-22), suggesting activation of the DNA damage response pathway. Interestingly, several other drugs that cause IDILI inhibit cell proliferation *in*

vitro as well (Basta-Kaim, et al., 2006, Chennamaneni, et al., 2012, Francavilla, et al., 1989, Rajabalian, et al., 2009).

Although DNA damage and cell cycle arrest do not always result in cell death, they can sensitize cells to the cytotoxic effects TNF (Gera, et al., 1993, Rodriguez, et al., 2007, Shih and Stutman, 1996). Therefore, activation of the DNA damage response pathway by TVX and perhaps other drugs with idiosyncratic liability might be a mechanism that sensitizes hepatocytes to TNF-induced apoptosis. The DNA damage response, including activation of cell cycle checkpoints leading to cell cycle arrest, is initiated through activation of the PI3K-like, serine-threonine protein kinases, ATM, ATR, and DNA-PK (Houtgraaf, et al., 2006, Yang, et al., 2003). Though activation of ATR is often associated with protection of cells against genotoxic stress, in certain circumstances ATR signaling can promote apoptosis (Joe, et al., 2006, Roos and Kaina, 2013, Yim, et al., 2006). Treatment of cells with TVX caused an increase in activated ATR in the nuclei (Figure 30), and inhibition of ATR attenuated the cytotoxicity induced by TVX/TNF cotreatment (Figure 31). In contrast, pharmacological inhibition of either ATM or DNA-PK did not alter the TVX/TNF-induced cytotoxicity. These results suggest that ATR, but not ATM or DNA-PK, contributes to cell death signaling initiated by TVX/TNF exposure.

Activation of the DNA damage response pathway can elicit cell cycle arrest and cytotoxicity through the activation of p53 (Haupt, et al., 2003, Lakin and Jackson, 1999). Activation of p53 occurs through a multitude of signaling events, including phosphorylation by ATR, which promotes p53 protein stabilization (Roos and Kaina, 2006). The activity of p53 as a transcription factor enables the expression of the

CDKN1A gene to promote cell cycle arrest through p21. It also transcribes the proapoptotic Bcl-2 family members p53 upregulated modulator of apoptosis (PUMA) and Noxa, which promote activation of caspase 9 and ultimately caspase 3, resulting in apoptosis (Haupt, et al., 2003). We have previously shown that both caspase 9 and caspase 3 are activated upon treatment with TVX/TNF (Beggs, et al., 2013). Nonetheless, neither p53 mRNA nor protein was increased by exposure to TVX, TNF or the combination (Figures 23-24). Furthermore, inhibition of p53-mediated transcription failed to affect TVX/TNF-induced cytotoxicity (Figure 25). These results suggest that p53 expression does not play a role in the TVX/TNF-induced cytotoxicity in HepG2 cells. Although this is surprising since p53 is typically involved in the DNA damage response, there are other examples in which cell cycle arrest, p21 upregulation and cytotoxicity resulting from DNA damage are p53-independent (Jeong, et al., 2010, Macleod, et al., 1995).

DNA damage activates the CDKN1A gene, resulting in expression of p21, which promotes cell cycle arrest through inhibition of the activity of the cyclin-dependent kinases (Gartel and Tyner, 2002). During the G1/S transition of the cell cycle, the inhibition of the Cdk2-Cyclin E complex by p21 prevents phosphorylation of the downstream target retinoblastoma (Rb) transcriptional repressor. In this hypophosphorylated state, Rb interacts with and suppresses the E2F family transcription factors, which regulate genes that promote entry into S phase and DNA synthesis (Bartek and Lukas, 2001). In a murine model of TVX/LPS-induced liver injury, the livers of mice treated with TVX/LPS had a 12-fold increase in CDKN1A expression compared to vehicle-treated animals (Shaw, et al., 2009b). Similarly, treatment of

HepG2 cells with TVX caused an increase in CDKN1A mRNA and p21 protein (Figure 26). TVX also induced G0/G1 cell cycle arrest (Figure 21). Arrest of the cell cycle in G0/G1 occurs to allow for repair damaged DNA and often prevents apoptosis. However, p21 protein expression is not increased in TVX/TNF-treated cells. One explanation for this discrepancy is that caspase 3 could be cleaving p21, which promotes apoptosis during DNA damage (Chai, et al., 2000, Zhang, et al., 1999). The temporal relationship between caspase activation and p21 suggests this could be occurring in HepG2 cells. TVX/TNF cotreatment activates caspase 3 at 8 hours (Beggs, et al., 2013), which occurs before p21 protein is significantly increased.

Another common cellular response to DNA damage is activation of MEK/ERK signaling. Activation of this pathway occurs in response to a multitude of DNA damaging agents and can play a role in altering the cell cycle and promoting apoptosis (Cagnol and Chambard, 2010). Treatment with TVX resulted in ERK activation starting 6 hours after treatment and lasting through 24 hours (Figure 27). In cells treated with either Veh or TNF, inhibition of ERK by U0126 resulted in an increase in the population of cells in the G0/G1 phase of the cell cycle and a decrease in the population of cells in S phase. However, ERK inhibition failed to modify the pronounced TVX- or TVX/TNF-induced decrease in the population of cells in S-phase (Figure 28). This suggests that ERK signaling normally promotes G1 to S progression in HepG2 cells but does not play a role in the TVX-induced cell cycle arrest. Despite its lack of influence on cell cycle arrest, ERK signaling did contribute to TVX/TNF-induced cell death, as evidenced by the pronounced decrease in cytotoxicity that occurred in the presence of U0126 treatment (Figure 29). Others have shown that ERK signaling can induce intrinsically

mediated apoptosis and transcription of proapoptotic Bcl-2 family members Bax, Bak, and PUMA, as well as postranslationally modify the antiapoptotic Bcl-2 by phosphorylation. All of these events can disrupt the mitochondrial membrane to facilitate cytochrome c release that precedes activation of caspase 9 and apoptosis (Cagnol and Chambard, 2010, Tamura, et al., 2004). Together these results suggest that ERK plays a role in the cytotoxic signaling induced by TVX/TNF.

Many of the changes observed were driven primarily by TVX. For example, treatment of HepG2 cells with TVX caused DNA damage. In response to this, ATR was activated in the nucleus and p21 expression was induced. These events preceded cell cycle arrest at the G1/S checkpoint and resulted in inhibition of cell proliferation. None of these events resulted in overt cell death within 24 hours of treatment with TVX alone; rather, cell death required the presence of both TVX and TNF. In the presence of TNF, the DNA damage caused by TVX was exacerbated and pronounced cytotoxicity occurred. Also, exposure to TVX induced activation of both ERK and ATR, and both of these contributed to cytotoxicity only when TNF was present. This suggests that another component of the TNF signaling pathway is required to evoke cell death through ATR and ERK. This component might be JNK, since TVX/TNF coexposure caused a prolonged activation of JNK that was involved in promoting cytotoxicity (Beggs, et al., 2013). Both ERK and JNK are able to disrupt mitochondrial homeostasis and promote cytochrome c release, resulting in an intrinsic apoptotic mode of cell death (Cagnol and Chambard, 2010, Win, et al., 2011). In cells treated with both TVX and TNF, cytochrome c release could require the actions of both JNK and ERK on

mitochondria, as the signaling of either one by itself might not be sufficient to disrupt the mitochondrial membrane enough to promote cell death.

Although these results indicate clearly that a drug associated with human IDILI can cause DNA damage that activates cell death signaling pathways, the relationship of the results to liver injury in vivo from exposure to this or other IDILI-associated drugs remains to be proven. It is of interest, however that several other drugs associated with human IDILI are able to induce cell cycle arrest in vitro (Basta-Kaim, et al., 2006, Chennamaneni, et al., 2012, Francavilla, et al., 1989, Rajabalian, et al., 2009). Our results suggest that DNA damage induced by certain drugs might act as a first insult that sensitizes cells to a secondary insult from cytokines such as TNF that are produced by an activated immune system. Knowledge of the mechanism(s) involved in the interaction between the DNA damage response and death receptor ligand signaling might enhance understanding of IDILI pathogenesis and susceptibility factors in human patients.

CHAPTER 4

Summary and Conclusions

4.1 Considerations for in vitro model development

The hypothesis tested in this dissertation is that TVX and TNF can interact to cause hepatocellular apoptosis involving NF-κB, JNK, caspase activation and components of the DNA damage response such as ERK and the PIKKs. These studies were focused on the hepatocyte in an attempt to parse out the critical cell death signaling events occurring in parenchymal tissue. This hypothesis was tested in the HepG2 human hepatoblastoma cell line. The high cost of obtaining primary human hepatocytes is a limiting factor that impedes their use for these studies. Primary human hepatocytes also are exposed to variable stimuli such as xenobiotics, genetic differences, environmental factor exposure, and disease status before they become available. These factors contribute to experimental variability (LeCluyse, 2001, Liguori, et al., 2005). The HepG2 cell line was chosen as a substitute because these cells propagate well in culture, they are currently used in preclinical testing and they are of human lineage. Studying a human cell line can help determine whether or not the interaction of TVX with inflammatory mediators to cause hepatotoxicity is only observed in rodents, and if this interaction has any significance to humans.

There are a number of differences between primary human hepatocytes and HepG2 cells. Most notably, these differences occur in the expression levels of genes involved in xenobiotic biotransformation and cell cycle regulation. Notably, HepG2 cells express lower levels of phase I metabolizing enzymes, particularly CYP 450 family enzymes (Rodriguez-Antona, et al., 2002). The difference in expression of phase II conjugating enzymes between HepG2 cells and primary human hepatocytes was not as significant, but decreased expression of some glucuronyltransferases was observed in

HepG2 cells (UGT1A6 and UGT2B15). Conversely, HepG2 cells had a greater expression of sulfotransferase enzymes compared to primary human hepatocytes (Liguori, et al., 2008). Increased expression of genes involved in cell cycle regulation included cell division cycle 2, cell division cycle associated 8, and cyclins E1 and A2, all of which were increased in HepG2 cells relative to primary human hepatocytes.

Although the expression of metabolizing enzymes is decreased in HepG2 cells, this is predominantly regarding phase I oxidation enzymes. TVX is mostly eliminated as the unaltered parent compound, and the remaining drug undergoes phase II reactions (Figure 4). This makes the lack of phase I enzymes in HepG2 cells less concerning. Also, because much of the drug is not metabolized the minor differences in phase II enzymes in HepG2 cells are less concerning. However, the differences in the expression levels of the cell cycle-related genes require more consideration and will be discussed later.

Lastly, it is important to note that TVX caused a number of similar changes in gene expression in both HepG2 cells and primary human hepatocytes that were not caused by other fluoroquinolones in either cell type (Liguori, et al., 2008). The regulation of this critical set of genes altered only by TVX was similar in both cell types. Overall the use of HepG2 cells in culture to study the interaction of TVX with TNF should allow for the identification of signaling elements involved in promoting cytotoxicity.

4.2 Summary of research

Initial concentration-response experiments demonstrated that neither TVX (up to 200 μM) nor TNF (up to 1000 ng/mL) caused any cytotoxicity. This corresponds with other reports studying these compounds individually in HepG2 cells (Hill, et al., 1995, Liguori, et al., 2008). However, exposure to TVX and TNF in combination did result in cytotoxicity in a concentration-dependent manner (Figure 9). This observation that increasing the concentration of either or both TVX and TNF resulted in a corresponding increase in cytotoxicity suggests that—in the context of LPS sensitizing the liver to injury—there might be a true dose-dependent relationship between drugs associated with IDILI and liver injury if inflammatory mediators are present (Figure 2). Cytotoxicity was not observed in cells treated with LVX in the presence of TNF (Figure 10). This result correlated with the finding that LVX did not interact with LPS to promote liver injury *in vivo* (Shaw, et al., 2007). This suggests that use of hepatocytes in culture to study the interaction of drug candidates with TNF to cause cytotoxicity *in vitro* might adequately distinguish between those drug candidates that have idiosyncratic liability from those that do not.

Based on the results obtained from the concentration-response studies, a combination treatment of 20 μM TVX and 4 ng/mL TNF was chosen for further investigation of this model. The concentration of TVX was chosen for its ability to cause a robust and repeatable cytotoxic interaction in the presence of TNF. 20 μM also is a concentration close to that observed in the plasma of patients undergoing TVX therapy, typically on the order of 2-10 μM (Melnik, et al., 1998, Teng, et al., 1996, Vincent, et al., 1997). Also, hepatocytes themselves could realistically be exposed to 20 μM TVX

considering that the liver is exposed to higher concentrations of TVX than every other organ during oral TVX pharmacotherapy (Fischman, et al., 1998). Similarly, the concentration of 4 ng/mL of TNF was chosen based on concentrations observed in humans who were administered LPS, as well as the plasma concentrations observed in the murine model of TVX/LPS-induced liver injury (Copeland, et al., 2005, Shaw, et al., 2007, Taudorf, et al., 2007). Together, these concentrations could approximate what might be observed in the livers of patients undergoing TVX therapy during a concurrent inflammatory stress.

To ascertain the type of cell death that occurred in response to TVX/TNF cotreatment, flow cytometric analysis of cell viability was performed. After 24 hours of treatment, TVX/TNF caused apoptotic cell death (Figure12). This treatment also caused a significant increase in the amount of early apoptotic cells that have external phosphatidylserine (PS) exposure but do not yet have compromised plasma membranes. Interestingly, TVX treatment alone caused a less severe but significant increase in the population of both early and late apoptotic cells. This indicated that treatment with TVX stressed the HepG2 cells in a way that did not result in overt cell death. Employing the TUNEL method revealed that DNA damage occurred in cotreated cells (Figure 14). Together, these studies demonstrated that TVX/TNF exposure induced apoptosis of HepG2 cells.

The observation that TVX/TNF cotreatment induced hepatocellular apoptosis *in vitro* contrasts with the liver histopathology findings *in vivo*. Although both TVX/LPS and TVX/TNF treatment caused some hepatocellular apoptosis, the liver injury observed in cotreated mice was predominantly oncotic necrosis (Shaw, et al., 2007, Shaw, et al.,

2009c). The oncotic necrosis that occurred after treatment *in vitro* was minimal, less than 2% for all treatments. One reason for this discrepancy might involve an early signaling event in apoptosis, such as the translocation of PS residues from the inner leaflet of the plasma membrane to the outer leaflet. This exposure of PS to the external environment occurs as a signal alerting neighboring cells that the PS exposed cell is about to undergo apoptosis; this stimulates the surrounding cells to phagocytose the dying cell (Fadok, et al., 1998, Martin, et al., 1995). However, PS exposure also activates the coagulation system (Heemskerk, et al., 2002, Lentz, 2003). The initiation of apoptosis in hepatocytes could be responsible for fibrin deposition and thrombi formation (Miyamoto, et al., 2004). As a consequence of clot formation, an impedance of blood flow could occur resulting in localized hypoxia and decreased adenosine triphosphate (ATP) formation (Malhi, et al., 2006). Decreased cellular ATP levels can ultimately promote oncotic necrosis (Leist, et al., 1997b, Tsujimoto, 1997).

It is possible that in the livers of mice treated with TVX and LPS, apoptosis is an initiating event that promotes coagulation system activation. Mice treated with both TVX and LPS had significant fibrin deposition in the sinusoids of the liver 4.5 hours after LPS administration (Shaw, et al., 2009c). The livers of mice treated with either TVX/LPS or TVX/TNF ultimately undergo oncotic necrosis that peaks between 15 and 21 hours after inflammagen administration (Shaw, et al., 2007). However, at an earlier time, the liver sections of mice treated with TVX and TNF predominantly display markers of apoptosis, including chromatin marginalization, TUNEL staining, and active caspase 3 (Shaw, et al., 2009a). These were observed 4 hours after TNF was administered, a time before fibrin deposition and coagulation system activation was observed. Collectively these

data suggest that cotreatment of mice with either TVX/LPS or TVX/TNF initiates apoptosis in the liver, and that the resulting exposure of PS to the outer leaflet of the plasma membrane could promote coagulation system activation that ultimately results in hypoxia-related oncotic necrosis at later times.

Caspase enzymes are significant contributors to cellular apoptosis, and their involvement in cell death was examined. Cell viability was measured in cells treated with the pancaspase inhibitor ZVAD. Inhibition of caspases using ZVAD resulted in a decrease in the percentage of early apoptotic cells in response to TVX and TVX/TNF treatment, as well as a decrease in the percentage of late apoptotic cells in response to TVX/TNF (Figure 12). Similarly, ZVAD completely prevented TVX/TNF-induced cytotoxicity as determined by membrane leakage (Figure 13). These results identified caspases as critical mediators of TVX/TNF-induced cytotoxicity.

