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CALCIUM-CALMODULIN COMPLEX INHIBITION
BY CISPLATIN AND EMESIS

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CALCIUM-CALMODULIN COMPLEX INHIBITION BY CISPLATIN AND EMESIS

By

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ABSTRACT

CALCIUM-CALMODULIN COMPLEX INHIBITION BY CISPLATIN AND EMESIS

By

Robert Karl Jarve

Cisplatin (8mg/kg) treatment of wistar rats demonstrated no change in nitric oxide synthase localization or its intensity for up to 5 days. However, immunohistochemical localization of L-citrulline and the Ca^{2+}-Calmodulin complex revealed decreased levels after only 3 days. An *in vitro* experiment using an analog of calmodulin, MeroCalmodulin-1, showed that *cis*-diammine-diaquacisplatinum (II), a hydrolyzed form of cisplatin, inhibited the calmodulin conformational shift from occuring through a direct interaction with the calmodulin molecule. These results indicate that distention of the stomach was due to inhibition of n-NOS activitation by a direct interaction between cisplatin and the calcium binding sites of the calmodulin molecule.

In Memory of My Brother
Daniel Peter Jarve

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Special Thanks go to Dr. S.K. Aggarwal for serving as my research advisor, and for having faith in my abilities. I would also like to thank Dr. R. Pax and Dr. A. Goyal for their valuable assistance. My thanks also go to Dr. Schindler for his help in the use of a spectrofluorometer and his laboratory facilities. My special thanks to Dr. D. Lansing Taylor for his kind gift of the MeroCalmodulin-1. Cisplatin was a gift of NIH chemistry branch. Diaqua-cisplatin was a gift from Dr. Broomhead of the Department of Medical Chemistry, Australian National University.

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INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum II), a broad spectrum anti-cancer drug has proven effective in the treatment of ovarian (1), head and neck (2) lung (3), breast (4), bladder, testicular cancers (5), and certain types of leukemia (6).

Draw backs of this chemotherapeutic drug are it' s severe toxic side effects, which include the production of free radicals, oxidative stress (7), lipid peroxidation (8), hypocalcemia, hypomagnesia (9),tetany, nausea, vomiting, ototoxicity, peripheral neuropathy, myelosuppression, nephrotoxicity (10), embryotoxicity (11), peptic ulcers, and distention of the stomach (12, 13). Nausea and vomiting being dose limiting factors in some patients receiving the drug with 60-80% of them having delayed emesis pronounced 48-72 h post-treatment (14, 15).

Distention of the stomach has been shown to parallel the nausea and vomiting that are associated with clinical use of cisplatin (16). Cisplatin induces inhibition of acetylcholine release from stomach nerve endings, which in turn results in a prolonged relaxation of stomach smooth muscle (13).

Studies on neuronal nitric oxide synthase (n-NOS) have shown that genetically engineered mice that have had the gene responsible for the production of n-NOS removed are normal except for bloating of the stomach due to prolonged constriction of the pyloric sphincter (17). Stomach smooth muscle contraction is acetylcholine dependent (18), while pyloric sphincter smooth muscle relaxation is nitric oxide dependent (19), and both acetylcholine release and nitric oxide synthesis are calmodulin dependent (20, 21).

Calmodulin's ability to bind to calcium has been shown to be nonspecific (22). Additionally, calmodulin has been shown to react with heavy metals like Cd^{2+}, Pb^{2+}, and Zn^{2+}, and these metal ions show biphasic activation curves (23). At low concentrations they can induce activation of calmodulin, however, at high concentrations there is no activation (23). Monovalent ions have also been shown to have an inhibitory effect on calmodulin's ability to bind to calcium (23). Once within the cell under low chloride ion concentrations cisplatin has been shown to hydrolyze into monoaqua and diaqua species that have a single or double positive charge respectively (12, 24-26).

Since normal stomach motility is influenced in part by the nitric oxide (NO) neurotransmitter and this in turn is regulated by the calcium-calmodulin complex (21) the present study was undertaken to evaluate whether cisplatin prevents n-NOS activation through an inhibitory interaction with

calmodulin similar to that seen with other heavy metal ions (23).

MATERIALS AND METHODS

Animals. Male and female Wistar rats (Charles Rivers Laboratories, Wilmington MA) weighing 175-200g were kept on a 12h light/ 12 h dark cycle with free access to laboratory animal feed and water in accordance with the Guide for Care and Use of Laboratory Animals. Rats were injected with a bolus ip injection of cisplatin (8mg/kg) in 0.85% NaCl,while the controls received the vehicle alone. Animals were either perfused with the fixatives or sacrificed via decapitation on the 4th or 5th day of treatment.

Tissue Collection. Normal and cisplatin treated rats were given a lethal dose of sodium pentabarbital and perfused with a mixture of 0.5% gluteraldehyde and 4% formaldehyde in 0.1M phosphate buffer (pH 7.4)using a Masterflex perfusion pump (Cole Parmer, Chicago IL). Tissues were then excised and postfixed at 4° C for 8-12 h in the same fixative (27). Tissues from decapitated animals were quickly excised and placed in a mixture of 0.5% gluteraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer(pH 7.4) at 4° C for 8 to 12 h.

After fixation all tissues were placed overnight in 0.1M phosphate buffer (pH 7.4) containing 15% sucrose, and

cryosectioned (7-10μm). Cryosections were picked up on gelatin coated coverslips.

