To the Graduate Council:

I am submitting herewith a thesis written by Jason Wayne Yarbrough entitled "Inhibitors of Intracellular Chlorine Regulation Induces Cisplatin Resistance in Canine Osteosarcoma Cells." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Kevin Hahn, Major Professor

We have read this thesis
and recommend its acceptance

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

INHIBITORS OF INTRACELLULAR CHLORIDE REGULATION INDUCES CISPLATIN RESISTANCE IN CANINE OSTEOSARCOMA CELLS

A Thesis

Presented for the Master of Science

Degree

The University of Tennessee, Knoxville

JASON WAYNE YARBROUGH

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DEDICATION

I dedicate this work to my parents whose love, support and guidance is immeasurable and my fiancé for her love and understanding.

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ABSTRACT

The objective of this study was to examine the role of ion transport mechanisms in clinical anticancer drug resistance. Reduction in intracellular accumulation of cisplatin is believed to be an early change in cisplatin-resistant cells, and may be dependent on the concentration of intracellular chloride (Cl^-) ions and intracellular pH. The primary aim of this study was to describe the modifying effects of NHMA (5-*N*,*N* hexamethylene; amiloride), a Na+/H+ antiport inhibitor, and/or SITS (4-acetamido-4';isothiocyanostilbene-2,2'-disulfonic acid), a HCO3-/Cl- transport inhibitor, in bicarbonate-containing or bicarbonate-free media on cisplatin (cis-diamminedichloroplatinum(II); CDDP) toxicity between known cisplatin-sensitive (COS31) and cisplatin-resistant (COS31/rCDDP) canine osteosarcoma cells. This study has shown that cell survival can be influenced by the inhibition of the Na^+-dependent HCO_3^-/Cl^- exchanger using SITS. The addition of SITS increases the intracellular Cl^- concentration in canine osteosarcoma cells cultured in a bicarbonate-containing media. In a bicarbonate-free media, the addition of SITS results in a decrease in the cytotoxic action of cisplatin.

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CHAPTER I

INTRODUCTION

Survival of dogs with osteosarcoma following cisplatin chemotherapy is directly affected by the accumulation of cisplatin in the cancer cell.[11-15,18,30-32] A decrease in cisplatin accumulation intracellularly can result in cisplatin resistance.[9,42] This drug resistance has been shown to be multifactorial.[9,42] A decrease in drug influx (passive or active transport into the cell), an increase drug efflux (mediated by a number of membrane-associated glycoproteins), or modifications in ion transport mechanisms which regulate pH (intracellular, pHi; extracellular, pHe) have been observed in cisplatin-resistant cell lines.[1,9,16,20,21,24,42]

The objective of this study was to examine the role of ion transport mechanisms in clinical anticancer drug resistance. Reduction in intracellular accumulation of cisplatin is believed to be an early change in cisplatin-resistant cells, and may be dependent on the concentration of intracellular chloride (Cl^-) ions and pHi. The primary aim of this study was to describe the modifying effects of NHMA (5-*N,N* hexamethylene; amiloride), a Na+/H+ antiport inhibitor, and/or SITS (4-acetamido-4';isothiocyanostilbene-2,2'-disulfonic acid), a HCO3-/Cl- transport inhibitor, in bicarbonate-containing or bicarbonate-free media on cisplatin (cis-diamminedichloroplatinum(II); CDDP) toxicity between known cisplatin-sensitive (COS31)[31] and cisplatin-resistant (COS31/rCDDP) [30] canine osteosarcoma cells.

The hypothesis to be tested was that a high intracellular Cl^- and a low pHi, mediated by inhibition of Na^+/H^+ antiport and HCO_3^-/Cl^- anion exchangers from NHMA and/or SITS, respectively, will lower the intracellular concentration of the cytotoxic aquated cisplatin species resulting in drug resistance.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Cancer treatment is often complicated by pathophysiological factors resulting from the microenvironmental heterogeneity of solid tumors. Properties such as tumor blood flow, tissue oxygenation and nutrient supply, pH distribution, and bioenergetic status modulate the response of cells to cancer therapy.[39] These microenvironmental factors may be involved in the development of resistant subpopulations of tumor cells.[39] To improve cell kill in solid tumors, therapies have been developed that target these resistant subpopulations of solid tumors.[17]