To confirm that these results were indicative of what is occurring in a non-transformed hepatocyte, the interaction of TVX with TNF to cause cytotoxicity was examined in primary murine hepatocytes. Similar to the results in the HepG2 cells, TVX, but not LVX, interacted with TNF to cause cytotoxicity in a concentration-dependent manner in the murine hepatocytes (Figure 19). Caspase enzymes also critically mediated this cell death suggesting similarities in the signaling pathways between primary hepatocytes and HepG2 cells.

TVX/TNF-induced cytotoxicity of HepG2 cells commenced between 8 and 12 hours of treatment and became significant at 20 hours (Figure 11). This suggested that critical signaling elements involved in promoting cell death were activated at or before 8 hours. As a result, the involvement of caspase enzymes and JNK were examined in

this time frame. Caspases 9 and 3 were activated at 8 hours, congruent with the onset of cytotoxicity, however caspase 8 did not become significantly activated until between 16 and 24 hours (Figure 15). This latter result was surprising, considering that sensitized hepatocytes undergo apoptotic signaling in response to TNF binding to TNFR1 that initiates caspase 8 activation through the extrinsic apoptotic pathway. These results suggested the cytotoxicity that occurred was intrinsically mediated apoptosis, and that the TNF-mediated signaling involved in the cytotoxicity most likely occurred through activation of one or several of the MAPKs.

Hepatocellular apoptosis involving TNF suggests that prolonged activation of the JNK MAPK is a significant contributor to cell death (Ding and Yin, 2004, Win, et al., 2011, Wullaert, et al., 2006). TNF exposure induced JNK activation in HepG2 cells at 1 hour (Figure 17). This effect was potentiated in the presence of TVX. At 8 hours of treatment, JNK activation in response to TNF had diminished. However, JNK was still activated in TVX/TNF-cotreated cells, indicating that exposure to both TVX and TNF caused a more robust and longer lasting activation of JNK. Interestingly, TVX exposure alone was able to activate JNK at 8 hours. JNK becomes activated in response to cellular stress, and similar to the observation that TVX increased the population of early apoptotic cells (Figure 12), these results together suggested that TVX mildly stressed the HepG2 cells without causing cell death after 24 hours.

JNK contributes to apoptosis by phosphorylating downstream c-Jun to induce transcription of proapoptotic genes as well as by phosphorylating antiapoptotic bcl family proteins on the mitochondria, which promotes caspase activation. The kinase activity is inhibited by SP600125. Exposure of cells to SP600125 attenuated TVX/TNF-

induced cytotoxicity, indicating that JNK contributed to the cell death (Figure 18). TNF also activates p38 in hepatocytes, and the kinase activity of p38 is inhibited by SB203580 (Bhat, et al., 1998). The role of p38 towards TVX/TNF-induced cytotoxicity was examined by treating cells with SB203580. Unlike inhibiting JNK, inhibition of p38 failed to change the cytotoxicity induced by TVX/TNF coexposure, indicating that p38 signaling does not promote cell death in this model (Figure 32).

The involvement of p38 in promoting liver injury in TVX/LPS-treated mice cannot be ignored completely. In the liver, p38 becomes activated in response to LPS ligation of TLR4 on Kupffer cells. p38 regulates TNF mRNA stabilization and translation, and can activate the TNF alpha converting enzyme (TACE) responsible for cleaving membrane-bound TNF to release soluble, active TNF (Deng, et al., 2009, Hitti, et al., 2006, Neininger, et al., 2002). In a model investigating the interaction of neutrophil elastase (NE) with tissue hypoxia, p38 caused an upregulation of proapoptotic genes and promoted hepatocellular cytotoxicity (Sparkenbaugh, et al., 2012). The interaction of NE with tissue hypoxia might occur in the livers of TVX/LPS-treated mice. In this model, fibrin deposition is observed and can cause local hypoxia. Neutrophils also are recruited to the liver, and genetic knock out of NE protected mice from TVX/LPS-induced hepatotoxicity. All of these effects in sinusoids should be taken into consideration, but p38 does not seem to be involved in the direct cytotoxic signaling induced by TVX/TNF in hepatocytes.

Prolonged JNK activation, as well as the activation of caspase enzymes in hepatocytes, often is associated with a disruption in NF-κB signaling (Wajant, et al., 2003, Wullaert, et al., 2007). TNF activates NF-κB-mediated upregulation of

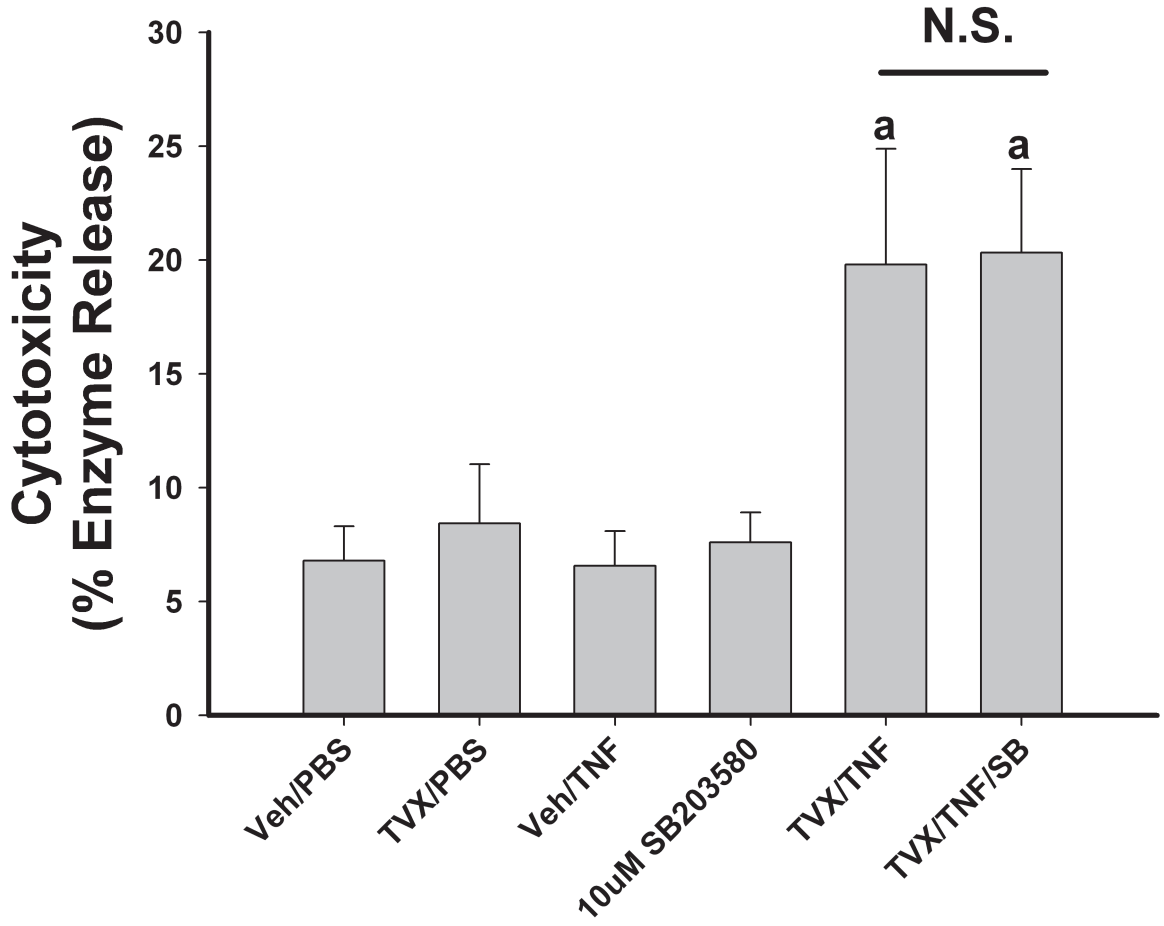


Figure 32. Inhibition of p38 does not affect TVX/TNF-induced cytotoxicity. Cells were treated with 20 μM TVX or Veh and 4 ng/mL TNF or PBS, as well as 10 μM SB203580 for 24 hours before cytotoxicity was measured. **a** Significantly different from Veh/PBS, TVX/PBS, Veh/TNF, and SB203580 treated groups. **N.S.** Not significantly different. Data represent the mean ± SEM of 3 separate experiments.

cytoprotective genes that neutralize oxidative stress from ROS and prevent caspase activation. The observation that a drug associated with IDILI might interfere with NF-κB signaling in response to TNF has been demonstrated with diclofenac. This study conducted in HepG2 cells revealed that diclofenac decreased the TNF-induced NF-κB activity (Fredriksson, et al., 2011). This corresponded to an apoptotic cell death that involved caspases and JNK.

To investigate if TVX could interfere with TNF-mediated upregulation of cytoprotective genes, mRNA expression of Bcl-xL and XIAP were examined, both of which are NF-κB responsive genes. These genes are involved in inhibiting the activities of caspases 9 and 3 (Hu, et al., 1998, Scott, et al., 2005, Shiozaki, et al., 2003). In response to TNF, only Bcl-xL mRNA was increased at 3.5 hours, and TVX did not alter the expression of this gene (Figure 33A). Exposure of cells to TNF did not alter XIAP expression either at 3.5 or 5 hours. However, at 5 hours of treatment there was a TVX-mediated decrease in XIAP expression (Figure 33B). Together, these results suggest that TVX has the potential to interfere with the upregulation of cytoprotective genes, but the HepG2 cell might not be the ideal system to study TNF-mediated NF-κB activation.

NF-κB is constitutively active in many tumor cell types, including HepG2 cells (Bargou, et al., 1997, Fujioka, et al., 2003, McElwee, et al., 2009, Takada, et al., 2005, Wang, et al., 1999). This constitutive activity might account for the lack of significant TNF-induced upregulation of cytoprotective genes in the HepG2 cells. Although gene array studies conducted in whole livers of animals did not identify TVX/LPS-associated changes in the expression of cytoprotective genes (Shaw, et al., 2009b), this might not be the case for isolated (non-transformed) hepatocytes. For example, in primary murine

A

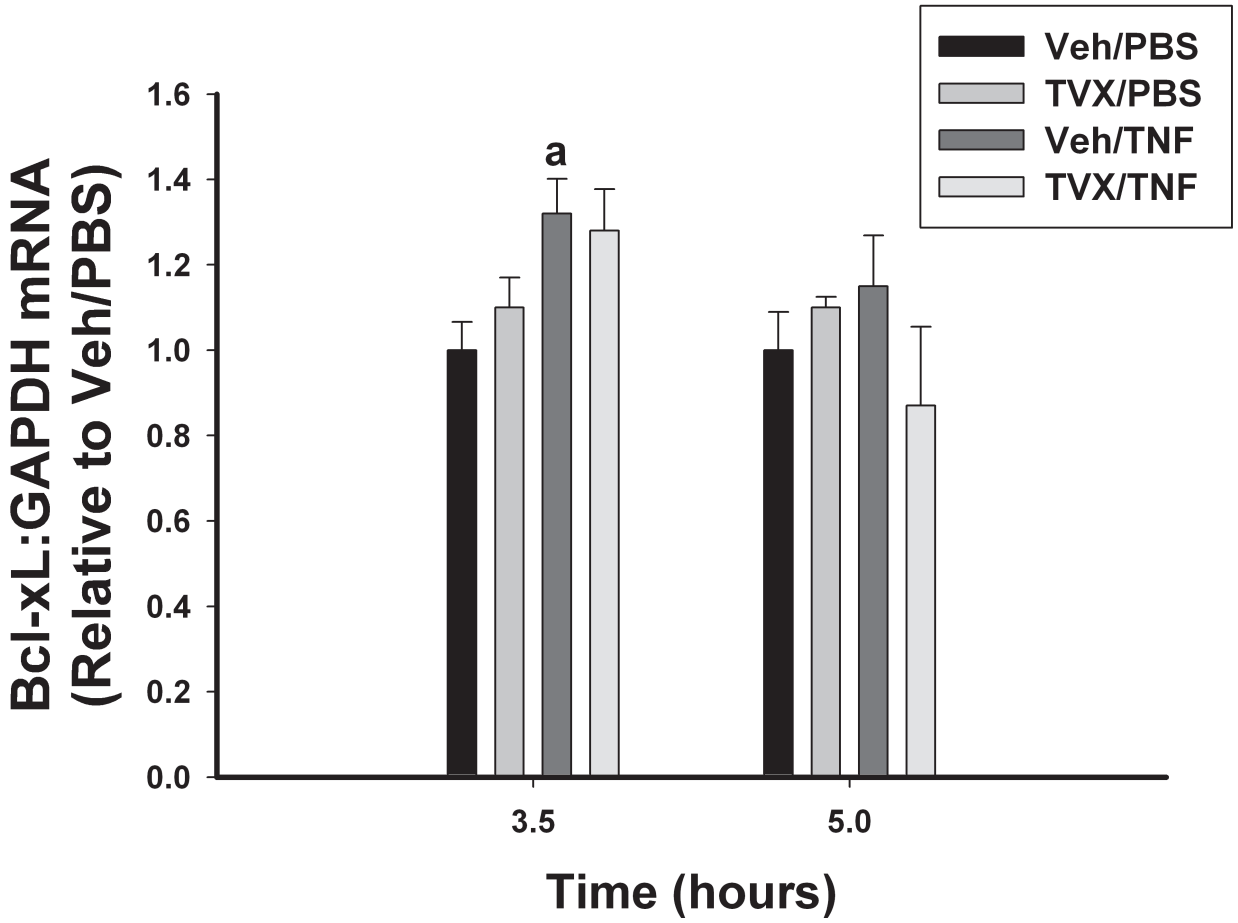
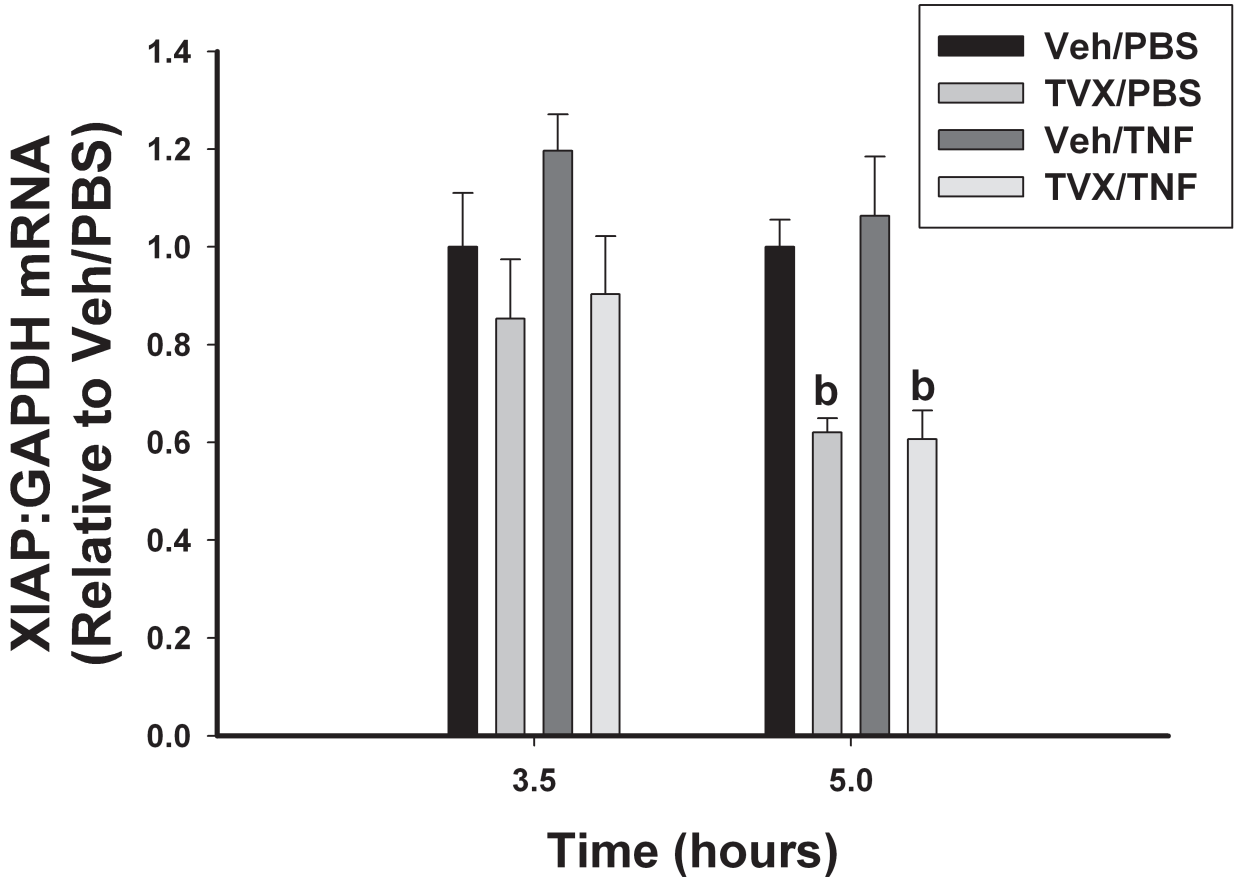


Figure 33. Treatment-induced changes in Bcl-xL and XIAP gene expression. Cells were treated with TVX or Veh and TNF or PBS for either 3.5 or 5 hours before mRNA was isolated. Relative mRNA expression was determined by qRT-PCR. (A) Bcl-xL and (B) XIAP mRNA expression relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **a** Significantly different from Veh/PBS-treated group.