Nitric Oxide Synthase Localization. Cryosections were washed in 50mM Tris (pH 7.4) for 10 min at room temperature, and incubated in 50mM Tris (pH 7.4) containing 1mg/ml NADPH (Sigma, USA), 0.3 mM Nitro Blue Tetrazoleum (Sigma, USA), and 0.2% Triton X-100 (Sigma, USA) at 37° C for 3 hours. Sections were dehydrated through a graded series of alcohols, cleared in Hemo-de, and mounted on glass slides with permount (27, 28) .

L-Citrulline Localization. Immunocytochemical localization of citrulline was done according to previously established protocols(27, 29). Cryosections from perfused fixed animals were rinsed for 10 min in 0.01M phosphate buffered saline (PBS) (pH 7.4) and treated with 5% normal goat serum (NGS) (Jackson Immunoresearch, West Grove PA) in 0.01M PBS (pH 7.4) for 10 min. These sections were then incubated overnight at room temperature with an antibody to L-citrulline (1/20,000 dilution) (Arnel Products Co., New York, NY) in 0.01M PBS (pH 7.4), containing 1% NGS, and 0.1% sodium azide. Such sections were rinsed in 0.01M PBS for 10 min,5% NGS for 10 min, and incubated for 30-60 min in a serum of biotinylated-goat anti-rabbit IgG (1/15 dilution) (Sigma) in 0.01M PBS (pH 7.4), containing 1% NGS, and 0.1% sodium azide. Sections were washed for 10 min in 0.01M PBS (pH 7.4) followed by a 10 min wash in 1% hydrogen peroxide in 0.1M PBS (pH 7.4) to block endogenous peroxidases. After washings, sections were

incubated 30-60 min in avidin-peroxidase solution(1/15 dilution) with 0.01M PBS containing 0.1% sodium azide, and exposed to freshly prepared 0.075% diaminobenzidine (DAB) with 0.0002% hydrogen peroxide in 50 mM Tris(pH 7.6). These sections were thoroughly rinsed in 50 mM Tris followed by 0.01M PBS and mounted on glass slides with glycerin jelly (27, 29). Control sections involved incubation in media without the primary or secondary antibody.

Ca^{2+}-Calmodulin Complex Localization. Care was taken to prepare all solutions for the following experiments with double distilled deionized water, and calcium free reagents. Immunocytochemical localization of the Ca^{2+}-calmodulin complex was done using the established protocol (30) with the minor modification that tissues were incubated in normal donkey serum (NDS) to minimize cross reactivity with rat tissue by the secondary antibody. Sections were washed with 0.01M PBS (pH 7.0) and incubated with a 1/50 dilution of mouse monoclonal anti-calmodulin antibody (Chemicon International Inc., Temecula CA) in 0.01M PBS (pH 7.0) containing 1% NDS, and 0.1% sodium azide for 60 min at 37° C. After primary incubation sections were washed with 0.01M PBS (pH 7.0) and incubated for 30 min in 5% NDS containing 0.01M PBS (pH 7.0). Tissues were then incubated for 60 min at 37° C with FITC-conjugated donkey anti-mouse IgG (1/200 dilution) (Jackson Immunoresearch, West Grove PA) in 0.01M PBS (pH 7.0), containing 1% NDS, and 0.1% sodium azide. Sections were then washed with 0.01 M PBS, and mounted on glass slides with slow

fade (Molecular Probes). Sections incubated in 0.01M PBS (pH 7.0) containing 5% NDS instead of the primary antibody were used as controls (30).

Photomicroscopy. Transmitted images were taken using a Zeiss Photomicroscope II with neutral density filters. Epifluorescent images were taken using a Zeiss 10 Laser Scanning Confocal Microscope (LSM) and a Matrix Multicolor computerized camera unit. All fluorescent images were taken in confocal mode, using a blue argon laser (488nm) for excitation and a long pass barrier filter of 520 nm.

Preparation of Hydrolyzed Cisplatin. Cisplatin (3mg) was added to 10ml of double distilled deionized water and incubated at 37° C for two weeks (25). The product of this hydrolysis was a mixture of monoaqua-cisplatin (cis-diammine-monoaqua-monochlorplatinum II) and diaqua-cisplatin (*cis*-diamine-diaquaplatinum II). Diaqua-cisplatin (31) was prepared by adding 2.5mg of a synthesized form of the molecule, to 10 ml of double distilled deionized water.

Calcium-Calmodulin Binding: *In vitro* Studies. MeroCalmodulin-1 (MeroCam) is a covalent adduct of calmodulin, isolated from bovine brain, and dye Mc4.19 that fluoresces when it undergoes a conformational shift associated with Ca^{2+} activation (32).

Lyophilized MeroCam was dissolved in double distilled deionized water to a concentration of 123 nM. Measurement of MeroCam's fluorescence was done using a Perkin Elmer 650-40 Fluorescence Spectrophotometer with a 150 Xenon power supply.

Each cuvette was filled with 1.0 ml of the solution. Excitation and emission slits were kept at 15 nm and 5 nm respectively. Excitation was at 532 nm and 608 nm, emission was monitored at 623 nm. Data was plotted using the ratio of 608 nm to 532 nm. Each data point was measured three different times without any variation.

Determination of calcium contamination of the MeroCam sample was done by taking 1 ml of a 123 nM solution of MeroCam and alternating concentrations by adding 50 µl of 1mM EGTA, 50 µl 1mM $CaCl_2$, 50µl of 1mM EGTA, and 100µl of 1mM $CaCl_2$.

Binding studies were conducted by increasing concetrations of 10µM diaqua-cisplatin, $CaCl_2$, double distilled deionized water, or the hydrolyzed product of cisplatin containing a mixture of monoaqua and diaqua-cisplatin (22, 32) at regular intervals in a 123nM MeroCalmodulin solution.