In hypoxic environments, such as those seen in solid tumors, glucose is preferentially metabolized by anaerobic glycolytic pathways resulting in excessive amounts of lactic acid.[2,41] Lactic acid and other ketogenic acids produced when fatty acids are utilized as alternative energy resources, are poorly cleared from the cell because of the limited vascular system in the solid tumors.[2,41] The accumulation of these acids result in a lower pHe that ranges from 6.6 to 7.0, an average of 0.5 pH units lower than normal tissues.[40] This acidic pHe can cause the pHi to fall below physiological pH ranges which results in cell death.[2,40,41]

The primary mechanism for maintaining pHi homeostasis in mammalian cells involves ion transport processes.[41]. To remain viable, tumor cells become dependent on these exchangers to maintain the pHi within physiological ranges.[22] Agents that inhibit

the regulation of these exchangers and thus change the pHi have been shown to have dose- and time-dependent cytotoxic effects on tumor cells.[22]

There are three major ion exchangers that are involved in keeping pHi within the normal physiological range. One exchanger predominates under conditions of cytoplasmic alkalinization and the other two predominate under conditions of cytoplasmic acidosis. Under normal physiological conditions, the cation-independent Cl^-/HCO_3^- exchanger works to decrease pHi following cytoplasmic alkalinization by allowing extracellular Cl^- to exchange with intracellular HCO_3^-.[5,7,20,21,37] The Cl^-/HCO_3^- exchanger is minimally active in solid tumor cells because these cells have a lower pHi than that found in normal cells.[5,22]

The Na^+/H^+ antiport exchanges extracellular Na^+ for intracellular H^+ in conditions where the pHi is extremely low.[22,26] In similar conditions, the Na^+-dependent HCO_3^-/Cl^- exchanger transports HCO_3^- into the cell in exchange for Cl^-.[26,29,38] This Cl^- is removed from the cell against a Cl^- concentration gradient because the inward flow of Na^+ allows $NaHCO_3$ to be exchanged for HCl.[20-22] Both of these acid-recovering exchangers appear to be minimally active at normal physiological pH.[5,19,43] The Na^+-dependent HCO_3^-/Cl^- exchanger is most active during periods of acidic pHi when the pHi is closer to that of normal physiological pH (e.g., 6.9).[22] It has been observed that as the pHe drops below a pH of 7.0, the activity of both ion exchangers is decreased. However, the Na^+/H^+ exchanger appears to be the most active at an acidic pHi of approximately 6.6.[5,6,10]

Studies using microelectrodes to measure the pH within solid tumors found that most tumors have pHe values lower than those found in normal tissue.[39] Most tumors

have a pHe range of 6.5 to 6.9, whereas the normal surrounding tissues have pHe values of 7.0 to 7.5.[40] Despite the lower pHe of tumor subpopulations, cells remain viable in these regions.[28] Tumor tissues with an acidic pHe can maintain a fairly constant pHi, which is similar to the pHi of normal tissue.[7] However, cells maintained at low pHe may exhibit a higher pHi due to the pHi regulatory mechanisms which function to neutralize intracellular acid and thereby maintain pHi homeostasis.

Manipulation of the pHe *in vitro* is one way to manipulate ion exchange and effect cell toxicity. It has been reported that KHT fibrosarcoma cells and EMT6 mouse mammary tumor cells exposed to an pHe of 7.2 and 6.8 for a 72 hour time period show a significant decrease in cell survivability.[41] When the relationship between pHe and pHi was studied in EMT6 cells, a linear relationship was observed between the two pH units. The pHi was seen to be alkaline at all pHe values but as the pHe continued to decline the pHi levels eventually fell into the acidic range.[20-22]

Ion transport inhibitors such as NHMA and SITS alter cisplatin toxicity by interfering with cellular homeostasis and disrupting ion balance in the cell.[1] Modifications in ion transport mechanisms have been observed in other cisplatin-resistant cell lines.[3] Although cisplatin is believed to enter cells by passive diffusion, other evidence suggests that cisplatin accumulation may involve a carrier-mediated mechanism dependent upon Na^+ concentration.[9,42] The regulatory mechanisms inhibited by SITS and NHMA are involved in H^+, HCO_3^-, and Cl^- transport.[1,20,21] Therefore, changes in the cellular concentration of these ions may alter cisplatin toxicity by altering pHi. A low intracellular Cl^- concentration is believed to contribute to cisplatin remaining in its toxic

form.[9,42] If Cl^- concentrations within the cell increase in the presence of NHMA and SITS, lower concentrations of the cytotoxic aquated cisplatin species may result. Therefore, it is possible that NHMA and SITS may cause cisplatin resistance by interfering with numerous ion-mediated cellular processes.