Figure 33 (cont'd)

B



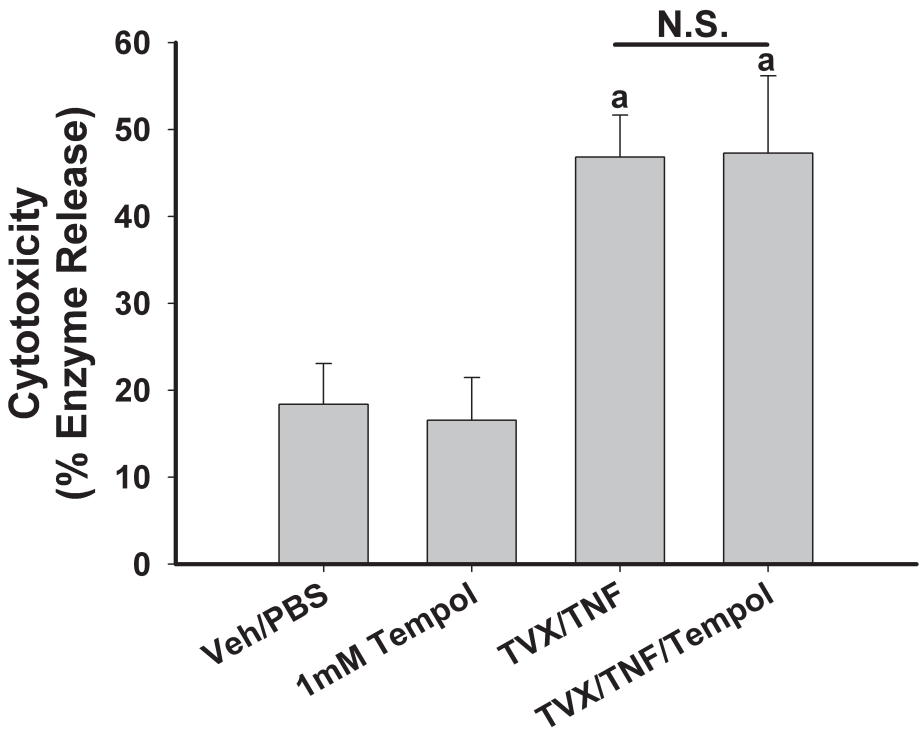
b Significantly different from corresponding group in the absence of TVX. Data represent the mean ± SEM of 3 separate experiments.

hepatocytes exposure to TNF caused an increase in genes associated with inhibition of caspase activation, and treatment with a topoisomerase inhibitor decreased the TNF-mediated increase in these genes (Hentze, et al., 2004). Therefore, in studies focused on the interaction of drugs with idiosyncratic liability and TNF on hepatocytes, the TNF-mediated upregulation of cytoprotective genes should not be ignored. However, HepG2 cells might not be an ideal system to focus on these studies.

Prolonged JNK activation is frequently associated with cellular oxidative stress (Papa, et al., 2004, Seki, et al., 2012, Wullaert, et al., 2006). Antioxidant treatment and the dichlorodihydrofluorescein diacetate method of measuring ROS (Eruslanov and Kusmartsev, 2009) were used to investigate the involvement of oxidative stress in promoting TVX/TNF-induced cytotoxicity. Cells were treated with a water-soluble antioxidant (tempol) or the lipid-soluble antioxidant (alpha-tocopherol), as well as N-acetylcysteine (NAC) that promotes glutathione synthesis (Buettner, 1993, Kelly, 1998, Wilcox, 2010). None of the antioxidants caused any change in the TVX/TNF-induced cytotoxicity (Figure 34A-C). Congruently, ROS production was not observed in response to any treatment (Figure 34D). These results suggest that oxidative stress did not occur in response to treatment and did not contribute to TVX/TNF-mediated cell death.

Prolonged JNK activation also occurs in response to genotoxic stress (Chen, et al., 1996, Roos and Kaina, 2006). Also, DNA damaging agents often cause intrinsically mediated apoptosis (Fogg, et al., 2011, Jendrossek, 2012, Roos and Kaina, 2006). Exposure of cells to both TVX and TNF resulted in prolonged JNK activation and intrinsic apoptosis, which presented the possibility that genotoxic stress might be

A



B

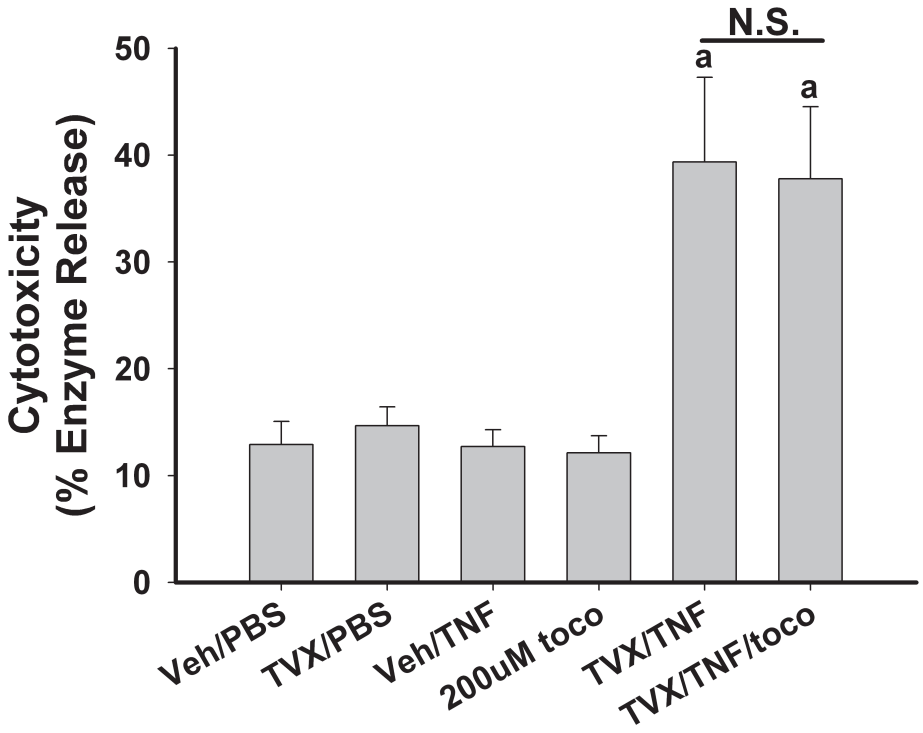
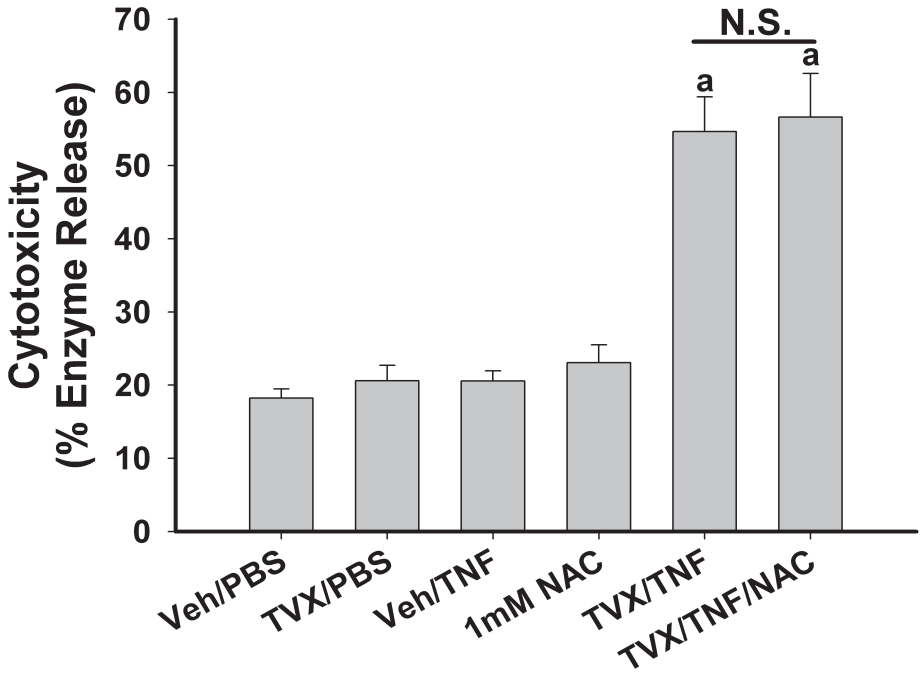


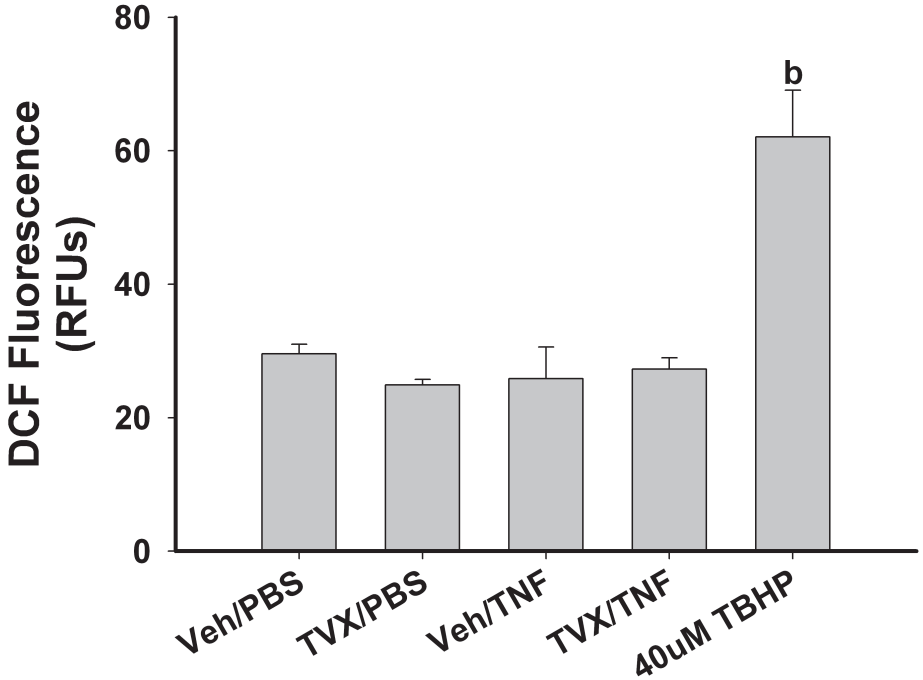
Figure 34. TVX/TNF-induced cytotoxicity does not involve oxidative stress.

Figure 34 (cont'd)

C



D



(A-C) Cells were treated simultaneously as indicated. Cells were exposed to different antioxidants including (A) Tempol, (B) α-tocopherol (toco), or (C) N-acetylcysteine

Figure 34 (cont'd)

(NAC). Cytotoxicity was measured 24 hours after treatment. **a** Significantly different from all non-TVX/TNF-treated groups. **N.S.** Not significantly different. Data represent the mean ± SEM of 3 separate experiments. (D) Cells were treated with TVX or Veh and TNF or PBS, or the prooxidant tert-butyl hydroperoxide (TBHP). Fluorescence was measured 6 hours after treatment. **b** Significantly different from all other treatment groups. Data represent the mean ± SEM of 4 separate experiments.

involved in the cell death that occurs in this model. Supporting this notion, topoisomerase IIα activity was inhibited by TVX, which can cause DNA damage (Poulsen, et. al., 2011). Other studies demonstrated that TVX induced cell cycle arrest, which is a common response to DNA damage (Holtom, et al., 2000, Thadepalli, et al., 2005, Zakeri, et al., 2000). For these reasons, the DNA damage response was investigated.

Exposure of HepG2 cells to both TVX and TNF resulted in DNA damage that occurred within 12 hours after treatment (Figure 20). This could be in response to the caspase 3 activation that occurred in cotreated cells at 8 hours, since caspase 3 can promote DNA damage (Andera, 2009, Fan, et al., 2005, Wullaert, et al., 2007). This DNA damage was more severe at 24 hours. At this time, DNA damage was also detected in cells exposed to TVX alone. This suggested that TVX caused DNA damage in HepG2 cells that became exacerbated in the presence of TNF. TVX also caused G0/G1 cell cycle arrest that resulted in decreased DNA synthesis at 12 hours (Figure 21). In addition, exposure of cells to TNF caused a more modest G0/G1 arrest. However, in cotreated cells TNF did not affect the TVX-induced cell cycle arrest. As a result of this cell cycle arrest, the proliferation of cells exposed to TVX was decreased (Figure 22).

These results suggested that TVX caused genotoxic stress in the HepG2 cells. In response to this, cells were arrested in the G0/G1 phase of the cell cycle, and proliferation was decreased. However, DNA damage was not detected in TVX exposed cells until 24 hours. Cell cycle arrest occurred at 12 hours in response to TVX, which was before DNA damage was detected in TVX exposed cells. Although γH2AX is

currently the most sensitive marker for detecting DNA damage, it is associated with DNA double strand breaks (Kinner, et al., 2008, Rogakou, et al., 1998, Sharma, et al., 2012). It could be that other types of DNA damage, such as single-strand breaks, occurred in response to TVX. Also, if not repaired properly single strand breaks in DNA can act as precursors to double strand breaks (Cortes-Ledesma and Aguilera, 2006, Kuzminov and Stahl, 1999).

Single strand breaks in DNA cause cell cycle arrest (Kitagawa, et al., 2007, Sertic, et al., 2012, Smith, et al., 2010). The idea that TVX might cause single stranded DNA lesions is something to be considered going forward. Alternatively, DNA damage as detected by γH2AX might not be required to induce cell cycle arrest. A study investigating the effects of another fluoroquinolone ciprofloxacin on a lymphoblastoid cell line demonstrated clearly that stabilized cleavage complex formation and cell cycle arrest occurred prior to γH2AX detection (Smart, et al., 2008).

p53 is prominently involved in cell cycle regulation and apoptotic signaling in response to DNA damage (Haupt, et al., 2003, Lakin and Jackson, 1999). Surprisingly, neither p53 transcript nor protein expression changed in response to TVX or TVX/TNF exposure (Figures 23-24). Exposure of cells to a p53 inhibitor also did not alter the cytotoxicity in response to TVX/TNF (Figure 25). This suggested that p53 was not involved in the responses mediated by TVX or TVX/TNF in HepG2 cells. Although many transformed cell lines have a mutated p53 gene, HepG2 cells express a functional, wild type p53 (Hosono, et al., 1991). To confirm that p53 was present and inducible in response to genotoxic stress, HepG2 cells were exposed to the topoisomerase inhibitor doxorubicin, and a prominent induction in p53 protein was

observed (Figure 35). Together, these results demonstrate that p53 is present and inducible in HepG2 cells but is not involved in the TVX/TNF-induced cytotoxicity.

The lack of p53 involvement also is surprising considering that CDKN1A gene expression was induced in a TVX-dependent manner (Figure 26). The induction of the CDKN1A gene that encodes p21 is largely regulated by p53-mediated transcription, and this is ultimately a manner in which p53 induces cell cycle arrest (Agarwal, et al., 1995, Bunz, et al., 1998, Waldman, et al., 1995). However, p21 induction and cell cycle arrest can occur independently of p53 (Aliouat-Denis, et al., 2005, Huang, et al., 2000, Huo, et al., 2004, Megyesi, et al., 1996). For example, the signal transducer and activator of transcription (STAT) family of transcription factors can induce p21 expression (Abbas and Dutta, 2009). Alternatively, a group of p53 homologs known as p63 and p73 also could be involved (Arrowsmith, 1999, Levrero, et al., 2000). Both of these transcription factors bind to the promoters of several p53 responsive genes, including p21. They also can promote apoptosis by inducing apoptotic gene expression or by localizing to the mitochondria to initiate intrinsic apoptosis (Roos and Kaina, 2006). Although p53 itself might not be involved in the TVX-mediated cell cycle arrest or the TVX/TNF-induced cytotoxicity, the involvement of p63 and p73 should be considered.