RESULTS

Nitric Oxide Synthase Localization. Using histochemical methods the presence of n-NOS was demontrated in the muscular plexus of stomach smooth muscle in both the cisplatin treated and normal animals (Figure 1). Nerve fibers of the muscular plexus are localized as dark blue striations within the smooth muscle of the stomach (Figure 1). In normal tissues macrophages could be demonstrated as intensely positive for nitric oxide synthase within the stomach (Figure 1), however, tissues from cisplatin treated animals did not show any nitric oxide synthase within their macrophages (Figure 1). Cross sections of the villi from the stomachs of both the normal and cisplatin treated animals demonstrated intense reaction (Figure 1).

L-Citrulline Localization. Immunohistochemical studies demonstrated an intense reaction for L-citrulline in the neural ganglia, muscular plexus, and nerve fibers within the smooth muscle of the stomach and pyloric sphincter, and the stomach villi of normal animals (Figure 2).

Pyloric sphincter and stomach tissues from cisplatin treated animals showed minimal to no localization of the L-citrulline within the neural plexus and the neural ganglia of

Figure 1. Light micrograph of NOS localization in the pyloric region of the stomach. In normal tissues (A) there is intense localization of macrophage nitric oxide synthase (M-NOS) within macrophages (curved arrow). Cisplatin treated tissues (B), however, show no localization for m-NOS. Note both normal tissues and cisplatin treated tissues show localization of n-NOS within the nerve fibers (arrows) of the stomach's smooth muscle, and within the cytoplasm of stomach villi (double arrows).

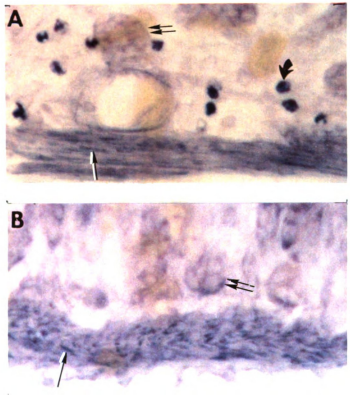
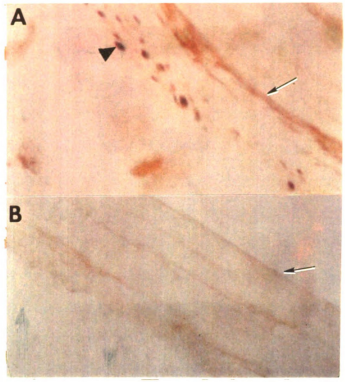

A
B

Figure 2. Light micrograph of immunohistochemical localization of L-citrulline, observed as intense brown staining only in the stomach smooth muscle of the normal rats (A), however, no such staining was observed after cisplatin treatment (B). Note: neural ganglia (arrowhead) and nerve fibers (arrow).


A
B

the smooth muscle (Figure 2). Localization of L-citrulline was also depressed within cross sections of the stomach villi of cisplatin treated tissues.

Ca^{2+}-Calmodulin Complex Localization. Normal animal tissues showed intense immunohistochemical localization of the Ca^{2+}-calmodulin complex in the smooth muscle of the pyloric sphincter as well as in the Brunner's Glands and both the longitudinal and circular muscle layers of the gastroduodenal junction (Figure 3). Intensity of nerve fibers within the deep muscular plexus was so great that nerve endings could not be easily distinguished from nerve fibers (Figure 3).

When compared to normal, tissues from cisplatin treated animals showed a marked decrease in the localization of the Ca^{2+}-calmodulin complex within the smooth muscle of the pyloric sphincter and the Brunner's Glands and longitudinal and circular muscle layers of the gastroduodenal junction (Figure 3). While nerve endings of the deep muscular plexus were labeled by the antibody, the nerve fibers themselves showed little to no labeling (Figure 3).

Calcium-Calmodulin Binding: *In vitro* Studies. After addition of 50 μM of EGTA the excitation ratio of the MeroCam solution decreased by one (Figure 4). This decrease was reversed by addition of 50μM of $CaCl_2$ (Figure 5). Fluorescence was alternately decreased and increased by subsequent additions of 50 μM of EGTA and 50 μM of $CaCl_2$ respectively (Figure 4).

Figure 3. Light micrographs indicating the presence of the Ca^{2+}-Calmodulin-complex taken using a Zeiss 10 Laser Scanning Confocal Microscope in confocal mode with a 488nm argon laser for the light source. Normal Tissue (A) demonstrating intense fluorescence within the gastroduodenal junction. Cisplatin treated tissues (B) showed a marked decrease in fluorescence. Note: circular muscle laryer (c), longitudinal muscle layer (l), nerve fibers (thin arrows), nerve endings (thick arrows), Brunner's glands (b).

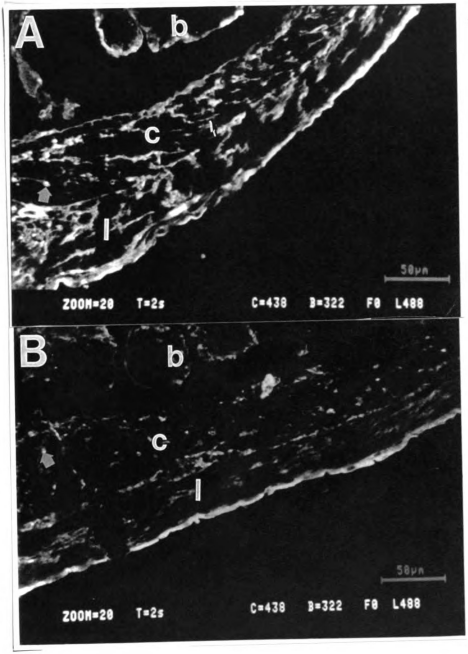

A
b
c
l
50µm
ZOOM=20 T=2s C=438 B=322 F0 L488
B
b
c
l
50µm
ZOOM=20 T=2s C=438 B=322 F0 L488

Figure 4. Graph showing the fluorescence of 123 nM MeroCam when treated with alternating doses of 1 mM EGTA and 1mM $CaCl_2$. Fluorescence of MeroCam decreased after the addition of EGTA to it. This decrease was reversed by subsequent addition of $CaCl_2$. This effect of EGTA was repeated through two more alternating additions of EGTA and $CaCl_2$.