Cisplatin in its native state is not reactive.[21,23,33] When cisplatin is dissolved into water, Cl^- are replaced by water molecules in a stepwise manner resulting in mono- and diaquated cisplatin.[21,22] The extent of this aquation is determined by the reaction time and the Cl^- concentration in the media.[33] *In vivo* this aquation reaction is thought to occur in the cytosol since the pHi and Cl^- concentrations are lower than that in the blood.[33]

It has been shown that cisplatin reacts differently in basic and acidic solutions.[20,23] In an acidic pH range of 5.5 to 7.2, cisplatin hydrolysis results in the formation of (chloro)(hydroxo), (chloro)(aqua) and (hydroxo)(aqua) species.[20,23] This is important since di(hydroxo) and (chloro)(hydroxo) are inert to substitution, but these, along with di(chloro), are capable of cell membrane penetration.[20,23] The metabolites (hydroxo)(aqua) and (chloro)(aqua) ions are reactive and ready to attack polar donor groups in the DNA. The Cl^- concentration plays a role in the active groups since Cl^- can reverse the hydrolysis of the (hydroxo)(aqua) and the (chloro)(aqua) to (chloro)(hydroxo).[20] This means that in an acidic medium, cisplatin is predominantly in an electroneutral form.[20] This explains the observation that an acidic pHe results in an increase in cisplatin uptake into the cell, and that the change in ion concentration inside the cell causes ion trapping of the drug.[20,21]

Because a reduction in intracellular accumulation of cisplatin is believed to be an early change in cisplatin-resistant cells and may be dependent on the concentration of intracellular Cl^- and pHi, the primary aim of this study was to describe the modifying effects of NHMA and/or SITS in bicarbonate-containing or bicarbonate-free media on cisplatin toxicity between known cisplatin-sensitive (COS31)[31] and cisplatin-resistant (COS31/rCDDP)[30] canine osteosarcoma cells.

CHAPTER III

METHODS

Study Design

All experiments were performed on cell lines derived from a naturally occurring canine osteosarcoma and known to retain the *in vitro* biologic, physiologic and immunologic properties that were observed *in vivo*.[30,31] Cells were maintained in either bicarbonate-containing or bicarbonate-free culture medium in the presence or absence of NHMA or SITS. The effect of these microenvironmental and cellular manipulations on the cytotoxicity of cisplatin was determined by measuring cell survival following the exposure of cells to varying concentrations of cisplatin, and by measuring intracellular Cl^- concentration (MEQ fluorescence detection). All experiments were performed in triplicate and repeated in a known cisplatin-resistant canine osteosarcoma cell line.[30]

Cell Culture Conditions

COS31 and COS31/rCDDP cells were grown and subcultured in 75 cm^2 cell culture flasks (Fisher Scientific, Pittsburgh PA). Dulbelcco's modification of Eagle's medium (DMEM) containing 3.7 grams of sodium bicarbonate per liter was used for maintaining viable cells and producing cell subcultures. The DMEM was supplemented with 10% unheated fetal bovine serum (FBS) and 1% [2mM] glutamic acid (Life Technologies, Grand Island, N.Y.,USA) for complete media. The media was adjusted to

a pH of 7.2 to 7.4. Cells were grown in an environment of 5% CO_2 and 95% air and kept at a constant temperature of 37 degrees C.