CDKN1A gene induction occurred in response to both TVX alone and TVX/TNF cotreatment. However, statistically significant p21 protein induction occurred only in response to TVX exposure (Figure 26). This suggested that TNF could be causing a posttranslational modification resulting in decreased p21 expression. One possibility is that p21 was cleaved by caspase 3 in cotreated cells. TNF can decrease p21 expression, and other studies revealed that cleavage of p21 by caspase 3 promotes

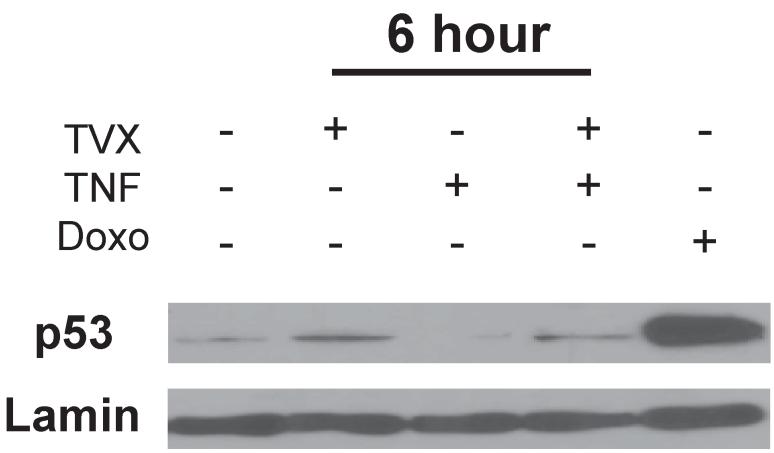


Figure 35. Doxorubicin-induced p53 protein induction in HepG2 cells. Cells were treated with 20 μM TVX or Veh and with 4 ng/mL TNF or PBS, or with 10 μM doxorubicin (Doxo). After 6 hours of treatment protein was isolated. p53 and Lamin were detected by western analysis.

apoptosis in arrested cells (Cao, et al., 2005, Chai, et al., 2000, Gartel and Tyner, 2002, Levkau, et al., 1998, Zhang, et al., 1999). Temporally, this seems like a reasonable possibility, since caspase 3 becomes activated at a time before p21 protein expression is increased.

Another significant signaling event that occurs in response to genotoxic stress is activation of ERK, which is involved in cell cycle regulation as well as promoting apoptosis (Cagnol and Chambard, 2010, Chang, et al., 2003, Tentner, et al., 2012). ERK was activated by TVX at 6 hours of treatment, and this TVX-induced ERK activation maintained to 24 hours (Figure 27). ERK also promoted cell cycle progression in HepG2 cells but was not involved in the TVX-induced cell cycle arrest (Figure 28). ERK is another p53-independent inducer of p21 expression (Beier, et al., 1999, Ciccarelli, et al., 2005). However, the observation that ERK inhibition promoted cell cycle arrest of HepG2 cells suggests ERK is not responsible for the p21 induction observed in TVX-treated cells.

Inhibition of ERK activation attenuated TVX/TNF-induced cytotoxicity (Figure 29). This result indicated that ERK activation is involved in the cytotoxicity related to TVX/TNF-cotreatment. However, the observation that ERK activation was similar in TVX alone- and TVX/TNF-treated groups suggested that ERK activation by itself was not sufficient to cause cytotoxicity. Some component of the TNF-induced signaling must contribute to the cytotoxicity involving ERK. It could be the case that both the prolonged activation of JNK, as well as ERK activation, are required to promote cell death fully in this model.

Both of these MAPKs are capable of phosphorylating antiapoptotic bcl family proteins on the mitochondria (Aoki, et al., 2002, Dhanasekaran and Reddy, 2008, Donovan, et al., 2002, Kim, et al., 2003, Schroeter, et al., 2003, Wang, et al., 2000, Zhang, et al., 2004). This initiates the intrinsic apoptotic pathway, which would be consistent with the TVX/TNF-induced cell death. It is interesting that inhibition of either JNK or ERK alone resulted in reduction of TVX/TNF-induced cytotoxicity but did not fully prevent cell death. This raises the possibility that the signaling of these two activated MAPKs together was necessary to induce maximal cytotoxicity in this system.

The DNA damage response is initiated by the PIKKs, ATM, ATR and DNA-PK. To investigate the contribution of each of these kinases in promoting cytotoxicity, pharmacological inhibitor studies were conducted. Inhibition of either ATM or DNA-PK did not modify the TVX/TNF-induced cell death. However, inhibition of ATR resulted in attenuation of cytotoxicity (Figure 31). Correspondingly, exposure to either TVX alone or TVX/TNF resulted in increased activated ATR in the nuclei of cells (Figure 30). This paralleled with the TVX-mediated cell cycle arrest, as ATR activation ultimately inhibits activation of Cdk2, which is critical for progression into S phase (Figure 8) (Sorensen and Syljuasen, 2012).

It is intriguing that TVX exposure resulted in ATR activation. Activation of ATR is associated with single stranded DNA damage that occurs in response to stalled DNA replication forks (Burrows and Elledge, 2008, Choi, et al., 2010, Dart, et al., 2004, Guo, et al., 2000, Ward and Chen, 2001, Zou, 2007). These stalled replication forks can occur when a DNA polymerase collides with topoisomerase cleavage complexes trapped on the DNA in response to topoisomerase inhibitors (Hong and Kreuzer, 2000,

Howard, et al., 1994, Larsen, et al., 1996, Pohlhaus and Kreuzer, 2005). Even though type II topoisomerases cleave both strands of DNA and can promote double strand breaks, the processing of these lesions can result in single stranded breaks in response to type II topoisomerase inhibitors (Rossi, et al., 2006).

In the event of replicative stress resulting from DNA replication fork stalling, activation of ATR stimulates homologous recombination that is required for the abolition of DNA replication blockage (Roos and Kaina, 2013, Saleh-Gohari, et al., 2005)(Roos and Kaina 2013, Saleh-Gohari 2005). It was a surprising discovery that ATR inhibition resulted in protection from TVX/TNF-treatment. Activation of ATR is typically associated with DNA repair and prevention of apoptosis. However, there are some documented instances in which ATR promotes apoptosis (Joe, et al., 2006, Liu, et al., 2011, Yim, et al., 2006).

4.3 Proposed mechanisms of TVX/TNF-induced hepatocellular apoptosis

It seems possible that TVX inhibits eukaryotic topoisomerases in HepG2 cells to create stabilized cleavage complexes on the DNA. On its own, topoisomerase inhibition isn't cytotoxic but becomes problematic when a DNA or RNA polymerase comes into contact with the complex. One hypothesis to explain the enhanced DNA damage induced by TVX in the presence of TNF is that TNF stimulates cellular transcription or DNA replication. The increased activity of polymerases causes collisions with TVX-mediated cleavage complexes that promote DNA damage. In response to replicative stress, ATR becomes activated and p21 induction occurs, both of which inhibit the activation of Cdks and promote cell cycle arrest. At this point, it is possible that homologous recombination to repair the stalled replication forks is occurring. Genotoxic stress also is associated with decreased global transcription and might result in decreased expression of the MAPK phosphatases (MPKs) that negatively regulate JNK and ERK, leading to their activation. The transcriptional interference also could result in decreased expression of cytotoprotective genes, such as XIAP.

In response to TNF, JNK activation occurs early, and JNK remains activated in response to cotreatment with TVX. The activation of both JNK and ERK might promote the release of cytochrome c from the mitochondria by phosphorylating the antiapoptotic bcl proteins in the mitochondrial outer membrane. Furthermore, ATR also is capable of promoting cytochrome c release by phosphorylating Bid. Release of cytochrome c then promotes activation of caspase 9, which cleaves and activates effector caspase 3.

The activation of these caspases is facilitated by the loss of caspase inhibitor proteins such as XIAP. In response to caspase 3 activation, DNA damage becomes

worse and p21 can be cleaved and inactivated. This loss of p21 might promote some aberrant attempts of cell division that could then worsen the DNA damage promoted by the TVX stabilized cleavage complex, colliding with more polymerases. A temporal relationship of all the critical findings in this model is depicted in Table 2. A diagram outlining these overlapping signaling pathways is displayed in Figure 36.

Time	Observation	TVX	TNF	TVX/TNF
1 hour	JNK activation	-	+	++
3.5 hour	Increase in Bcl-Xl mRNA	-	+	+
5 hour	Decrease in XIAP mRNA	+	-	+
6 hour	ERK activation	+	-	+
	ATR activation	+	-	+
	p21 mRNA induction	+	-	+
8 hour	JNK activation	+	-	+
	Caspase 9 activation	-	-	+
	Caspase 3 activation	-	-	+++
12 hour	Cell cycle arrest	+++	+	+++
	p21 protein induction	+	-	-
	DNA damage	-	-	++
20 hour	Significant plasma membrane leakage	-	-	++
24 hour	ERK activation	+	-	+
	DNA damage	+	-	+++
	Caspase 8 activation	-	-	+
	Decreased DNA content	++	-	++

Table 2. Timecourse of critical responses observed in HepG2 cells exposed to TVX and TNF. A "+" denotes a positive observation was made. More +'s indicate a stronger observation. A "-" denotes no observation was made.

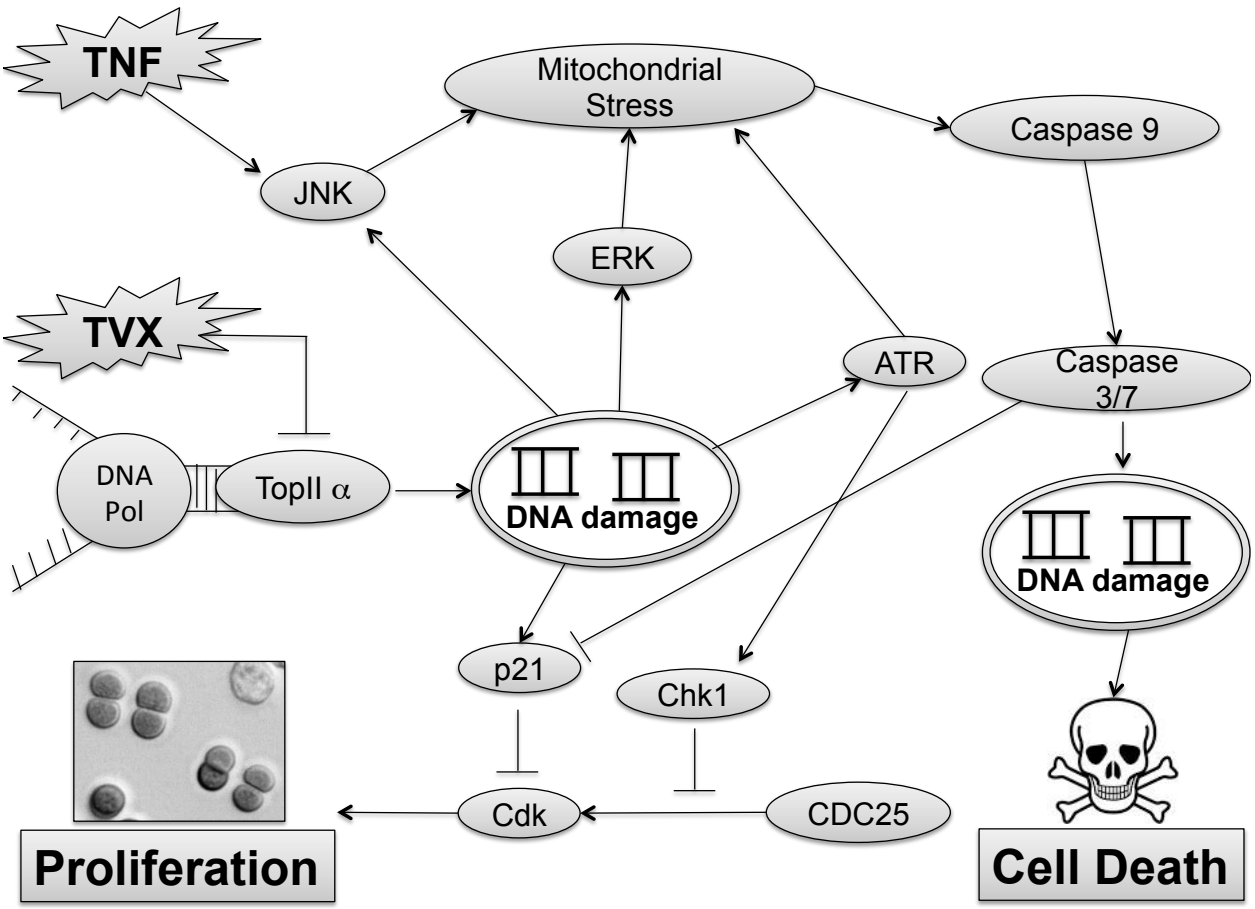


Figure 36. Proposed signaling mechanisms involved in HepG2 cell cycle arrest and apoptosis. TVX inhibits topoisomerase IIα (Top IIα) ahead of the DNA replication fork formed by DNA polymerase (DNA pol), which results in modest DNA damage. ATR becomes activated and p21 expression is increased, and these events cause cell cycle arrest. In the combined presence of both TVX and TNF, the kinase activities of ERK, JNK, and ATR all promote mitochondrial stress that result in caspase 9 activation, which leads to the activation of caspase 3. Caspase 3 then cleaves p21 and inactivates it, resulting in aberrant DNA synthesis, causing more DNA damage. Caspase 3 also causes DNA damage that eventually results in cell death.

4.4 Major findings and implications

Altogether, the aforementioned data identify that TVX/TNF-mediated hepatocellular cytotoxicity is apoptotic and dependent on caspase activity. Particularly, the apoptosis seems to be intrinsically mediated and is not initiated by caspase 8. The JNK and ERK MAPKs as well as ATR contribute to the cytotoxic interaction of TVX and TNF. Exposure of HepG2 cells to TVX alone causes genotoxic stress that activates the DNA damage response. This includes the formation of DNA double strand breaks and activated ATR in the nuclei, as well as an induction of the Cdk inhibitor p21.

In response to this, G0/G1 cell cycle arrest ensues, which results in decreased cell proliferation. However, none of these events result in overt cytotoxicity within 24 hours; that is, TVX by itself is not cytotoxic. The addition of TNF exacerbates the TVX-induced DNA damage. The combined exposure to TVX and TNF also results in prolonged activation of JNK, as well as a degradation of p21 protein. Both of these events promote cellular apoptosis.

These results suggest that the interaction of drugs with IDILI potential and TNF to cause hepatocellular toxicity could involve members of the MAPK family, as well as caspase activation. The DNA damage response also could be a contributing factor involved in the cytotoxic response generated by some xenobiotics associated with IDILI. As mentioned previously, a number of drug with idiosyncratic liability cause cell cycle arrest *in vitro*. This cell cycle arrest could be a sensitivity factor in the presence of TNF, as other studies have demonstrated that TNF-mediated apoptosis occurred preferentially in arrested cells. Collectively, these factors could be taken into

consideration to develop new screening protocols that might reliably detect the potential of new drug candidates to cause IDILI.

In response to an inflammatory stress, drugs that are capable of inducing genotoxic stress and mediating cell cycle arrest could sensitize cells to inflammatory mediators such at TNF. The liver is particularly susceptible, as it harbors a unique environment composed of cells that promote inflammation and that can contribute to tissue injury in response to an inflammatory stimulus. Another factor that could make the liver unusually susceptible to this interaction of inflammatory mediators with drugs that cause IDILI is the manner in which it recovers from injury. In response to noxious stimuli, the liver is capable of regenerating by initiating parenchymal cell division. If drugs associated with IDILI prevent hepatocellular proliferation, this could interfere with adaptation to injury and be a contributing factor in the progression to ALF.

4.5 Knowledge gaps and proposed future studies

There are many queries that remain unanswered in regard to TVX and TNF interacting to kill hepatocytes. An important observation that is lacking in substantial information is that the TVX/TNF-induced apoptosis appears to be mediated by caspases 9 and 3. This heavily implicates mitochondrial dysfunction in the role of this cytotoxicity. As mentioned, both ERK and JNK are able to disrupt the mitochondrial membrane to promote cytochrome C release that results in caspase 9 activation.

It would be helpful to know whether or not JNK and/or ERK are contributing to the activation of caspase 9. The activity of caspase 9 should be examined during inhibition of either JNK or ERK or both to see if these MAPKs do play a role in promoting activation of this caspase. Mitochondrial membrane potential also could be measured in the presence and absence of JNK/ERK inhibition. It would be interesting to see if TVX alone causes a change in mitochondrial membrane potential. If JNK and ERK alter mitochondrial membrane potential, it is possible that TVX could affect this since TVX is capable of activating both JNK and ERK.