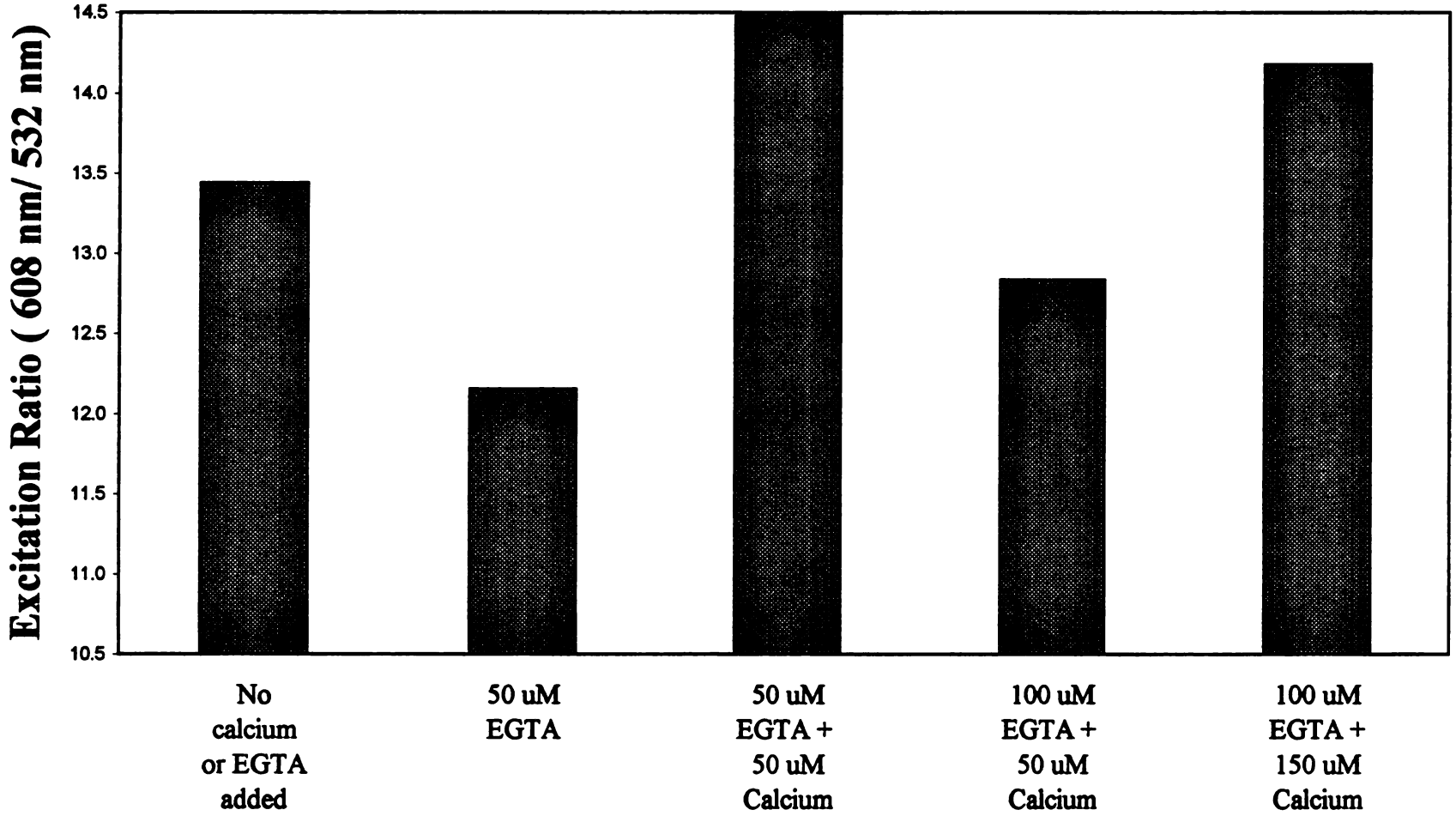
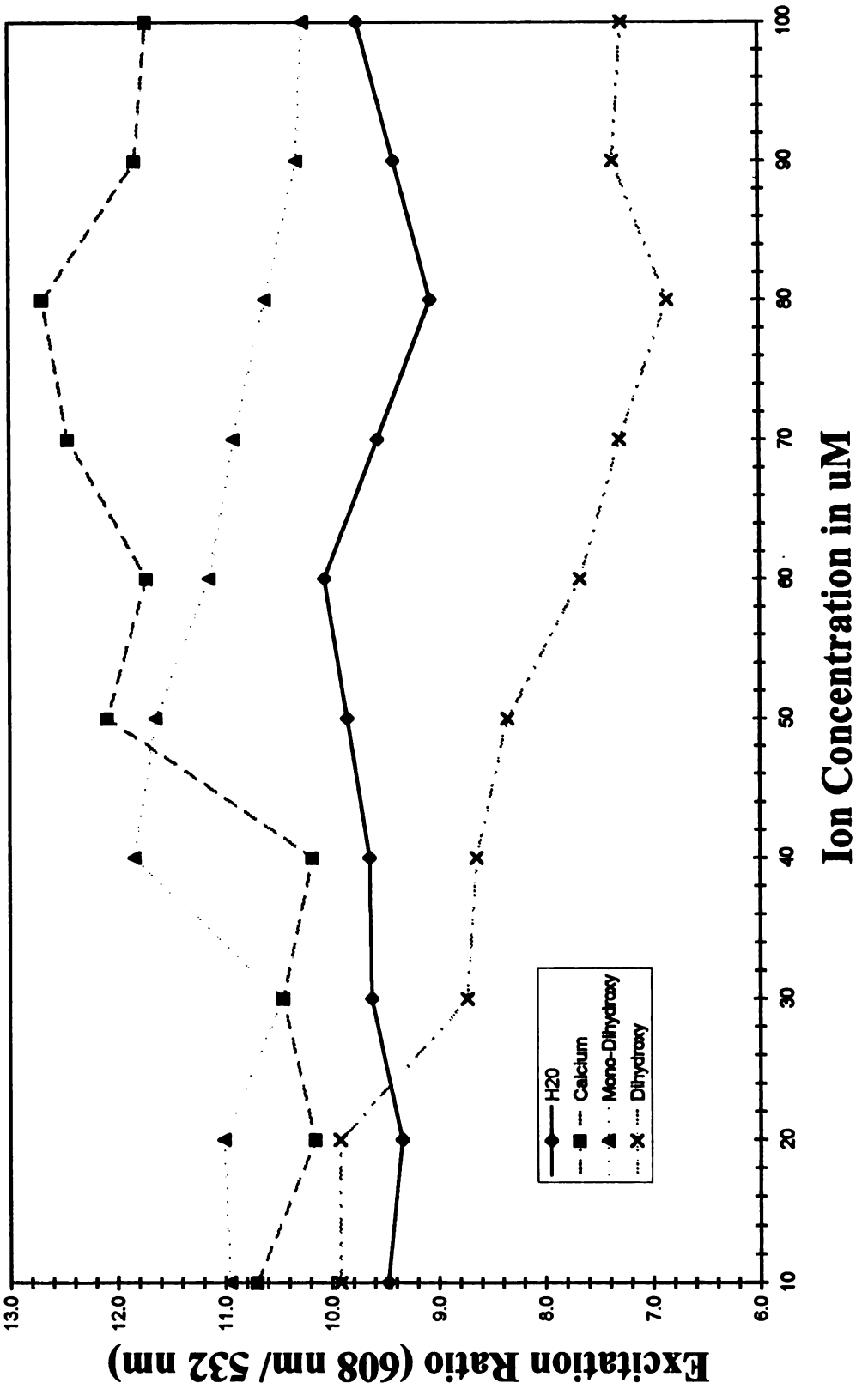