Cells were removed from culture flasks when they were at approximately 90% confluency. Spent media was aspirated using a sterile pipet and a vacuum hose. Ten ml of Hank's Balanced Salt Solution (HBSS) was then added to the flask to remove the traces of the serum-containing media. Once the flask was rinsed with HBSS, the salt solution was removed by aspiration. Four ml of 25% Trypsin- 1mM EDTA was added to the flask, the flask capped, and incubated for five minutes at 37 degrees C. The flask was gently tapped on the side with the palm of the hand to make sure all cells had lifted free from the surface of the tissue flask. Under a type IIA hood 8 ml of complete media was added to the flask. Complete media containing FBS was used to inactivate the trypsin. The cells were then removed from the tissue culture flask and placed into a 15ml centrifuge tube. The cells were centrifuged at 1000-x g for five minutes in an 18 degrees C centrifuge. The media was removed by gentle aspiration, being careful not to disturb the cells pellet at the bottom of the tube. Cells were resuspended in 6 ml of complete media and a cell count performed.

The cells were counted with a hemocytometer. Forty µl of the resuspended cells were placed into a 5ml glass test tube. Eighty µl of 0.4% trypan blue was added and the two samples gently mixed. A 9.1µl aliquot of the cell-dye preparation was added to each side of the hemocytometer, avoiding air bubbles and overfilling of the hemocytometer. Viable cells, which did not take up the dye, were counted and the cell concentration was calculated using the equation:

$$(\#\ Cells/\ 10)*3*10^4 = Cells/ml$$

The # Cells is the total number of cells counted on the hemocytometer grid. This number was divided by ten because of the ten quadrants evaluated on the hemocytometer, resulting in an average number of cells per quadrant on the hemocytometer. The concentration ratio (3) was used since the final solution contained one part cells and media and two parts trypan blue. Finally 10^4 was used to convert the number of cells counted to cells per ml.

Drugs and Drug Preparation

Cisplatin, SITS, and NHMA were purchased (Sigma Chemical Company, Saint Louis, MO). Cisplatin (300 μg) was dissolved in 1 ml of serum free media. Dilutions were made from this stock solution using complete media. SITS was prepared as a 10mM solution in serum-free media. NHMA was prepared as a 1mM solution in dimethyl sulfoxide (DMSO). Dilutions from these stock solutions were made using complete media. All solutions were prepared the same day as the experiment and were kept protected from light.

6-methoxy-N-ethylacridinium chloride (MEQ) is a cell-impermeable compound that must be converted to 6-methoxy-N-ehtyl-1,2-dihydroquinoline (DiH-MEQ) to be permeable. The yield of DiH-MEQ is approximately quantitative to the initial amount of MEQ prepared. Five mg of MEQ was placed into a 5ml glass test tube and 6 mg of sodium borohydride was placed into a second glass test tube. The MEQ test tube was covered with aluminum foil to protect it from light. Under a ventilation hood, 0.1ml of

distilled water was added to the MEQ and 50 µl of distilled water was added to the sodium borohydride. The MEQ solution was flushed with a slow stream of nitrogen gas for one minute. While maintaining the nitrogen gas flow, 10 µl of sodium borohydride was slowly added to the MEQ solution. The MEQ/sodium borohydride mixture was kept under the nitrogen gas flow for an additional 30 minutes. During this time period any remaining sodium borohydride was destroyed through the addition of acetone.

Following the conversion of MEQ to DiH-MEQ, 0.5ml of the solvent chloroform and 0.5ml of water were added to the DiH-MEQ mixture. The mixture was vortexed thoroughly for 30 seconds to allow the water and organic layer to intermingle. After vortexing the layers were allowed to separate and the organic layer was removed by glass pipet, avoiding the aspiration of the aqueous layer, and placed into a clean 5ml glass test tube. The procedure was repeated with another 0.5ml of chloroform and the organic layer from both procedures was combined in the new glass test tube. With the organic layer in a new test tube, 100mg of magnesium sulfate was added as a drying agent and mixed thoroughly with a glass pipet. The test tube was placed under slow nitrogen flow and allowed it to sit for five minutes.

The organic layer was transferred to a glass container and sealed tightly. The organic layer was removed from the test tube with a glass pipet and transferred to a glass container, without disturbing the settled magnesium sulfate. The organic extract was evaporated using a nitrogen gas flow placed above the solvent. The remaining solid DiH-MEQ was stored in the dark in a tightly sealed container under nitrogen. The solid DiH-MEQ can be stored under these conditions at –70 degrees C for two weeks.