It also would be important to identify the pathway involved in TVX-mediated p21 upregulation. The likely candidate could be the p53 homologs, p63 or p73, as these are both capable of inducing CDKN1A transcription in response to genotoxic stress. If it is discovered that one of these p53 homologs is involved, their involvement in the upregulation of proapoptotic genes such as Bax also should be investigated (De Laurenzi and Melino, 2000, Levrero, et al., 2000, Momii, et al., 2007). Lastly, it would be interesting to see if the lack of p21 protein expression in TVX/TNF-treated cells is in response to caspase 3 activation. This would determine if the lack of p21 protein

induction in cotreated cells is in response to the caspase activation that also occurs, or if some other posttranslational modification is responsible.

To identify if TVX inhibits topoisomerase enyzmes in hepatocytes, the trapped in agarose DNA immunostaining (TARDIS) assay could be utilized. This assay allows for the visualization and quantification of topoisomerase-DNA complexes within cells (Cowell, et al., 2011). The hypothesis would be that TVX inhibits topoisomerase IIα to form a cleavage complex with the DNA. It has been suggested that TNF is capable of increasing topoisomerase activity in cells (Baloch, et al., 1995, Debernardis, et al., 1996, Utsugi, et al., 1990). Accordingly, it would be interesting if there were more complex formations in response to both TVX and TNF. The increased activity of topoisomerases induced by TNF might increase the interaction of TVX with the enzyme, promoting the formation of more stabilized cleavage complex's. This would identify a role for TNF to potentiate the TVX-mediated topoisomerase inhibition and might explain why DNA damage was more prominent and occurred earlier in cotreated cells compared to TVX treatment alone.

It might be prudent to investigate whether TVX induces single stranded lesions in DNA. Although inhibition of type II topoisomerases results in double stranded breaks in DNA, there have been instances in which this process resulted in single stranded breaks. ATR activation is associated with single stranded DNA at stalled replication forks. The observation that ATR is activated in response to TVX suggests that single stranded DNA could be present in cells. An unlikely alternative to explain the presence of single stranded breaks, if they are present, is that TVX might inhibit type I topoisomerases, which results in single strand lesions.

The ability of TVX to interfere with transcription of critical genes involved in the regulation of MAPK activity and caspase activation should be investigated. As mentioned previously, HepG2 cells might not be the ideal system to study the TNF-induced-NF-κB-mediated expression of cytoprotective genes. However, in primary hepatocytes this might not be the case and should be considered. An NF-κB luciferase reporter assay could indicate whether or not NF-κB is activated in response to TNF and if TVX interferes with this activation.

In HepG2 cells, the relative expression of MKPs should be examined in the presence of TVX. Since TVX induces genotoxic stress, which can result in decreased global transcription, the activation of both JNK and ERK by TVX might be in response to a downregulation in MKPs. In particular, MKP-1 and MKP-7 should be studied as both of these regulate JNK and ERK phosphorylation (Boutros, et al., 2008, Masuda, et al., 2010, Mizuno, et al., 2004). The expression of GADD45β should also be measured. This gene is TNF-inducible and prevents both ERK and JNK activation by inhibiting MKK7 upstream of JNK (Larsen, et al., 2006, Papa, et al., 2004, Wullaert, et al., 2006).

Lastly, concerns remain about whether or not these events induced by TVX and TNF in the HepG2 cells are applicable to primary hepatocytes and the liver as a whole. It is encouraging that TVX and TNF interacted to cause caspase-dependent cell death in primary murine hepatocytes. However, HepG2 cells have increased expression of a number of genes involved in cell cycle regulation, and they proliferate readily in culture. This is in contrast to hepatocytes in culture and in the liver, which are quiescent and resting in G0 of the cell cycle (Fausto, 2000). However, in response to tissue damage a drastic shift from quiescence into cellular proliferation occurs. As much as 70% of the

original organ mass can be regenerated (Fausto, 2000, Michalopoulos, 2010). It could be possible that TVX might interfere with the regeneration of the liver by inhibiting proliferation.

Interestingly, studies examining the molecular events involved in liver injury have identified TNF, NF-κB, and JNK as critical mediators that promote liver regeneration (Iimuro, et al., 2007, Kirillova, et al., 1999, Plumpe, et al., 2000, Yamada, et al., 1998). In these studies, transcriptional activity of NF-κB and AP-1, as well as STAT3, were increased. All of this correlated to increased bromodeoxyuridine (BrdU) incorporation, indicating the hepatocytes were undergoing DNA synthesis. It also was identified that TNF-induced IL-6 expression through NF-κB was critical to the activation of STAT3. Downstream of JNK, the action of c-Jun/AP-1 transcription factor is critical to liver regeneration, and is involved in downregulating p21 (Stepniak, et al., 2006). This is interesting, considering that the livers of mice treated with TVX/LPS had increased CDKN1A expression. If p21 protein expression is not decreased in response to TVX/LPS treatment, it could counteract the proliferative signaling induced by JNK and NF-κB, hindering the livers ability to recover from injury.

In response to injury invoked by TVX/LPS cotreatment in mice, it would seem that TVX might prevent regenerative DNA synthesis from occuring. Alternatively, if the process of DNA replication is successfully initiated, this might result in greater genotoxic stress if the TVX is bound to topoisomerases on the DNA. DNA polymerases could collide with the ternary complexes formed by TVX inhibiting topoisomerases, increasing the genotoxic stress. Congruent with this thinking, there is a threshold level of injury that can occur in the liver before repair processes are inhibited altogether (Anand, et al.,

2004). If initial attempts to promote cell division and tissue repair result in exacerbating the DNA damage in the liver, the regeneration might not occur at all.

To examine whether or not TVX has the ability to prevent hepatocellular division *in vivo*, BrdU incorporation and immunohistochemical staining for proliferating cell nuclear antigen (PCNA) can be conducted. It would be interesting to learn if TVX is capable of preventing the injury-mediated regeneration. As a follow up to this, the effects of TVX on regeneration after partial hepatectomy also could be studied. This model is used to study the regeneration process of the liver. If TVX prevented or delayed the regeneration of liver tissue, it would indicate the TVX-mediated cell cycle arrest *in vitro* is similarly observed *in vivo*. However, caution should be used when administering TVX to partially hepatectomized mice, as hepatectomy is associated with increased circulating TNF. The combination of TVX and TNF might result in liver failure.

One final *in vivo* study that could correlate the findings to the *in vitro* work would be to administer a caspase inhibitor to mice treated with TVX and LPS. As mentioned before, the histopathology suggests that at an early time, apoptotic cell death is observed in cells that appear predominantly oncotic at later times. Caspase inhibition *in vivo* might prevent the liver injury altogether. PS exposure to the outer leaflet of the plasma membrane has been determined to be both dependent and independent of caspase activation (Cohen, et al., 2004, Ferraro-Peyret, et al., 2002, Mandal, et al., 2002). Therefore, coagulation system activation in response to PS exposure might not be prevented during caspase inhibition. If coagulation still occurred during caspase inhibition, this could still promote oncotic necrosis.

Overall, it is important to relate the findings of this dissertation to occurrences observed in functioning livers. To validate the significance of the critical signaling pathways identified in this project, the involvement of JNK, caspases, ERK and ATR should be studied in models examining the interaction of other drugs associated with IDILI with TNF. While the studies compiled in this dissertation offer insight into molecular signaling events that are occurring in hepatocytes in response to the interaction of TVX with TNF, the mechanism might be different in liver tissue composed of quiescent hepatocytes. If the combination of TVX and LPS is initiating tissue injury that is prompting liver cell division for regeneration, the use of proliferating hepatocytes *in vitro* could be a reliable means of studying this interaction of drugs associated with IDILI and TNF.

REFERENCES

REFERENCES

Abbas, T. and Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer.* **9** (6), 400-14.

Agarwal, M. L., et al. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci U S A.* **92** (18), 8493-7.

Aggarwal, B. B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol.* **3** (9), 745-56.

Aithal, G. P., et al. (2004). Hepatic adducts, circulating antibodies, and cytokine polymorphisms in patients with diclofenac hepatotoxicity. *Hepatology.* **39** (5), 1430-40.

Albertini, S., et al. (1995). Genotoxicity of 17 gyrase- and four mammalian topoisomerase II-poisons in prokaryotic and eukaryotic test systems. *Mutagenesis.* **10** (4), 343-51.

Aliouat-Denis, C. M., et al. (2005). p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res.* **3** (11), 627-34.

Anand, S. S., et al. (2004). Dose-dependent liver tissue repair in chloroform plus thioacetamide acute hepatotoxicity. *Environ Toxicol Pharmacol.* **18** (2), 143-8.

Andera, L. (2009). Signaling activated by the death receptors of the TNFR family. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* **153** (3), 173-80.

Anderson, V. E., et al. (1998). Topoisomerase IV catalysis and the mechanism of quinolone action. *J Biol Chem.* **273** (28), 17879-85.

Anderson, V. E., et al. (2000). Action of quinolones against Staphylococcus aureus topoisomerase IV: basis for DNA cleavage enhancement. *Biochemistry.* **39** (10), 2726-32.

Aoki, H., et al. (2002). Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. *J Biol Chem.* **277** (12), 10244-50.

Arrowsmith, C. H. (1999). Structure and function in the p53 family. *Cell Death Differ.* **6** (12), 1169-73.

Aviles, A., et al. (1984). Hepatic injury during doxorubicin therapy. *Arch Pathol Lab Med.* **108** (11), 912-3.

Bajt, M. L., et al. (2004). Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine. *Toxicol Sci.* **80** (2), 343-9.

Ballet, F. (2010). Back to basics for idiosyncratic drug-induced liver injury: dose and metabolism make the poison. *Gastroenterol Clin Biol.* **34** (6-7), 348-50.

Baloch, Z., et al. (1995). Modulation of topoisomerase activities by tumor necrosis factor. *Cell Immunol.* **160** (1), 98-103.

Ban, J. O., et al. (2011). Troglitazone, a PPAR agonist, inhibits human prostate cancer cell growth through inactivation of NFkappaB via suppression of GSK-3beta expression. *Cancer Biol Ther.* **12** (4), 288-96.

Bargou, R. C., et al. (1997). Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest.* **100** (12), 2961-9.

Barrett, J. F., et al. (1989). Use of in vitro topoisomerase II assays for studying quinolone antibacterial agents. *Antimicrob Agents Chemother.* **33** (10), 1697-703.

Bartek, J. and Lukas, J. (2001). Pathways governing G1/S transition and their response to DNA damage. *FEBS Lett.* **490** (3), 117-22.

Barton, C. C., et al. (2000). Lipopolysaccharide augments aflatoxin B(1)-induced liver injury through neutrophil-dependent and -independent mechanisms. *Toxicol Sci.* **58** (1), 208-15.

Basta-Kaim, A., et al. (2006). Inhibitory effect of antipsychotic drugs on the Con A- and LPS-induced proliferative activity of mouse splenocytes: a possible mechanism of action. *J Physiol Pharmacol.* **57** (2), 247-64.

Beggs, K. M., et al. (2013). Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor-alpha Interaction. *Toxicol Sci.*

Beier, F., et al. (1999). The Raf-1/MEK/ERK pathway regulates the expression of the p21(Cip1/Waf1) gene in chondrocytes. *J Biol Chem.* **274** (42), 30273-9.

Bell, L. N. and Chalasani, N. (2009). Epidemiology of idiosyncratic drug-induced liver injury. *Semin Liver Dis.* **29** (4), 337-47.

Bemelmans, M. H., et al. (1996). Increased concentrations of tumour necrosis factor (TNF) and soluble TNF receptors in biliary obstruction in mice; soluble TNF receptors as prognostic factors for mortality. *Gut.* **38** (3), 447-53.

Bennett, B. L., et al. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A.* **98** (24), 13681-6.

Bergmeyer, H. U. and Horder, M. (1980). International federation of clinical chemistry. Scientific committee. Expert panel on enzymes. IFCC document stage 2, draft 1; 1979-11-19 with a view to an IFCC recommendation. IFCC methods for the measurement of catalytic concentration of enzymes. Part 3. IFCC method for alanine aminotransferase. *J Clin Chem Clin Biochem.* **18** (8), 521-34.

Bhat, N. R., et al. (1998). Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. *J Neurosci.* **18** (5), 1633-41.

Bhatti, S., et al. (2011). ATM protein kinase: the linchpin of cellular defenses to stress. *Cell Mol Life Sci.* **68** (18), 2977-3006.

Bjornsson, E. (2010). Review article: drug-induced liver injury in clinical practice. *Aliment Pharmacol Ther.* **32** (1), 3-13.

Boutros, T., et al. (2008). The MAP kinase phosphatase-1 MKP-1/DUSP1 is a regulator of human liver response to transplantation. *Am J Transplant.* **8** (12), 2558-68.

Bradham, C. A., et al. (1998). Mechanisms of hepatic toxicity. I. TNF-induced liver injury. *Am J Physiol.* **275** (3 Pt 1), G387-92.

Breedveld, F. (1998). New tumor necrosis factor-alpha biologic therapies for rheumatoid arthritis. *Eur Cytokine Netw.* **9** (3), 233-8.

Brenner, D. A. (1998). Signal transduction during liver regeneration. *J Gastroenterol Hepatol.* **13 Suppl** S93-5.

Brighty, K. E. and Gootz, T. D. (1997). The chemistry and biological profile of trovafloxacin. *J Antimicrob Chemother.* **39 Suppl B** 1-14.

Buchweitz, J. P., et al. (2002). Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy? *J Pharmacol Exp Ther.* **300** (2), 460-7.

Buettner, G. R. (1993). The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys.* **300** (2), 535-43.

Bunz, F., et al. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science.* **282** (5393), 1497-501.

Burrows, A. E. and Elledge, S. J. (2008). How ATR turns on: TopBP1 goes on ATRIP with ATR. *Genes Dev.* **22** (11), 1416-21.

Cabal-Hierro, L. and Lazo, P. S. (2012). Signal transduction by tumor necrosis factor receptors. *Cell Signal.* **24** (6), 1297-305.

Cadalbert, L., et al. (2005). Conditional expression of MAP kinase phosphatase-2 protects against genotoxic stress-induced apoptosis by binding and selective dephosphorylation of nuclear activated c-jun N-terminal kinase. *Cell Signal.* **17** (10), 1254-64.

Cagnol, S., et al. (2006). Prolonged activation of ERK1,2 induces FADD-independent caspase 8 activation and cell death. *Apoptosis.* **11** (3), 337-46.

Cagnol, S. and Chambard, J. C. (2010). ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. *FEBS J.* **277** (1), 2-21.

Cao, W., et al. (2005). TNF-alpha promotes Doxorubicin-induced cell apoptosis and anti-cancer effect through downregulation of p21 in p53-deficient tumor cells. *Biochem Biophys Res Commun.* **330** (4), 1034-40.

Chai, F., et al. (2000). Involvement of p21(Waf1/Cip1) and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. *Carcinogenesis.* **21** (1), 7-14.

Chamulitrat, W., et al. (1995). Tumor necrosis factor-alpha and nitric oxide production in endotoxin-primed rats administered carbon tetrachloride. *Life Sci.* **57** (24), 2273-80.

Chang, F., et al. (2003). Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). *Int J Oncol.* **22** (3), 469-80.

Chen, H. J., et al. (2000). Acute eosinophilic hepatitis from trovafloxacin. *N Engl J Med.* **342** (5), 359-60.

Chen, W., et al. (2002). Stimulus-specific requirements for MAP3 kinases in activating the JNK pathway. *J Biol Chem.* **277** (51), 49105-10.

Chen, Y. R., et al. (1996). The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem.* **271** (50), 31929-36.

Chene, P. (2001). The role of tetramerization in p53 function. *Oncogene.* **20** (21), 2611-7.

Chennamaneni, S., et al. (2012). COX inhibitors Indomethacin and Sulindac derivatives as antiproliferative agents: synthesis, biological evaluation, and mechanism investigation. *Eur J Med Chem.* **56** 17-29.

Choi, J. H., et al. (2010). Reconstitution of RPA-covered single-stranded DNA-activated ATR-Chk1 signaling. *Proc Natl Acad Sci U S A.* **107** (31), 13660-5.

Chosay, J. G., et al. (1997). Neutrophil margination and extravasation in sinusoids and venules of liver during endotoxin-induced injury. *Am J Physiol.* **272** (5 Pt 1), G1195-200.

Chow, J. C., et al. (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem.* **274** (16), 10689-92.

Ciccarelli, C., et al. (2005). p21WAF1 expression induced by MEK/ERK pathway activation or inhibition correlates with growth arrest, myogenic differentiation and onco-phenotype reversal in rhabdomyosarcoma cells. *Mol Cancer.* **4** 41.