Excitation Ratio (608 nm/ 532 nm)
14.5
14.0
13.5
13.0
12.5
12.0
11.5
11.0
10.5
No calcium or EGTA added
50 uM EGTA
50 uM EGTA + 50 uM Calcium
100 uM EGTA + 50 uM Calcium
100 uM EGTA + 150 uM Calcium

Figure 5. Graph showing the fluorescence of 123 nM MeroCam when treated with varying concentrations of calcium, diaqua-cisplatin, double distilled deionized water, and a mixture containing monoaqua- & diaqua-cisplatin,. Note that the fluorescence of MeroCam decreased after diaqua-cisplatin treatment with the greatest decrease obtained at a concentration of 80μM.


Excitation Ratio (608 nm/ 532 nm)
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
10
20
30
40
50
60
70
80
90
100
Ion Concentration in uM
H20
Calcium
Mono-Dihydroxy
Dihydroxy

MeroCam when subjected to increasing concentrations of calcium showed a two and a half point increase in its excitation ratio, when compared with the baseline solution which received only double distilled deionized water, with a peak in fluorescence at 80μM of $CaCl_2$ (Figure 5).

MeroCam that received increasing concentrations of mono- and diaqua-cisplatin showed an initial two point excitation ratio peak at 40μM but the excitation ratio declined afterwards ending with only a half point increase in the excitation ratio when compared to the baseline solution (Figure 5).

With varying concentrations of diaqua-cisplatin fluorescence decreased dramatically with a two and a half point decrease in it's excitation ratio when compared with the baseline solution (Figure 5)

DISCUSSION

The mechanisms of action of cisplatin are far from clear. It is generally accepted that cisplatin binds to DNA to prevent it's replication or transcription, yet repair mechanisms have been shown to reverse this process (33). Microtubular and microfilamentous depolymerization has also been suggested as a means to inhibit cytokinesis (11), however, these mechanisms do not explain the toxicities associated with this drug. Of these, nausea and vomiting are dose limiting factors (13). We have learned to control these symptoms in part through diuresis and the use of antiemetic drugs but the cause of these toxicities still eludes us. Cisplatin treatment causes distention of the stomach in rodents as they do not show antiemetic responses (16). Distention of the stomach has been shown to parallel the nausea and vomiting that are associated with the clinical use of cisplatin (16) . Thus, alleviation of stomach distention may hold the key to preventing the symptoms of nausea and vomiting associated with it, however, the mechanism by which cisplatin induces distention of the stomach needs to be worked out before this can be achieved.