Experiments

Experiment 1: Influence of pH on cisplatin toxicity in COS31 and COS31/rCDDP cells.

Approximately 1800 cells from either cell line were placed in each well of a 96-well flat-bottom plate (Falcon Chemical Company) in complete media, adjusted to a pH of 6.4 or 7.4. Cisplatin was added to each well at a concentration varying from 0.0 to 2.5 μg/ml. The final volume of each treatment well was 200μl and all experiments were performed in quadruplicate. Cells were incubated for 4 days at 37 degrees C in an atmosphere of 5% CO_2 and 95% air.

Cell survivability at pH 6.4 and pH 7.4 was determined using the Cell Titer 96@AQueous One Solution Cell Proliferation Assay. Briefly, following 4 days of incubation, 40μl of the cell titer solution was added to each well. The plates were incubated at 37 degrees C for four hours. Before the plates were read all wells were cleared of any air bubbles which could produce artifactual results. The optical density at 490 nm (OD_{490}) of the metabolized fluorochrome, which is the product of the Cell Titer after mitochondria metabolism, was measured using a 96-well plate reader (EL-312, Biokinetic Reader, Bio-Tex Instruments, Winooski, VT). Cell survival was calculated as a fraction of OD_{490} of each well compared with untreated (control) cell OD_{490}, multiplied by 100.

Experiment 2: LD_{50} of cisplatin in COS31 and COS31/rCDDP cells.

A standard curve of cisplatin toxicity in both cell lines at a pH of 7.4 and the LD_{50} (the concentration of drug which kills 50% of the cells) was determined for the COS31 and COS31/rCDDP cell lines. Approximately 1800 cells were plated in each well of a 96-well culture plate and exposed to cisplatin at dosages ranging from 0.0 to 3.0 μg/ml for two to four days. Cell survivability of cisplatin treated cells was determined using the Cell Titer 96@AQueous One Solution Cell Proliferation Assay. Cell survival was calculated as a fraction of the OD_{490} of each well compared with untreated (control) cell OD_{490}, multiplied by 100. All experiments were conducted in triplicate.

Experiment 3: Influence of NHMA on cisplatin toxicity in COS31 and COS31/rCDDP cells.

To determine the effect of NHMA, an inhibitor of Na^+/H^+ exchange, on cisplatin toxicity, 10μM NHMA (a concentration shown by others to significantly inhibit ion exchange)[20] was added to each well of a 96-well culture plate containing approximately 1800 cells per well. Cells were exposed to cisplatin at dosages ranging from 0.0 to 2.5 μg/ml for 4 days. Cell survivability of NHMA and cisplatin treated cells was determined using the Cell Titer 96@AQueous One Solution Cell Proliferation Assay. Cell survival was calculated as a fraction of the OD_{490} of each well compared with untreated (control) cell OD_{490}, multiplied by 100. All experiments were conducted in triplicate.

Experiment 4: Influence of SITS on cisplatin toxicity in COS31 and COS31/rCDDP cells.

To determine the effect of SITS, an inhibitor of HCO_3^-/Cl^- ion exchange on cisplatin toxicity, cells from both lines were exposed to SITS at dosages ranging from 0 to 2.0mM. SITS was added to each well of a 96-well culture plate containing approximately 1800 cells per well. Cell survivability of SITS treated cells was determined using the Cell Titer 96@AQueous One Solution Cell Proliferation Assay. Cell survival was calculated as a fraction of the OD_{490} of each well as compared to untreated (control) cell OD_{490} multiplied by 100. All experiments were conducted in triplicate.

Once a concentration of SITS was found that did not significantly affect cell survivability, a second experiment was performed comparing SITS' affect of cisplatin. Approximately 1800 cells per well, of a 96-well culture plate, were exposed to a set SITS concentration of 0.05mM, and also varying dosages of cisplatin (0.0 to 2.5 μg/ml) for four days. Cell survivability of the SITS and cisplatin treated cells was determined using the Cell Titer 96@AQueous One Solution Cell Proliferation Assay. Cell survival was calculated as a fraction of the OD_{490} of each well compared with untreated (control) cell OD_{490}, multiplied by 100. All experiments were conducted in triplicate.

Experiment 5: Influence of bicarbonate-free media on cisplatin toxicity in COS31 and COS31/rCDDP cells.