Clay, K. D., et al. (2006). Brief communication: severe hepatotoxicity of telithromycin: three case reports and literature review. *Ann Intern Med.* **144** (6), 415-20.

Cohen, Z., et al. (2004). Caspase inhibition decreases both platelet phosphatidylserine exposure and aggregation: caspase inhibition of platelets. *Thromb Res.* **113** (6), 387-93.

Copeland, S., et al. (2005). Acute inflammatory response to endotoxin in mice and humans. *Clin Diagn Lab Immunol.* **12** (1), 60-7.

Corsini, A., et al. (2012). Current challenges and controversies in drug-induced liver injury. *Drug Saf.* **35** (12), 1099-117.

Cortes-Ledesma, F. and Aguilera, A. (2006). Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange. *EMBO Rep.* **7** (9), 919-26.

Cosgrove, B. D., et al. (2009). Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. *Toxicol Appl Pharmacol.* **237** (3), 317-30.

Cowell, I. G., et al. (2011). An overview of the visualisation and quantitation of low and high MW DNA adducts using the trapped in agarose DNA immunostaining (TARDIS) assay. *Mutagenesis.* **26** (2), 253-60.

Cuschieri, J. and Maier, R. V. (2005). Mitogen-activated protein kinase (MAPK). *Crit Care Med.* **33** (12 Suppl), S417-9.

Czaja, M. J., et al. (1994). Lipopolysaccharide-neutralizing antibody reduces hepatocyte injury from acute hepatotoxin administration. *Hepatology.* **19** (5), 1282-9.

Dalvie, D. K., et al. (1997). Excretion and metabolism of trovafloxacin in humans. *Drug Metab Dispos.* **25** (4), 423-7.

Daly, A. K., et al. (2009). HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet.* **41** (7), 816-9.

Dart, D. A., et al. (2004). Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase. *J Biol Chem.* **279** (16), 16433-40.

De Laurenzi, V. and Melino, G. (2000). Evolution of functions within the p53/p63/p73 family. *Ann N Y Acad Sci.* **926** 90-100.

De Smaele, E., et al. (2001). Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. *Nature.* **414** (6861), 308-13.

Debernardis, D., et al. (1996). Endogenous tumor necrosis factor enhances topoisomerase II inhibitors activity in human ovarian cancer cell lines. *J Pharmacol Exp Ther.* **279** (1), 84-90.

Dembry, L. M., et al. (1999). Fluoroquinolone Antibiotics: Adverse Effects and Safety Profiles. *Infectious Diseases in Clinical Practice.* **8** (9), 421-428.

Deng, X., et al. (2006). Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. *J Pharmacol Exp Ther.* **319** (3), 1191-9.

Deng, X., et al. (2009). Inflammatory stress and idiosyncratic hepatotoxicity: hints from animal models. *Pharmacol Rev.* **61** (3), 262-82.

Derijard, B., et al. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science.* **267** (5198), 682-5.

Dhanasekaran, D. N. and Reddy, E. P. (2008). JNK signaling in apoptosis. *Oncogene.* **27** (48), 6245-51.

Ding, W. X. and Yin, X. M. (2004). Dissection of the multiple mechanisms of TNF-alpha-induced apoptosis in liver injury. *J Cell Mol Med.* **8** (4), 445-54.

Donovan, N., et al. (2002). JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. *J Biol Chem.* **277** (43), 40944-9.

Drlica, K. (1999). Mechanism of fluoroquinolone action. *Curr Opin Microbiol.* **2** (5), 504-8.

Dugan, C. M., et al. (2010). A mouse model of severe halothane hepatitis based on human risk factors. *J Pharmacol Exp Ther.* **333** (2), 364-72.

Eghtesadi-Araghi, P., et al. (2008). Halothane hepatitis in Iran: a review of 59 cases. *World J Gastroenterol.* **14** (34), 5322-6.

Ehrhardt, H., et al. (2013). Cell cycle-arrested tumor cells exhibit increased sensitivity towards TRAIL-induced apoptosis. *Cell Death Dis.* **4** e661.

Esmon, C. T. (1999). Possible involvement of cytokines in diffuse intravascular coagulation and thrombosis. *Baillieres Best Pract Res Clin Haematol.* **12** (3), 343-59.

Fadok, V. A., et al. (1998). The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ.* **5** (7), 551-62.

Fan, T. J., et al. (2005). Caspase family proteases and apoptosis. *Acta Biochim Biophys Sin (Shanghai).* **37** (11), 719-27.

Fausto, N. (2000). Liver regeneration. *J Hepatol.* **32** (1 Suppl), 19-31.

Favata, M. F., et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem.* **273** (29), 18623-32.

Ferraro-Peyret, C., et al. (2002). Caspase-independent phosphatidylserine exposure during apoptosis of primary T lymphocytes. *J Immunol.* **169** (9), 4805-10.

Fischman, A. J., et al. (1998). Pharmacokinetics of [18F]trovafloxacin in healthy human subjects studied with positron emission tomography. *Antimicrob Agents Chemother.* **42** (8), 2048-54.

Fogg, V. C., et al. (2011). Mitochondria in cancer: at the crossroads of life and death. *Chin J Cancer.* **30** (8), 526-39.

Francavilla, A., et al. (1989). Effect of cimetidine, ranitidine, famotidine and omeprazole on hepatocyte proliferation in vitro. *J Hepatol.* **8** (1), 32-41.

Fredriksson, L., et al. (2011). Diclofenac inhibits tumor necrosis factor-alpha-induced nuclear factor-kappaB activation causing synergistic hepatocyte apoptosis. *Hepatology.* **53** (6), 2027-41.

Freudenberg, M. A. and Galanos, C. (1990). Bacterial lipopolysaccharides: structure, metabolism and mechanisms of action. *Int Rev Immunol.* **6** (4), 207-21.

Friedl, J., et al. (2002). Induction of permeability across endothelial cell monolayers by tumor necrosis factor (TNF) occurs via a tissue factor-dependent mechanism: relationship between the procoagulant and permeability effects of TNF. *Blood.* **100** (4), 1334-9.

Fujioka, S., et al. (2003). Function of nuclear factor kappaB in pancreatic cancer metastasis. *Clin Cancer Res.* **9** (1), 346-54.

Ganey, P. E., et al. (2004). Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. *Chem Biol Interact.* **150** (1), 35-51.

Ganey, P. E., et al. (2007). Role of the coagulation system in acetaminophen-induced hepatotoxicity in mice. *Hepatology.* **46** (4), 1177-86.

Gantner, F., et al. (1995). Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. *Hepatology.* **21** (1), 190-8.

Gartel, A. L. and Tyner, A. L. (2002). The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther.* **1** (8), 639-49.

Gera, J. F., et al. (1993). Inhibition of DNA repair with aphidicolin enhances sensitivity of targets to tumor necrosis factor. *J Immunol.* **151** (7), 3746-57.

Gonzalez, M. A. and Selwyn, A. P. (2003). Endothelial function, inflammation, and prognosis in cardiovascular disease. *Am J Med.* **115 Suppl 8A** 99S-106S.

Gortz, B., et al. (2005). Tumour necrosis factor activates the mitogen-activated protein kinases p38alpha and ERK in the synovial membrane in vivo. *Arthritis Res Ther.* **7** (5), R1140-7.

Greenblatt, M. S., et al. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.* **54** (18), 4855-78.

Guo, Z., et al. (2000). Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. *Genes Dev.* **14** (21), 2745-56.

Gurumurthy, P., et al. (1984). Lack of relationship between hepatic toxicity and acetylator phenotype in three thousand South Indian patients during treatment with isoniazid for tuberculosis. *Am Rev Respir Dis.* **129** (1), 58-61.

Hamaguchi, N., et al. (2010). In vitro and in vivo therapeutic efficacy of the PPAR-gamma agonist troglitazone in combination with cisplatin against malignant pleural mesothelioma cell growth. *Cancer Sci.* **101** (9), 1955-64.

Hamdi, M., et al. (2005). DNA damage in transcribed genes induces apoptosis via the JNK pathway and the JNK-phosphatase MKP-1. *Oncogene.* **24** (48), 7135-44.

Han, D., et al. (2009). Redox regulation of tumor necrosis factor signaling. *Antioxid Redox Signal.* **11** (9), 2245-63.

Hanlon, A. M., et al. (2002). Signaling from cytokine receptors that affect Th1 responses. *Front Biosci.* **7** d1247-54.

Hassan, F., et al. (2008). Lipopolysaccharide augments the in vivo lethal action of doxorubicin against mice via hepatic damage. *Clin Exp Immunol.* **151** (2), 334-40.

Haupt, S., et al. (2003). Apoptosis - the p53 network. *J Cell Sci.* **116** (Pt 20), 4077-85.

Hautekeete, M. L., et al. (1999). HLA association of amoxicillin-clavulanate--induced hepatitis. *Gastroenterology.* **117** (5), 1181-6.

Hayakawa, J., et al. (2003). The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. *J Biol Chem.* **278** (23), 20582-92.

He, K., et al. (2004). Incorporation of an oxygen from water into troglitazone quinone by cytochrome P450 and myeloperoxidase. *Drug Metab Dispos.* **32** (4), 442-6.

Heatwole, V. M. (1999). TUNEL assay for apoptotic cells. *Methods Mol Biol.* **115** 141-8.

Heemskerk, J. W., et al. (2002). Platelet activation and blood coagulation. *Thromb Haemost.* **88** (2), 186-93.

Heine, G. F., et al. (2008). Multiple mechanisms contribute to inhibit transcription in response to DNA damage. *J Biol Chem.* **283** (15), 9555-61.

Hentze, H., et al. (2004). Topoisomerase inhibitor camptothecin sensitizes mouse hepatocytes in vitro and in vivo to TNF-mediated apoptosis. *Hepatology.* **39** (5), 1311-20.

Hewett, J. A. and Roth, R. A. (1993). Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol Rev.* **45** (4), 382-411.

Hewett, J. A. and Roth, R. A. (1995). The coagulation system, but not circulating fibrinogen, contributes to liver injury in rats exposed to lipopolysaccharide from gram-negative bacteria. *J Pharmacol Exp Ther.* **272** (1), 53-62.

Hiasa, H., et al. (1996). DNA strand cleavage is required for replication fork arrest by a frozen topoisomerase-quinolone-DNA ternary complex. *J Biol Chem.* **271** (42), 26424-9.

Hickson, I., et al. (2004). Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res.* **64** (24), 9152-9.

Hill, D. B., et al. (1995). In vitro tumor necrosis factor cytotoxicity in Hep G2 liver cells. *Hepatology.* **21** (4), 1114-9.

Hirano, F., et al. (2003). Tumor necrosis factor alpha (TNF-alpha)-induced RANTES chemokine expression via activation of NF-kappaB and p38 MAP kinase: roles of TNF-alpha in alcoholic liver diseases. *J Hepatol.* **38** (4), 483-9.

Hitti, E., et al. (2006). Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element. *Mol Cell Biol.* **26** (6), 2399-407.

Ho, J. S., et al. (1996). Identification of factors from rat neutrophils responsible for cytotoxicity to isolated hepatocytes. *J Leukoc Biol.* **59** (5), 716-24.

Holtom, P. D., et al. (2000). Inhibitory effects of the quinolone antibiotics trovafloxacin, ciprofloxacin, and levofloxacin on osteoblastic cells in vitro. *J Orthop Res.* **18** (5), 721-7.

Honda, R. and Yasuda, H. (2000). Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. *Oncogene.* **19** (11), 1473-6.

Hong, G. and Kreuzer, K. N. (2000). An antitumor drug-induced topoisomerase cleavage complex blocks a bacteriophage T4 replication fork in vivo. *Mol Cell Biol.* **20** (2), 594-603.

Hoshino, K., et al. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol.* **162** (7), 3749-52.

Hosono, S., et al. (1991). Molecular analysis of the p53 alleles in primary hepatocellular carcinomas and cell lines. *Oncogene.* **6** (2), 237-43.

Houtgraaf, J. H., et al. (2006). A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovasc Revasc Med.* **7** (3), 165-72.

Howard, M. T., et al. (1994). Disruption of a topoisomerase-DNA cleavage complex by a DNA helicase. *Proc Natl Acad Sci U S A.* **91** (25), 12031-5.

Hsu, H., et al. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell.* **81** (4), 495-504.

Hsu, H., et al. (1996). TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity.* **4** (4), 387-96.

Hu, Y., et al. (1998). Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci U S A.* **95** (8), 4386-91.

Huang, L., et al. (2000). Activation of the p21WAF1/CIP1 promoter independent of p53 by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) through the Sp1 sites. *Oncogene.* **19** (50), 5712-9.

Huang, Y. S. (2007). Genetic polymorphisms of drug-metabolizing enzymes and the susceptibility to antituberculosis drug-induced liver injury. *Expert Opin Drug Metab Toxicol.* **3** (1), 1-8.

Huo, J. X., et al. (2004). p53-independent induction of p21(waf1/cip1) contributes to the activation of caspases in GTP-depletion-induced apoptosis of insulin-secreting cells. *Cell Death Differ.* **11** (1), 99-109.

Hussaini, S. H. and Farrington, E. A. (2007). Idiosyncratic drug-induced liver injury: an overview. *Expert Opin Drug Saf.* **6** (6), 673-84.

Iimuro, Y., et al. (2007). Role of innate immune response in liver regeneration. *J Gastroenterol Hepatol.* **22 Suppl 1** S57-8.

Irmler, M., et al. (1997). Inhibition of death receptor signals by cellular FLIP. *Nature.* **388** (6638), 190-5.

Jackson, S. P. and Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature.* **461** (7267), 1071-8.

Jakubowska-Mucka, A., et al. (2012). Synergistic cytotoxic effect of sulindac and pyrrolidine dithiocarbamate against ovarian cancer cells. *Oncol Rep.* **27** (4), 1245-50.

Javelaud, D. and Besancon, F. (2001). NF-kappa B activation results in rapid inactivation of JNK in TNF alpha-treated Ewing sarcoma cells: a mechanism for the anti-apoptotic effect of NF-kappa B. *Oncogene.* **20** (32), 4365-72.

Jendrossek, V. (2012). The intrinsic apoptosis pathways as a target in anticancer therapy. *Curr Pharm Biotechnol.* **13** (8), 1426-38.

Jensen, B. S., et al. (1999). Inhibition of T cell proliferation by selective block of Ca(2+)-activated K(+) channels. *Proc Natl Acad Sci U S A.* **96** (19), 10917-21.

Jeong, J. H., et al. (2010). p53-independent induction of G1 arrest and p21WAF1/CIP1 expression by ascofuranone, an isoprenoid antibiotic, through downregulation of c-Myc. *Mol Cancer Ther.* **9** (7), 2102-13.

Jiao, P., et al. (2013). MK-2206 induces cell cycle arrest and apoptosis in HepG2 cells and sensitizes TRAIL-mediated cell death. *Mol Cell Biochem.*

Jin, Z., et al. (2002). Enhanced sensitivity of G1 arrested human cancer cells suggests a novel therapeutic strategy using a combination of simvastatin and TRAIL. *Cell Cycle.* **1** (1), 82-9.

Joe, Y., et al. (2006). ATR, PML, and CHK2 play a role in arsenic trioxide-induced apoptosis. *J Biol Chem.* **281** (39), 28764-71.

Jupp, O. J., et al. (2001). Type II tumour necrosis factor-alpha receptor (TNFR2) activates c-Jun N-terminal kinase (JNK) but not mitogen-activated protein kinase (MAPK) or p38 MAPK pathways. *Biochem J.* **359** (Pt 3), 525-35.

Kamata, H., et al. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell.* **120** (5), 649-61.

Kaplowitz, N. (2005). Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov.* **4** (6), 489-99.

Kassahun, K., et al. (2001). Studies on the metabolism of troglitazone to reactive intermediates in vitro and in vivo. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem Res Toxicol.* **14** (1), 62-70.

Kelly, G. S. (1998). Clinical applications of N-acetylcysteine. *Altern Med Rev.* **3** (2), 114-27.

Kim, G. S., et al. (2003). Leptin induces apoptosis via ERK/cPLA2/cytochrome c pathway in human bone marrow stromal cells. *J Biol Chem.* **278** (24), 21920-9.

Kim, H. S., et al. (2009). Doxorubicin exerts cytotoxic effects through cell cycle arrest and Fas-mediated cell death. *Pharmacology.* **84** (5), 300-9.

Kinner, A., et al. (2008). Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res.* **36** (17), 5678-94.

Kirillova, I., et al. (1999). Tumor necrosis factor induces DNA replication in hepatic cells through nuclear factor kappaB activation. *Cell Growth Differ.* **10** (12), 819-28.