Normal stomach motility is controlled in part by two different neurotransmitter systems, one using acetylcholine

and the other NO. Both systems are initiated by an action potential induced depolarization of the nerve's plasma membrane that triggers the opening of calcium gated channels (20). The subsequent increase in cytosolic calcium causes an increase in the formation of the calcium-calmodulin complex. In the case of stomach smooth muscle contraction this complex phosphorylates synapsin I, which in turn causes the uncoupling of acetylcholine vesicles from the cytoskeletal structure of stomach nerve terminals (20). Once free from the cytoskeletal structure the vesicles proceed to the synaptic cleft where they are released, opening the sodium channels of the surrounding smooth muscle. This in turn triggers contraction of myofibrils causing muscle contraction (Figure 6a) (20).

The calcium-calmodulin complex causes pyloric sphincter relaxation, however, by activating n-NOS (21). Once activated n-NOS converts L-arginine into L-citrulline and NO (29). NO is then immediately released into the synaptic cleft where it easily diffuses into the surrounding smooth muscle and activates guanyline cyclase(18, 20). This in turn causes an increase in the levels of cGMP which leads to smooth muscle relaxation.

Inhibition of acetycholine activity and accumulation of acetylcholine vesicles in the nerve terminals of stomach smooth muscle has been demonstrated after cisplatin treatment (13). Histochemical localization of NOS showed that levels of the enzyme in the smooth muscle of both the pyloric

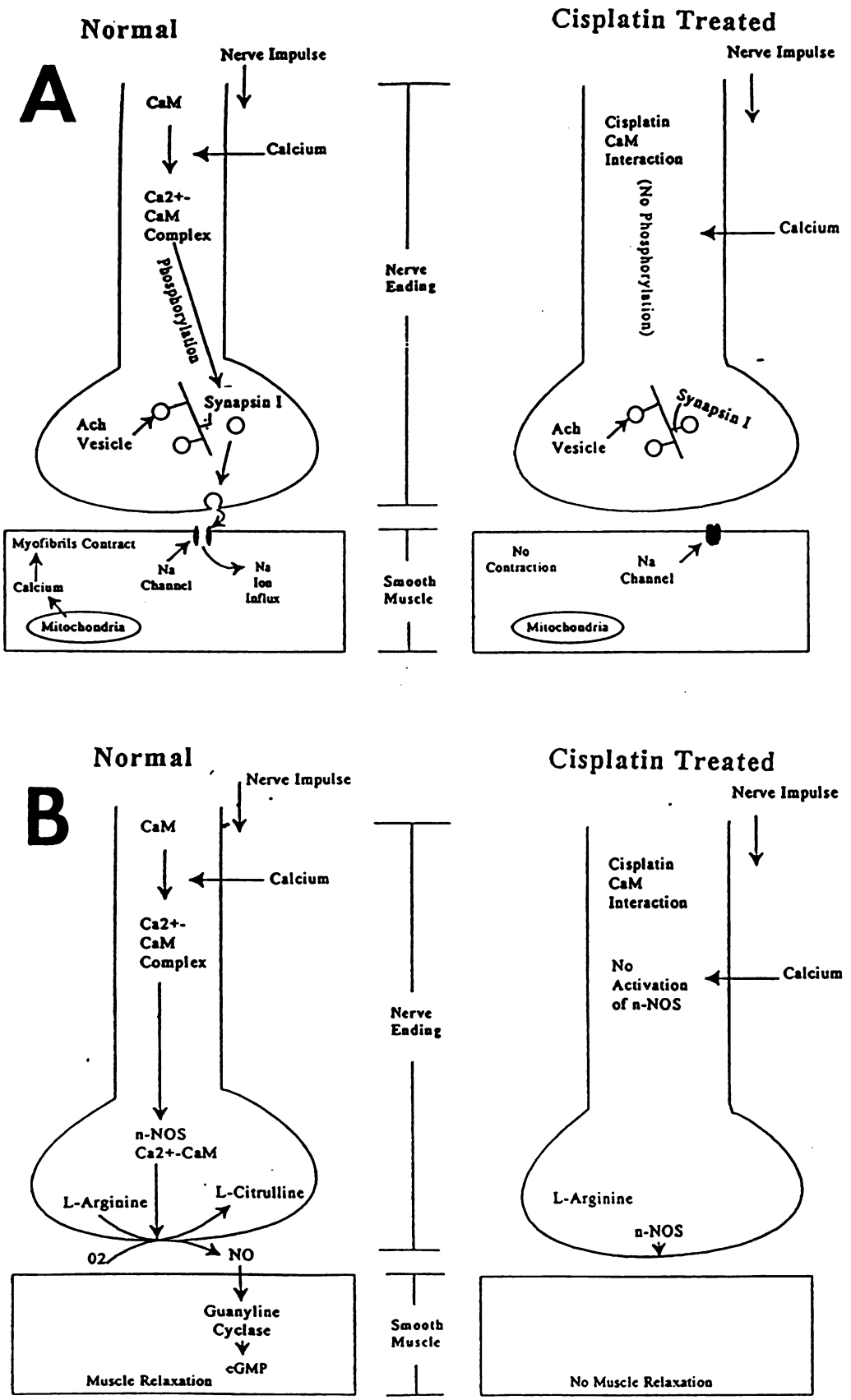
sphincter and stomach were unaffected by cisplatin treatment, however, the level of L-citrulline present in the neural ganglia and muscular plexus of the smooth muscle from cisplatin treated tissues was depressed compared to normal animals. Thus, cisplatin treatment appears to be inhibiting the activation of n-NOS in the pyloric sphincter.

Acetylcholine release and n-NOS activation are regulated by the calcium-calmodulin complex (20, 21), and our studies show that cisplatin depresses the level of this complex. It is possible that this disruption of the calcium-calmodulin complex is responsible for the inhibition of Ach release and NO production that are seen with cisplatin treatment.