To determine the effect of the absence of extracellular HCO_3^- on the cytotoxic action of cisplatin, cells from both lines were cultured in bicarbonate-containing and bicarbonate-free DMEM. In place of sodium bicarbonate, a 1% HEPES solution was

added to the media and the pH was adjusted to 7.4. As before, cells were exposed to cisplatin at dosages ranging from 0.0 to 2.5 μg/ml for 4 days. Cell survivability of various treatment groups was determined using the Cell Titer 96@AQueous One Solution Cell Proliferation Assay. Cell survival was calculated as a fraction of the OD_{490} of each well compared with untreated (control) cell OD_{490}, multiplied by 100. All experiments were conducted in triplicate.

Experiment 6: Influence of SITS with either bicarbonate-containing or -free media on cisplatin toxicity in COS31 and COS31/rCDDP cells.

Experiment 5 was repeated with the exception that both cell lines were cultured with or without concurrent exposure to an LD_{10} concentration of SITS.

Experiment 7: Influence of SITS on intracellular Cl^- concentration in cisplatin-treated COS31 cells.

Intracellular Cl^- was measured with the MEQ [6-methoxy-N-ethylquinolinium chloride] (Molecular Probes, Eugene, OR) assay and was performed to determine whether exposure of cells to the HCO_3^-/Cl^- ion exchange inhibitor SITS would alter intracellular Cl^- ion concentration. Alterations in intracellular Cl^- would support the hypothesis that pHi influences intracellular Cl^- concentration thus inhibiting the aquation reaction of cisplatin to its toxic form resulting in an increase in cell survival.

Eight $75cm^2$ tissue culture flasks were prepared with each flask containing 4 million cells obtained from stock COS31 cell cultures. The cells in all eight flasks were

exposed to a cisplatin concentration of 0.3125 μg/ml. The cells in four flasks were also exposed to a SITS concentration of 0.05mM. All flasks were incubated at 37 degrees C for four days.

Cells were harvested from each flask by trypsinization using previously described methods and centrifuged in a 50ml conical centrifuge tube for five minutes at 1000 x g. The media was removed by gentle aspiration and the cells resuspended in three ml of serum-free media. A cell count was performed. Approximately 10^6 cells were placed into a 1.5ml Eppendorf tube and the volume adjusted to 1ml using serum-free media. The cells were washed with serum free media three times. DiH-MEQ was dissolved into 2.5ml of DMSO producing a 40mM stock solution of DiH-MEQ. From this stock solution 100μl was placed into 900μl of serum free DMEM. Ten μl (4 mM) was then added to each Eppendorf tube containing cells and incubated for 10 minutes. The samples were centrifuged for 3 minutes at 1000 x g. The media was carefully aspirated and the cells were washed twice with 1ml of serum free DMEM. The cells were resuspended in 1ml of serum-free DMEM and incubated at 37 degree C for 15 minutes.

A Shimadzu flourometer (Shimadzu Corporation, Kyoto, Japan) with an excitation wavelength of 344 nm and emission spectra of 390 nm to 530 nm (maxima, 440 nm) was used to measure fluorescence of the resultant sample following the addition of 20μM of nigericin or 20μM tributyltin. Nigericin and tributyltin were used to quench the cell of its intracellular chloride.

Intracellular Cl^- concentration was calculated using the following formula:

$$(Fo/F) - 1 = Ksv[Q]$$

where Fo is the fluorescence intensity without halide or other quenching ions; F is the fluorescence intensity in the presence of quencher; [Q] is the concentration of quencher; and Ksv is the Stern-Volmer constant ($19M^{-1}$) which was provided by Molecular Probe.

Statistical Methods

Differences in proliferative rate and intracellular concentration of Cl^- among the various experimental groups were compared with appropriate control groups using One-way ANOVA multiple comparison using Tukey equation. Significant differences between the various treatment groups was determined by use of the Student-Newman-Keuls mean separation test or by the Students *t*-test with a confidence level set at 95%.[34] Unless otherwise stated, the results were expressed as the mean of three replicates per treatment group and +/- standard error of the mean was calculated. Curve fitting was done by the least-squares method and was tested by an analysis of variance (SAS, SAS Systems, Cary, NC). LD_{50} concentrations were then determined through the use of EXCEL program:

$$\text{Dose} = D_L - ((P_O-P_L)/(P_H-P_L))*(D_L-D_H)$$

where Dose is the drug concentration at a selected percentage survival (P_O). P_L and P_H represent the known lower and higher percent servival, respectivly, in the given experiment. D_L and D_H represent the known drug concentrations for the lower and higher survival percentages, P_L and P_H, respectively.