Kitagawa, T., et al. (2007). Genome-wide analysis of cellular response to bacterial genotoxin CdtB in yeast. *Infect Immun.* **75** (3), 1393-402.

Kitteringham, N. R., et al. (1995). Detection of autoantibodies directed against human hepatic endoplasmic reticulum in sera from patients with halothane-associated hepatitis. *Br J Clin Pharmacol.* **40** (4), 379-86.

Klaunig, J. E., et al. (1981). Mouse liver cell culture. I. Hepatocyte isolation. *In Vitro.* **17** (10), 913-25.

Kobayashi, K. and Tsukamoto, I. (2001). Prolonged Jun N-terminal kinase (JNK) activation and the upregulation of p53 and p21(WAF1/CIP1) preceded apoptosis in hepatocytes after partial hepatectomy and cisplatin. *Biochim Biophys Acta.* **1537** (1), 79-88.

Komarov, P. G., et al. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science.* **285** (5434), 1733-7.

Kozlov, S. V., et al. (2011). Autophosphorylation and ATM activation: additional sites add to the complexity. *J Biol Chem.* **286** (11), 9107-19.

Krause, D., et al. (2001). Transient activation of Jun N-terminal kinases and protection from apoptosis by the insulin-like growth factor I receptor can be suppressed by dicumarol. *J Biol Chem.* **276** (22), 19244-52.

Kusters, S., et al. (1997). In vivo evidence for a functional role of both tumor necrosis factor (TNF) receptors and transmembrane TNF in experimental hepatitis. *Eur J Immunol.* **27** (11), 2870-5.

Kuzminov, A. and Stahl, F. W. (1999). Double-strand end repair via the RecBC pathway in Escherichia coli primes DNA replication. *Genes Dev.* **13** (3), 345-56.

Kyosseva, S. V. (2004). Mitogen-activated protein kinase signaling. *Int Rev Neurobiol.* **59** 201-20.

Lakin, N. D. and Jackson, S. P. (1999). Regulation of p53 in response to DNA damage. *Oncogene.* **18** (53), 7644-55.

Lamb, J. A., et al. (2003). JunD mediates survival signaling by the JNK signal transduction pathway. *Mol Cell.* **11** (6), 1479-89.

Lammert, C., et al. (2008). Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals. *Hepatology.* **47** (6), 2003-9.

Larsen, A. K., et al. (1996). The roles of DNA topoisomerase II during the cell cycle. *Prog Cell Cycle Res.* **2** 229-39.

Larsen, C. M., et al. (2006). Growth arrest- and DNA-damage-inducible 45beta gene inhibits c-Jun N-terminal kinase and extracellular signal-regulated kinase and decreases IL-1beta-induced apoptosis in insulin-producing INS-1E cells. *Diabetologia.* **49** (5), 980-9.

Larsen, F. S. and Bjerring, P. N. (2011). Acute liver failure. *Curr Opin Crit Care.* **17** (2), 160-4.

Lasser, K. E., et al. (2002). Timing of new black box warnings and withdrawals for prescription medications. *JAMA.* **287** (17), 2215-20.

Lavin, M. F. and Kozlov, S. (2007). ATM activation and DNA damage response. *Cell Cycle.* **6** (8), 931-42.

Lazarczyk, D. A., et al. (2001). Trovafloxacin hepatotoxicity. *Dig Dis Sci.* **46** (4), 925-6.

LeCluyse, E. L. (2001). Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci.* **13** (4), 343-68.

Lee, H. J., et al. (2005). Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. *Toxicol Appl Pharmacol.* **203** (2), 124-31.

Lee, M. S., et al. (2007). The novel combination of chlorpromazine and pentamidine exerts synergistic antiproliferative effects through dual mitotic action. *Cancer Res.* **67** (23), 11359-67.

Lee, W. M. (2003). Acute liver failure in the United States. *Semin Liver Dis.* **23** (3), 217-26.

Lee, W. M. (2012). Recent developments in acute liver failure. *Best Pract Res Clin Gastroenterol.* **26** (1), 3-16.

Leist, M., et al. (1994). Murine hepatocyte apoptosis induced in vitro and in vivo by TNF-alpha requires transcriptional arrest. *J Immunol.* **153** (4), 1778-88.

Leist, M., et al. (1997a). Tumor necrosis factor-induced apoptosis during the poisoning of mice with hepatotoxins. *Gastroenterology.* **112** (3), 923-34.

Leist, M., et al. (1997b). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med.* **185** (8), 1481-6.

Leitner, J. M., et al. (2010). Hepatotoxicity of antibacterials: Pathomechanisms and clinical. *Infection.* **38** (1), 3-11.

Lentz, B. R. (2003). Exposure of platelet membrane phosphatidylserine regulates blood coagulation. *Prog Lipid Res.* **42** (5), 423-38.

Levine, C., et al. (1998). DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. *Biochim Biophys Acta.* **1400** (1-3), 29-43.

Levkau, B., et al. (1998). Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. *Mol Cell.* **1** (4), 553-63.

Levrero, M., et al. (2000). The p53/p63/p73 family of transcription factors: overlapping and distinct functions. *J Cell Sci.* **113 (Pt 10)** 1661-70.

Li, J. and Uetrecht, J. P. (2010). The danger hypothesis applied to idiosyncratic drug reactions. *Handb Exp Pharmacol.* (196), 493-509.

Liguori, M. J., et al. (2005). Microarray analysis in human hepatocytes suggests a mechanism for hepatotoxicity induced by trovafloxacin. *Hepatology.* **41** (1), 177-86.

Liguori, M. J., et al. (2008). Trovafloxacin-induced gene expression changes in liver-derived in vitro systems: comparison of primary human hepatocytes to HepG2 cells. *Drug Metab Dispos.* **36** (2), 223-33.

Liu, S., et al. (2011). ATR autophosphorylation as a molecular switch for checkpoint activation. *Mol Cell.* **43** (2), 192-202.

Liu, Z. X. and Kaplowitz, N. (2002). Immune-mediated drug-induced liver disease. *Clin Liver Dis.* **6** (3), 755-74.

Locksley, R. M., et al. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell.* **104** (4), 487-501.

Lu, J., et al. (2012). Amiodarone exposure during modest inflammation induces idiosyncrasy-like liver injury in rats: role of tumor necrosis factor-alpha. *Toxicol Sci.* **125** (1), 126-33.

Lu, Y. and Cederbaum, A. I. (2010). CYP2E1 potentiation of LPS and TNFalpha-induced hepatotoxicity by mechanisms involving enhanced oxidative and nitrosative stress, activation of MAP kinases, and mitochondrial dysfunction. *Genes Nutr.* **5** (2), 149-67.

Lubasch, A., et al. (2000). Comparative pharmacokinetics of ciprofloxacin, gatifloxacin, grepafloxacin, levofloxacin, trovafloxacin, and moxifloxacin after single oral administration in healthy volunteers. *Antimicrob Agents Chemother.* **44** (10), 2600-3.

Lucena, M. I., et al. (2000). Trovafloxacin-induced acute hepatitis. *Clin Infect Dis.* **30** (2), 400-1.

Luyendyk, J. P., et al. (2003). Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. *J Pharmacol Exp Ther.* **307** (1), 9-16.

Luyendyk, J. P., et al. (2005). Coagulation-mediated hypoxia and neutrophil-dependent hepatic injury in rats given lipopolysaccharide and ranitidine. *J Pharmacol Exp Ther.* **314** (3), 1023-31.

MacKenzie, S. H. and Clark, A. C. (2012). Death by caspase dimerization. *Adv Exp Med Biol.* **747** 55-73.

Macleod, K. F., et al. (1995). p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev.* **9** (8), 935-44.

Maddox, J. F., et al. (2010). Bacterial- and viral-induced inflammation increases sensitivity to acetaminophen hepatotoxicity. *J Toxicol Environ Health A.* **73** (1), 58-73.

Malhi, H., et al. (2006). Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology.* **43** (2 Suppl 1), S31-44.

Malhi, H. and Gores, G. J. (2008). Cellular and molecular mechanisms of liver injury. *Gastroenterology.* **134** (6), 1641-54.

Mandal, D., et al. (2002). Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. *FEBS Lett.* **513** (2-3), 184-8.

Martin, S. J., et al. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med.* **182** (5), 1545-56.

Masuda, K., et al. (2010). MKP-7, a JNK phosphatase, blocks ERK-dependent gene activation by anchoring phosphorylated ERK in the cytoplasm. *Biochem Biophys Res Commun.* **393** (2), 201-6.

McElwee, M. K., et al. (2009). Copper activation of NF-kappaB signaling in HepG2 cells. *J Mol Biol.* **393** (5), 1013-21.

Megyesi, J., et al. (1996). The p53-independent activation of transcription of p21 WAF1/CIP1/SDI1 after acute renal failure. *Am J Physiol.* **271** (6 Pt 2), F1211-6.

Melnik, G., et al. (1998). Hepatobiliary elimination of trovafloxacin and metabolites following single oral doses in healthy volunteers. *Eur J Clin Microbiol Infect Dis.* **17** (6), 424-6.

Menegazzi, R., et al. (1994). Evidence that tumor necrosis factor alpha (TNF)-induced activation of neutrophil respiratory burst on biologic surfaces is mediated by the p55 TNF receptor. *Blood.* **84** (1), 287-93.

Menzel, R. and Gellert, M. (1994). The biochemistry and biology of DNA gyrase. *Adv Pharmacol.* **29A** 39-69.

Michalopoulos, G. K. (2010). Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am J Pathol.* **176** (1), 2-13.

Micheau, O. and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell.* **114** (2), 181-90.

Minero, V. G., et al. (2013). JNK activation is required for TNFalpha-induced apoptosis in human hepatocarcinoma cells. *Int Immunopharmacol.* **17** (1), 92-8.

Miyamoto, Y., et al. (2004). Apoptotic hepatocellular carcinoma HepG2 cells accelerate blood coagulation. *Hepatol Res.* **29** (3), 167-172.

Mizuno, R., et al. (2004). Inhibition of MKP-1 expression potentiates JNK related apoptosis in renal cancer cells. *J Urol.* **172** (2), 723-7.

Moll, U. M. and Petrenko, O. (2003). The MDM2-p53 interaction. *Mol Cancer Res.* **1** (14), 1001-8.

Momii, Y., et al. (2007). p73gamma transactivates the p21 promoter through preferential interaction with the p300/CBP-associated factor in human prostate cancer cells. *Oncol Rep.* **18** (2), 411-6.

Mondal, N. and Parvin, J. D. (2001). DNA topoisomerase IIalpha is required for RNA polymerase II transcription on chromatin templates. *Nature.* **413** (6854), 435-8.

Moore, F. D., Jr., et al. (1991). Tumor necrosis factor and endotoxin can cause neutrophil activation through separate pathways. *Arch Surg.* **126** (1), 70-3.

Murphy, E. J., et al. (2000). Troglitazone-induced fulminant hepatic failure. Acute Liver Failure Study Group. *Dig Dis Sci.* **45** (3), 549-53.

Nakano, H. (2004). Signaling crosstalk between NF-kappaB and JNK. *Trends Immunol.* **25** (8), 402-5.

Nam, E. A., et al. (2011). Thr-1989 phosphorylation is a marker of active ataxia telangiectasia-mutated and Rad3-related (ATR) kinase. *J Biol Chem.* **286** (33), 28707-14.

Neininger, A., et al. (2002). MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. *J Biol Chem.* **277** (5), 3065-8.

Nguyen, N. T. and Vierling, J. M. (2011). Acute liver failure. *Curr Opin Organ Transplant.* **16** (3), 289-96.

Nightingale, S. L. (1999). From the Food and Drug Administration. *JAMA.* **282** (1), 19.

Nishina, H., et al. (2004). Physiological roles of SAPK/JNK signaling pathway. *J Biochem.* **136** (2), 123-6.

Nowsheen, S. and Yang, E. S. (2012). The intersection between DNA damage response and cell death pathways. *Exp Oncol.* **34** (3), 243-54.

O'Grady, J. G., et al. (1993). Acute liver failure: redefining the syndromes. *Lancet.* **342** (8866), 273-5.

Obermayer-Straub, P., et al. (2000). Autoimmune hepatitis. *J Hepatol.* **32** (1 Suppl), 181-97.

Ohira, H., et al. (2003). Adhesion molecules and CXC chemokines in endotoxin-induced liver injury. *Fukushima J Med Sci.* **49** (1), 1-13.

Okayasu, R., et al. (1998). Wortmannin inhibits repair of DNA double-strand breaks in irradiated normal human cells. *Radiat Res.* **149** (5), 440-5.

Ostapowicz, G., et al. (2002). Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann Intern Med.* **137** (12), 947-54.

Outman, W. R. and Nightingale, C. H. (1989). Metabolism and the fluoroquinolones. *Am J Med.* **87** (6C), 37S-42S.

Pannu, H. K., et al. (2001). Acute liver failure due to trovafloxacin: CT findings. *Emergency Radiology.* **8** (2), 108-110.

Papa, S., et al. (2004). Linking JNK signaling to NF-kappaB: a key to survival. *J Cell Sci.* **117** (Pt 22), 5197-208.

Parada-Turska, J., et al. (2006). Effect of glutamate receptor antagonists and antirheumatic drugs on proliferation of synoviocytes in vitro. *Eur J Pharmacol.* **535** (1-3), 95-7.

Parrilla-Castellar, E. R., et al. (2004). Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. *DNA Repair (Amst).* **3** (8-9), 1009-14.

Peasland, A., et al. (2011). Identification and evaluation of a potent novel ATR inhibitor, NU6027, in breast and ovarian cancer cell lines. *Br J Cancer.* **105** (3), 372-81.

Pereira, N. A. and Song, Z. (2008). Some commonly used caspase substrates and inhibitors lack the specificity required to monitor individual caspase activity. *Biochem Biophys Res Commun.* **377** (3), 873-7.

Platz, E. A. and De Marzo, A. M. (2004). Epidemiology of inflammation and prostate cancer. *J Urol.* **171** (2 Pt 2), S36-40.

Plumpe, J., et al. (2000). NF-kappaB determines between apoptosis and proliferation in hepatocytes during liver regeneration. *Am J Physiol Gastrointest Liver Physiol.* **278** (1), G173-83.

Pognonec, P. (2010). ERK and cell death: overview. *FEBS J.* **277** (1), 1.

Pohlhaus, J. R. and Kreuzer, K. N. (2005). Norfloxacin-induced DNA gyrase cleavage complexes block Escherichia coli replication forks, causing double-stranded breaks in vivo. *Mol Microbiol.* **56** (6), 1416-29.

Polo, S. E. and Jackson, S. P. (2011). Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev.* **25** (5), 409-33.

Pommier, Y., et al. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol.* **17** (5), 421-33.

Poulsen, K. L., et al. (2011). Trovafloxacin promotes DNA double-strand breaks in a macrophage cell line. *The Toxicologist.* **120** (2), 188.

Price, C. J., et al. (2003). Human cellular inflammation in the pathology of acute cerebral ischaemia. *J Neurol Neurosurg Psychiatry.* **74** (11), 1476-84.

Racanelli, V. and Rehermann, B. (2006). The liver as an immunological organ. *Hepatology.* **43** (2 Suppl 1), S54-62.

Rajabalian, S., et al. (2009). Diclofenac inhibits proliferation but not NGF-induced differentiation of PC12 cells. *Pak J Pharm Sci.* **22** (3), 259-62.

Rangnekar, A. S. and Fontana, R. J. (2011). An update on drug induced liver injury. *Minerva Gastroenterol Dietol.* **57** (2), 213-29.

Rasola, A., et al. (2010). Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition. *Proc Natl Acad Sci U S A.* **107** (2), 726-31.

Roberts, R. A., et al. (2007). Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. *Toxicol Sci.* **96** (1), 2-15.

Robles, S. J., et al. (1999). Permanent cell cycle arrest in asynchronously proliferating normal human fibroblasts treated with doxorubicin or etoposide but not camptothecin. *Biochem Pharmacol.* **58** (4), 675-85.

Rodriguez, R., et al. (2007). TNF triggers mitogenic signals in NIH 3T3 cells but induces apoptosis when the cell cycle is blocked. *Eur Cytokine Netw.* **18** (4), 172-80.

Rodriguez-Antona, C., et al. (2002). Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica.* **32** (6), 505-20.

Rogakou, E. P., et al. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* **273** (10), 5858-68.

Roos, W. P. and Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol Med.* **12** (9), 440-50.

Roos, W. P. and Kaina, B. (2013). DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. *Cancer Lett.* **332** (2), 237-48.

Rossi, R., et al. (2006). The dispersal of replication proteins after Etoposide treatment requires the cooperation of Nbs1 with the ataxia telangiectasia Rad3-related/Chk1 pathway. *Cancer Res.* **66** (3), 1675-83.