Cisplatin hydrolyzes into monoaqua (monovalent) and diaqua (divalent) species rapidly within the cell and cisplatin treatment has been shown to cause hypocalcemia (6, 13). Monovalent and divalent ions have been shown to interfere with calmodulin's ability to bind to calcium, and the monovalent and divalent species of cisplatin have been shown to be the most toxic forms of the drug (12, 24, 26, 34).

In vitro tests on the effects of cisplatin's monoaqua and diaqua species show that addition of diaqua cisplatin causes a distinct and immediate decrease in the amount of calmodulin undergoing a conformational shift, where as increased calcium levels cause an increase in the level of calmodulin undergoing a conformational shift associated with

Figure 6. Schematic representation of the interaction of acetylcholine (A) and nitric oxide (B) with the gastrointestinal smooth muscle and a possible mechanism by which cisplatin interacts with it. Ach: Acetylcholine; CaM: Calmodulin; cGMP: cyclic guanidine monophosphate.


Normal
Cisplatin Treated
A
Nerve Impulse
CaM
Calcium
Ca2+-
CaM
Complex
Phosphorylation
Synapsin I
Ach
Vesicle
Nerve
Ending
Myofibrils Contract
Calcium
Na
Channel
Na
Ion
Influx
Mitochondria
Smooth
Muscle
Cisplatin
CaM
Interaction
(No Phosphorylation)
No
Contraction
Na
Channel
B
n-NOS
Ca2+-CaM
L-Arginine
L-Citrulline
02
NO
Guanyline
Cyclase
cGMP
Muscle Relaxation
No
Activation
of n-NOS
No Muscle Relaxation

calcium binding (22, 23, 32). These results suggest that diaqua cisplatin with its divalent charge is inhibiting the ability of calmodulin to undergo it's conformational shift by competitively binding to the sites where calcium normally would. Thus, it is possible that n-NOS activation and acetylcholine vesicle release are prevented due to a competitive inhibition of the calmodulin molecule.

Without normal n-NOS activation and acetycholine vessicle release stomach motility would be compromised. Specifically, stomach smooth muscle would undergo prolonged relaxation (13), and pyloric sphincter smooth muscle would become hypercontractile (17) with the end result being stomach distention probably inducing nausea and vomiting (13, 17, 35). This is in fact the situation that occurs with cisplatin treatment (13). Thus, a treatment that could reverse the effect of cisplatin on calmodulin might be extremely beneficial in the therapeutic application of the drug.

A possible method for the alleviation of the nausea and vomiting associated with the drug's toxic effects on stomach motility is the artificial elevation of calcium levels prior to and during cisplatin treatment. In rodents such treatments have been shown to reduce stomach distention and ulceration, and return normal contractility to stomach smooth muscle (13). This ability of artificially high calcium levels to reduce cisplatin induced distention of the stomach

is most likely due to a competition between the increased calcium and diaqua cisplatin for calmodulin's binding sites.

REFERENCES

1. Mangioni, C., Colombo, N., Epis, A., Landoni, F., Redaelli, L., Vassena, L., and Torri, W. Different platinum schedules in the treatment of epithelial ovarian cancer. *In:* M. Nicolini (ed.) Platinum and other metal coordination compounds in cancer chemotherapy, pp. 386. Padua: Martinus Nijhoff Publishing, 1987.

2. Choksi, A. and Hong, W. K. Chemotherapy of head and neck cancer. *In:* M. Nicolini (ed.) Platinum and Other Metal Coordination compounds in cancer chemotherapy, pp. 375-385. Padua: Martinus Nijhoff Publishing, 1987.

3. Paccagnella, A., Favaretto, G., and Fiorentino, M. V. Chemotherapy of lung cancer. *In:* M. Nicolini (ed.) Platinum and other metal coordination compounds in cancer chemotherapy., pp. 207. Padua: Martinus Nijhoff Publishing, 1987.

4. Pronzato, P., Amoroso, D., Bertelli, G., Gallotti, P., and Rosso, R. Combination of cisplatin and 5-fluorouracil in the treatment of advanced breast cancer. *In:* M. Nicolini (ed.) Platinum and other metal coordination comounds in cancer chemotherapy., pp. 499. Padua: Martinus Nijhoff Publishing, 1987.

5. Stoter, G., Williams, S. W., and Einhorn, L. H. Genitourinary tumors. *In:* H. M. Pinedo (ed.) Cancer Chemotherapy Annual, pp. 317. New York: Elsevier North-Holland, 1981.

6. Rosenberg, B. Fundamental studies with cisplatin., Cancer. *55:* 2303-2316, 1985.

7. Douple, E. B. *Cis*-Diamminedichloroplatinum (II): Effects of a representative metal coordination complex on mammalian cells. *In:* L. A. Dethlefsen (ed.) Cell Cycle Effects of Drugs, pp. 215. New York: Pergamon Press, 1985.

8. Bompart, G. Cisplatin-induced changes in cytochrome P-450, lipid peroxidation and drug-metabolizing enzyme activities in rat kidney cortex., Toxicol. Letts. *48:* 193-199, 1989.

9. Aggarwal, S. K. and Fadool, J. M. Effects of cisplatin and carboplatin on neurohypophysis, parathyroid and their role in nephrotoxicity. *In:* M. Nicolini (ed.) Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy, pp. 235-247. Padua: Martinus Nijhoff Publishing, 1987.

10. Hacker, M. P. and Roberts, J. D. Cisplatin Efficacy and toxicity: are they separable? *In:* M. Nicolini (ed.) Platinum and other metal coordination compounds in cancer chemotherapy., pp. 163. Padua: Martineus Nijhoff Publishing, 1987.