CHAPTER IV

RESULTS

Experiments

Experiment 1: *Influence of pH on cisplatin toxicity in COS31 cells.*

Significant differences between cell survival were observed between cells cultured at pH 6.4 versus pH 7.4 for varying concentrations of cisplatin. These differences were observed when cisplatin concentration ranged from 0.156 μg/ml to 1.25 μg/ml (Figure 1).*

Experiment 2: *LD_{50} of cisplatin in COS31 and COS31/rCDDP cells.*

The two cell lines were significantly different through out all drug concentrations. For a reference point an LD50 was determined for each cell line and the two drug concentrations were statistically compared. The LD_{50} of cisplatin for the COS31 cell line (0.24 μg/ml) was significantly greater than the LD_{50} for the COS31/rCCDP cell line (0.02 μg/ml) (Figure 2).

Experiment 3: *Influence of NHMA on cisplatin toxicity in COS31 cells.*

At varying concentrations of NHMA, cell survival was minimally affected at concentrations less than 10 μM (Figure 3). When a NHMA concentration of 2.5 μM was used and cells concurrently exposed to varying concentrations of cisplatin, cell survival[1]

* All experiments may be found in the Appendix

was significantly decreased, through out the varying drug concentrations, compared to non-NHMA treated cells (Figure 4).

Experiment 4: *Influence of SITS on cisplatin toxicity in COS31 and COS31/rCDDP cells.*

COS31 cells were exposed to varying concentrations of SITS (Figure 5). Interestingly, as SITS concentration increased, cell proliferation significantly increased. Therefore, a concentration of SITS, which did not result in significant cell proliferation (e.g., <110% of control) was selected for further experiments (0.05 mM). Cell survival was significantly decreased in SITS-treated COS31 cells at lower cisplatin concentrations (i.e., 0.078 - 0.315 μg/ml) but not at higher cisplatin concentrations (i.e., > 0.3125 μg/ml) (Figure 6). Cell survival in COS31/rCDDP was not significantly affected by presence of SITS (Figure 7).

Experiment 5: *Influence of bicarbonate-free media on cisplatin toxicity in COS31 and COS31/rCDDP cells.*

Cell survival was significantly decreased in COS31 and COS31/rCDDP cells in bicarbonate-free media at higher cisplatin concentrations in COS31 cells and at lower cisplatin concentrations in COS31/rCDDP cells compared with equivalent cisplatin concentrations from bicarbonate-containing media controls (Figures 8 and 9).

Experiment 6: *Influence of SITS with bicarbonate-free media on cisplatin toxicity in COS31 and COS31/rCDDP cells.*

For COS31 cells cultured in bicarbonate-free media, cell survival was significantly increased, in the presence of SITS, at a cisplatin concentration range of 0.0 to 0.6125 μg/ml, compared to SITS free cells (Figures 10). COS31/rCDDP cells cultured in bicarbonate-free media showed a significant increase in cell survival in the presence of SITS at a cisplatin range of 0.0 to 0.156 μg/ml, compared to similar concentrations of cisplatin without SITS (Figure 11).

Experiment 7: *Influence of SITS on intracellular Cl^- concentration in cisplatin-treated COS31 cells.* Chloride ion concentration was significantly increased in COS31 cells upon the addition of 0.05 mM SITS (Figure 12). The average Cl^- concentration increased from 21.85 mM in untreated COS31 cells to 36.89 mM in cisplatin-treated cells. Upon the addition of SITS to cisplatin-treated cells, the average Cl^- concentration increased to 47.95 mM.

CHAPTER V

DISCUSSION

This study has shown that cell survival can be influenced by the inhibition of the Na^+-dependent HCO_3^-/Cl^- ion exchanger using SITS. The addition of SITS increases the intracellular Cl^- ion concentration in canine osteosarcoma cells cultured in a bicarbonate-containing media and showed a decrease in cell survival at lower drug concentrations. In a bicarbonate-free media, the addition of SITS results in decreased cytotoxic action of cisplatin.