Roth, R. A., et al. (1997). Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? *Toxicol Appl Pharmacol.* **147** (2), 300-11.

Roth, R. A., et al. (2003). Inflammation and drug idiosyncrasy--is there a connection? *J Pharmacol Exp Ther.* **307** (1), 1-8.

Roth, R. A. and Ganey, P. E. (2011). Animal models of idiosyncratic drug-induced liver injury--current status. *Crit Rev Toxicol.* **41** (9), 723-39.

Saitoh, M., et al. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.* **17** (9), 2596-606.

Saleh-Gohari, N., et al. (2005). Spontaneous homologous recombination is induced by collapsed replication forks that are caused by endogenous DNA single-strand breaks. *Mol Cell Biol.* **25** (16), 7158-69.

Sass, G., et al. (2001). Inducible nitric oxide synthase is critical for immune-mediated liver injury in mice. *J Clin Invest.* **107** (4), 439-47.

Schlayer, H. J., et al. (1987). Enhancement of neutrophil adherence to isolated rat liver sinusoidal endothelial cells by supernatants of lipopolysaccharide-activated monocytes. Role of tumor necrosis factor. *J Hepatol.* **5** (3), 311-21.

Schroeter, H., et al. (2003). c-Jun N-terminal kinase (JNK)-mediated modulation of brain mitochondria function: new target proteins for JNK signalling in mitochondrion-dependent apoptosis. *Biochem J.* **372** (Pt 2), 359-69.

Schutze, S., et al. (2008). Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. *Nat Rev Mol Cell Biol.* **9** (8), 655-62.

Schwabe, R. F. (2006). Cell death in the liver-all roads lead to JNK. *Gastroenterology.* **131** (1), 314-6.

Scott, F. L., et al. (2005). XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *EMBO J.* **24** (3), 645-55.

Segel, G. B., et al. (2011). The paradox of the neutrophil's role in tissue injury. *J Leukoc Biol.* **89** (3), 359-72.

Seki, E., et al. (2012). A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches. *Gastroenterology.* **143** (2), 307-20.

Seok, J. H., et al. (2008). Long-term Activation of c-Jun N-terminal Kinase through Receptor Interacting Protein is Associated with DNA Damage-induced Cell Death. *Korean J Physiol Pharmacol.* **12** (4), 185-91.

Sertic, S., et al. (2012). NER and DDR: classical music with new instruments. *Cell Cycle.* **11** (4), 668-74.

Sharma, A., et al. (2012). Histone H2AX phosphorylation: a marker for DNA damage. *Methods Mol Biol.* **920** 613-26.

Shaw, P. J., et al. (2007). Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. *Toxicol Sci.* **100** (1), 259-66.

Shaw, P. J., et al. (2009a). Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. *Toxicol Sci.* **111** (2), 288-301.

Shaw, P. J., et al. (2009b). Coexposure of mice to trovafloxacin and lipopolysaccharide, a model of idiosyncratic hepatotoxicity, results in a unique gene expression profile and interferon gamma-dependent liver injury. *Toxicol Sci.* **107** (1), 270-80.

Shaw, P. J., et al. (2009c). The role of the hemostatic system in murine liver injury induced by coexposure to lipopolysaccharide and trovafloxacin, a drug with idiosyncratic liability. *Toxicol Appl Pharmacol.* **236** (3), 293-300.

Shaw, P. J., et al. (2009d). Trovafloxacin enhances the inflammatory response to a Gram-negative or a Gram-positive bacterial stimulus, resulting in neutrophil-dependent liver injury in mice. *J Pharmacol Exp Ther.* **330** (1), 72-8.

Shaw, P. J., et al. (2009e). Tumor necrosis factor alpha is a proximal mediator of synergistic hepatotoxicity from trovafloxacin/lipopolysaccharide coexposure. *J Pharmacol Exp Ther.* **328** (1), 62-8.

Shi, Y., et al. (2003). Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature.* **425** (6957), 516-21.

Shih, S. C. and Stutman, O. (1996). Cell cycle-dependent tumor necrosis factor apoptosis. *Cancer Res.* **56** (7), 1591-8.

Shim, J. H., et al. (2005). TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev.* **19** (22), 2668-81.

Shiozaki, E. N., et al. (2003). Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell.* **11** (2), 519-27.

Sidana, A., et al. (2012). Mechanism of growth inhibition of prostate cancer xenografts by valproic acid. *J Biomed Biotechnol.* **2012** 180363.

Smart, D. J. (2008). Genotoxicity of topoisomerase II inhibitors: an anti-infective perspective. *Toxicology.* **254** (3), 192-8.

Smart, D. J., et al. (2008). Ciprofloxacin-induced G2 arrest and apoptosis in TK6 lymphoblastoid cells is not dependent on DNA double-strand break formation. *Cancer Biol Ther.* **7** (1), 113-9.

Smart, D. J. and Lynch, A. M. (2012). Evaluating the genotoxicity of topoisomerase-targeted antibiotics. *Mutagenesis.* **27** (3), 359-65.

Smith, J., et al. (2010). The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res.* **108** 73-112.

Sorensen, C. S. and Syljuasen, R. G. (2012). Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. *Nucleic Acids Res.* **40** (2), 477-86.

Sparkenbaugh, E. M., et al. (2012). Hypoxia sensitization of hepatocytes to neutrophil elastase-mediated cell death depends on MAPKs and HIF-1alpha. *Am J Physiol Gastrointest Liver Physiol.* **302** (7), G748-57.

Sriskandan, S. and Cohen, J. (1999). Gram-positive sepsis. Mechanisms and differences from gram-negative sepsis. *Infect Dis Clin North Am.* **13** (2), 397-412.

Stadheim, T. A., et al. (2001). Inhibition of extracellular signal-regulated kinase (ERK) mediates cell cycle phase independent apoptosis in vinblastine-treated ML-1 cells. *Cancer Res.* **61** (4), 1533-40.

Stassen, J. M., et al. (2004). The hemostatic system. *Curr Med Chem.* **11** (17), 2245-60.

Stepniak, E., et al. (2006). c-Jun/AP-1 controls liver regeneration by repressing p53/p21 and p38 MAPK activity. *Genes Dev.* **20** (16), 2306-14.

Stohr, B. A. and Kreuzer, K. N. (2001). Repair of topoisomerase-mediated DNA damage in bacteriophage T4. *Genetics.* **158** (1), 19-28.

Suffredini, A. F., et al. (1995). Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration. *J Immunol.* **155** (10), 5038-45.

Supino, R., et al. (1977). Effect of daunorubicin and adriamycin on nucleic acid synthesis of serum stimulated mouse embryo fibroblasts. *Tumori.* **63** (1), 31-42.

Takada, Y., et al. (2005). Zerumbone abolishes NF-kappaB and IkappaBalpha kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis, and downregulation of invasion. *Oncogene.* **24** (46), 6957-69.

Takeda, K. and Akira, S. (2004). TLR signaling pathways. *Semin Immunol.* **16** (1), 3-9.

Tamura, Y., et al. (2004). The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria. *FEBS Lett.* **569** (1-3), 249-55.

Tang, F., et al. (2002). The absence of NF-kappaB-mediated inhibition of c-Jun N-terminal kinase activation contributes to tumor necrosis factor alpha-induced apoptosis. *Mol Cell Biol.* **22** (24), 8571-9.

Tartaglia, L. A., et al. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell.* **74** (5), 845-53.

Taudorf, S., et al. (2007). Human models of low-grade inflammation: bolus versus continuous infusion of endotoxin. *Clin Vaccine Immunol.* **14** (3), 250-5.

Taylor, R. C., et al. (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol.* **9** (3), 231-41.

Temple, R. J. and Himmel, M. H. (2002). Safety of newly approved drugs: implications for prescribing. *JAMA.* **287** (17), 2273-5.

Teng, R., et al. (1996). Multiple-dose pharmacokinetics and safety of trovafloxacin in healthy volunteers. *J Antimicrob Chemother.* **37** (5), 955-63.

Tentner, A. R., et al. (2012). Combined experimental and computational analysis of DNA damage signaling reveals context-dependent roles for Erk in apoptosis and G1/S arrest after genotoxic stress. *Mol Syst Biol.* **8** 568.

Thadepalli, H., et al. (2005). Antitumor activity of trovafloxacin in an animal model. *In Vivo.* **19** (1), 269-76.

Tracy, R. P. (2003). Emerging relationships of inflammation, cardiovascular disease and chronic diseases of aging. *Int J Obes Relat Metab Disord.* **27 Suppl 3** S29-34.

Tran, A., et al. (1991). Etoposide (VP 16-213) induced hepatitis. Report of three cases following standard-dose treatments. *J Hepatol.* **12** (1), 36-9.

Tsujimoto, Y. (1997). Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes. *Cell Death Differ.* **4** (6), 429-34.

Tukov, F. F., et al. (2007). The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. *Toxicol Sci.* **100** (1), 267-80.

Uetrecht, J. (2006). Evaluation of which reactive metabolite, if any, is responsible for a specific idiosyncratic reaction. *Drug Metab Rev.* **38** (4), 745-53.

Uetrecht, J. (2007). Idiosyncratic drug reactions: current understanding. *Annu Rev Pharmacol Toxicol.* **47** 513-39.

Uetrecht, J. and Naisbitt, D. J. (2013). Idiosyncratic adverse drug reactions: current concepts. *Pharmacol Rev.* **65** (2), 779-808.

Usui, M. and Kawarada, Y. (1998). [Endotoxin and its binding protein in organ failure]. *Nihon Geka Gakkai Zasshi.* **99** (8), 504-9.

Utsugi, T., et al. (1990). Potentiation of topoisomerase inhibitor-induced DNA strand breakage and cytotoxicity by tumor necrosis factor: enhancement of topoisomerase activity as a mechanism of potentiation. *Cancer Res.* **50** (9), 2636-40.

Vanderlinde, R. E. (1985). Measurement of total lactate dehydrogenase activity. *Ann Clin Lab Sci.* **15** (1), 13-31.

Ventura, J. J., et al. (2003). c-Jun NH(2)-terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor. *Mol Cell Biol.* **23** (8), 2871-82.

Vermes, I., et al. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods.* **184** (1), 39-51.

Vincent, J., et al. (1997). Pharmacokinetics and safety of trovafloxacin in healthy male volunteers following administration of single intravenous doses of the prodrug, alatrofloxacin. *J Antimicrob Chemother.* **39 Suppl B** 75-80.

Visvanathan, K., et al. (2007). Regulation of Toll-like receptor-2 expression in chronic hepatitis B by the precore protein. *Hepatology.* **45** (1), 102-10.

Vogelstein, B., et al. (2000). Surfing the p53 network. *Nature.* **408** (6810), 307-10.

Vos, S. M., et al. (2011). All tangled up: how cells direct, manage and exploit topoisomerase function. *Nat Rev Mol Cell Biol.* **12** (12), 827-41.

Wajant, H., et al. (2003). Tumor necrosis factor signaling. *Cell Death Differ.* **10** (1), 45-65.

Waldman, T., et al. (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* **55** (22), 5187-90.

Walgren, J. L., et al. (2005). Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit Rev Toxicol.* **35** (4), 325-61.

Wallach, D., et al. (1999). Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol.* **17** 331-67.

Wang, C., et al. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature.* **412** (6844), 346-51.

Wang, J. C. (2002). Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol.* **3** (6), 430-40.

Wang, J. E., et al. (2003). Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. *Shock.* **20** (5), 402-14.

Wang, W., et al. (1999). The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. *Clin Cancer Res.* **5** (1), 119-27.

Wang, W. H., et al. (2004). Sustained activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase pathways by hepatitis B virus X protein mediates apoptosis via induction of Fas/FasL and tumor necrosis factor (TNF) receptor 1/TNF-alpha expression. *Mol Cell Biol.* **24** (23), 10352-65.

Wang, X., et al. (2000). Requirement for ERK activation in cisplatin-induced apoptosis. *J Biol Chem.* **275** (50), 39435-43.

Ward, I. M. and Chen, J. (2001). Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem.* **276** (51), 47759-62.

Watkins, P. B. (2005). Idiosyncratic liver injury: challenges and approaches. *Toxicol Pathol.* **33** (1), 1-5.

Watkins, P. B. and Seeff, L. B. (2006). Drug-induced liver injury: summary of a single topic clinical research conference. *Hepatology.* **43** (3), 618-31.

Waxler, B., et al. (1994). Anesthetic agents modify tissue proteinase inhibitor content and tumor behavior. *J Lab Clin Med.* **123** (1), 53-8.

Wei, F., et al. (2011). Extracellular signal-regulated kinases modulate DNA damage response - a contributing factor to using MEK inhibitors in cancer therapy. *Curr Med Chem.* **18** (35), 5476-82.

Westwick, J. K., et al. (1994). Tumor necrosis factor alpha stimulates AP-1 activity through prolonged activation of the c-Jun kinase. *J Biol Chem.* **269** (42), 26396-401.

Wicovsky, A., et al. (2007). Sustained JNK activation in response to tumor necrosis factor is mediated by caspases in a cell type-specific manner. *J Biol Chem.* **282** (4), 2174-83.

Wilcox, C. S. (2010). Effects of tempol and redox-cycling nitroxides in models of oxidative stress. *Pharmacol Ther.* **126** (2), 119-45.

Willmott, C. J., et al. (1994). The complex of DNA gyrase and quinolone drugs with DNA forms a barrier to transcription by RNA polymerase. *J Mol Biol.* **242** (4), 351-63.

Wilstermann, A. M. and Osheroff, N. (2003). Stabilization of eukaryotic topoisomerase II-DNA cleavage complexes. *Curr Top Med Chem.* **3** (3), 321-38.

Win, S., et al. (2011). c-Jun N-terminal kinase (JNK)-dependent acute liver injury from acetaminophen or tumor necrosis factor (TNF) requires mitochondrial Sab protein expression in mice. *J Biol Chem.* **286** (40), 35071-8.

Witkamp, R. and Monshouwer, M. (2000). Signal transduction in inflammatory processes, current and future therapeutic targets: a mini review. *Vet Q.* **22** (1), 11-6.

Wullaert, A., et al. (2006). Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. *Biochem Pharmacol.* **72** (9), 1090-101.

Wullaert, A., et al. (2007). Hepatic tumor necrosis factor signaling and nuclear factor-kappaB: effects on liver homeostasis and beyond. *Endocr Rev.* **28** (4), 365-86.

Yamada, Y., et al. (1998). Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor. *Hepatology.* **28** (4), 959-70.

Yanagawa, Y., et al. (2002). Activation of extracellular signal-related kinase by TNF-alpha controls the maturation and function of murine dendritic cells. *J Leukoc Biol.* **71** (1), 125-32.

Yang, J., et al. (2003). ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. *Carcinogenesis.* **24** (10), 1571-80.

Yee, S. B., et al. (2003). The role of Kupffer cells and TNF-alpha in monocrotaline and bacterial lipopolysaccharide-induced liver injury. *Toxicol Sci.* **71** (1), 124-32.

Yim, H., et al. (2006). Cleavage of Cdc6 by caspase-3 promotes ATM/ATR kinase-mediated apoptosis of HeLa cells. *J Cell Biol.* **174** (1), 77-88.

Yoshida, K., et al. (2002). Activation of SAPK/JNK signaling by protein kinase Cdelta in response to DNA damage. *J Biol Chem.* **277** (50), 48372-8.

Zakeri, S. M., et al. (2000). Effects of trovafloxacin on the IL-1-dependent activation of E-selectin in human endothelial cells in vitro. *Immunopharmacology.* **48** (1), 27-34.

Zhang, C. L., et al. (2004). Cytochrome c release from oridonin-treated apoptotic A375-S2 cells is dependent on p53 and extracellular signal-regulated kinase activation. *J Pharmacol Sci.* **96** (2), 155-63.

Zhang, Y., et al. (1999). Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. *Oncogene.* **18** (5), 1131-8.

Zhou, B. B. and Elledge, S. J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature.* **408** (6811), 433-9.

Zimmerman, H. J. (1993). Hepatotoxicity. *Dis Mon.* **39** (10), 675-787.

Zou, L. (2007). Single- and double-stranded DNA: building a trigger of ATR-mediated DNA damage response. *Genes Dev.* **21** (8), 879-85.

Zou, W., et al. (2009a). Sulindac metabolism and synergy with tumor necrosis factor-alpha in a drug-inflammation interaction model of idiosyncratic liver injury. *J Pharmacol Exp Ther.* **331** (1), 114-21.

Zou, W., et al. (2009b). Hepatotoxic interaction of sulindac with lipopolysaccharide: role of the hemostatic system. *Toxicol Sci.* **108** (1), 184-93.