11. Aggarwal, S. K. and Fadool, J. M. Cisplatin and carboplatin induced changes in the neurohypophysis and parathyroid, and their role in nephrotoxicity., Anti-Cancer Drugs. *4:* 149, 1993.

12. Aggarwal, S. K. A histochemical approach to the mechanism of action fo cisplatin and its analogues, J. Histochem. Cytochem. *41:* 1053-1076, 1993.

13. Aggarwal, S. K., San Antonio, J. D., Sokhansanj, A., and Miller, C. M. Cisplatin induced peptic ulcer, vagotomy adrenal and calcium modulation., Anti-Cancer Drugs. *5:* 177-93, 1994.

14. Kris, M. B., Gralla, R. J., Clark, R. A., and al., e. Incidence, course and severity of delayed nausea and vomiting following the administration of high dose cisplatin., J. Clin. Oncol. *3:* 1738-84, 1985.

15. Richtsmeier, W. J. and Mazur, E. M. The use of metaclopromide in treatment of cisplatin-induced emesis., Otolaryngol. Head Neck Surg. *95:* 358-9, 1986.

16. Roos, I. A. G., Fairlie, D., and Whitehouse, M. W. A peculiar toxicity manifested by platinum (II) amines in rats: gastric distension after intraperitoneal administration., Chem.-Biol. Interact. *35:* 111-117, 1981.

17. Huang, P. L. and Dawson, T. M. Targeted Disruption of the neuronal nitric oxide synthase gene., Cell. *75:* 1273, 1993.

18. Schiller, L. R. Peristalsis, Sci. Am. Sci. Med. *1:* 38-47, 1994.

19. Soediono, P. and Burnstock, G. Contribution of ATP and nitric oxide to NANC inhibitory transmission in rat pyloric sphincter., Br. J. Pharmacol. *113:* 681-686, 1994.

20. Kendell, E. R., Schwartz, J. H., and Jessell, T. M. Principles in Neural Science, 3rd edition. Norwalk: Appleton and Lange, 1991.

21. Sessa, W. C. The Nitric Oxide Synthase Family of Proteins, J. Vasc. Res. *31:* 131-143, 1994.

22. Chao, S. H., Suzuki, Y., Zysk, J. R., and Cheung, W. Y. Activation of calmodulin by various metal cations as a function of ionic radius., Mol. Pharmacol. *26:* 75-82, 1984.

23. Klee, C.B. Interaction of calmodulin with Ca^{2+} and target proteins. *In:* P. Cohen and C. B. Klee (eds.), Calmodulin, Vol. 5, pp. 35-56. Amsterdamn: Elsevier, 1988.

24. Jones, M. M., Basinger, M. A., Beaty, J. A., and Holscher, M. A. The relative nephrotoxicity of cisplatin, *cis*-[$Pt(NH_3)_2(guanisine)_2]^{2+}$, and the hydrolysis product of cisplatin in the rat., Cancer Chemother. Pharmacol. *29:* 29-32, 1991.

25. Litterst, C. L. and Schweitzer, V. G. Covalent Binding of platinum to kidney sublcellular fractions from guinea pig strains sensitive or resistant to cisplatin toxicity. *In:* M. Nicolini (ed.) Platinum and other metal cooordination compounds in cancer chemotherapy., pp. 207. Padua: Martinus Nijhoff Publishing, 1987.

26. Mistry, P., Lee, C., and McBrien, D. C. H. Intracellular metabolites of cisplatin in the rat kidney., Cancer Chemother. Pharmacol. *24:* 73-79, 1989.

27. Zdanski, C. J., Prazma, J., Petrusz, P., Grossman, G., Raynor, E., Smith, T. L., and Pillsbury, H. C. Nitric oxide synthase is an active enzyme in the spiral ganglion cells of the rat cochlea., Hear. Res. *79:* 39-47, 1994.

28. Cobbs, C. S., Brenman, J. E., Aldape, K. D., Bredt, D. S., and Israel, M. A. Expression of nitric oxide synthase in human central nervous system tumors., Cancer Res. *55:* 727-738, 1995.

29. Petrusz, P., Merchenthaler, I., Weinberg, R. J., Grossman, G., and Ordronneau, P. Characterization of an antiserum to citrulline for use in immunocytochemistry., J. Histochem. Cytochem. *41:* 1126, 1993.

30. Mitsuyama, F. and Kanno, T. Localization of Ca^{2+}-calmodulin to the kinetochore of C6 glioma cells: an investigation of the anti-tumor effects of calmodulin antagoinsts in the treatment of brain tumors., Neurol. Res. *15:* 131-135, 1993.

31. Aggarwal, S. K., Broomhead, J. A., Fairlie, D. P., and Whitehouse, M. W. Platinum Drugs: Combined anti-lymphoproliferative and nephrotoxicity assay in rats, Cancer. Chemother. Pharmacol. *4:* 249-258, 1980.

32. Hahn, K. H., Waggoner, A. S., and Taylor, D. L. A calcium-sensitive fluorescent analog of calmodulin based on a novel calmodulin-binding fluorophore., J. Biol. Chem. *265:* 20335-20345, 1990.

33. Andrews, P. and Howell, S. Cellular pharmacology of cisplatin: perspectives on mechanism of acquired resistance, Cancer Cells. *2:* 35, 1990.

34. Jennerwein, M. and Andrews, P. A. Effect of intracellular chloride on the cellular pharmacodynamics of *cis*-diamminedichloroplatinum (II)., Drug Met. Disp. *23:* 178-184, 1995.

35. Vander, A. J., Sherman, J. H., and Luciano, D. S. Human Physiology: The Mechanisms of Body Function, 5th edition, p. 536. New York: McGraw-Hill Publishing Co., 1990.