Significant differences were observed between cells cultured at pH 6.4 versus pH 7.4 for varying concentrations of cisplatin. However, no significant difference was observed between the slopes of two distribution curves. These results suggested that pHe may increase cisplatin uptake into cells and trap the drug intracellularly, and decrease cell survival. This finding is supported by others in the EMT6 mouse mammary carcinoma cell line with other anticancer drugs.[20] *In vivo,* the high Cl^- ion concentration in the plasma prevents the hydrolysis of the Cl^- ion platinum ligands. This allows cisplatin to remain in its electroneutral forms of di(chloro) and (chloro)(hydroxo) which is not reactive to the cell. Once cisplatin has entered the lower Cl^- ion concentration of the cytoplasm it is hydrolyzed to its aqua forms which are inert to substitutions and are the active agents for attack of polar donor groups in the DNA target molecule.[21,23,33]

The LD_{50} of cisplatin for the COS31 cell line (0.24μg/ml) was significantly greater than the LD_{50} for the COS31/rCDDP cell line (0.02μg/ml). In a previous study by

Shoeib (1998)[30-32], the opposite effect was observed; suggesting that continued passages of both lines resulted in altered sensitivity to cisplatin and that other mechanisms of drug resistance may exist. Since a difference in the LD_{50} existed, it is possible to state that two different osteosarcoma cell lines, with different sensitivity to cisplatin's cytotoxic effects, were evaluated in this study.

At varying concentrations of NHMA, cell survivability was seen to decrease until NHMA concentrations fell bellow 10μM. When a NHMA concentration of 2.5μM was used and cells were concurrently exposed to varying concentrations of cisplatin, cell survival was significantly decreased compared to non-NHMA treated cells. These results support a hypothesis that as NHMA shuts down the Na^+/H^+ antiport, a resulting increase in intracellular H^+ causes a decrease in pHi. This decrease in pHi results in increased in activity of Na^+-dependent HCO_3^-/Cl^- ion channels, and a decreased intracellular Cl^- ion concentration and thus aquation of the cisplatin (i.e., increased toxic action). Since it was shown in this study that exposure of cells to NHMA was directly cytotoxic, one possible explanation is that a decrease in intracellular Na^+ concentration affects cell survivability.

As cells in bicarbonate-containing media were exposed to an increasing concentration of SITS, cell proliferation increased in COS31 cells. It is known that inhibitors of HCO_3^-/Cl^- exchange channels also alter the function of other membrane associated channels.[20,21] These channels have effects on secondary messengers which promote or inhibit cell cycle regulation.[21] A concentration of 0.05mM SITS was observed to have the least affect on COS31 cell proliferation and was used to determine whether SITS alters cisplatin toxicity in bicarbonate-containing media conditions. Cell

survival was significantly decreased in SITS-treated (LD_{10} concentration) COS31 cells at lower cisplatin concentrations (i.e. 0.078 – 0.3125 μg/ml) but not at higher cisplatin concentrations (i.e. > 0.3125μg/ml) nor at any concentration in COS31/rCDDP cells.

When cisplatin enters the cell it undergoes an aquation reaction resulting in the loss of two Cl ions, thus increasing intracellular Cl^- concentration. In bicarbonate-containing media, the Cl^- ion is exchanged with a HCO_3^- ion thus maintaining the Cl^- deficient intracellular state. When SITS is added and Na^+-dependent ion exchange is inhibited, Cl^- ion concentration rises but does not return to pre-cisplatin levels. However, it was noted in the COS31 cells that at lower cisplatin concentrations, cell survival was significantly decreased rather than increased when SITS was added. Although this result is contrary to the existing literature [20,21], it is likely that other HCO_3^-/Cl^- ion exchange mechanisms exist, obviating the SITS affects of ion transport inhibition occurring at the Na^+ dependent ion exchangers.

Cell survival was significantly decreased in COS31 and COS31/rCDDP cells in bicarbonate-free media at higher cisplatin concentrations in COS31 cells and at lower cisplatin concentrations in COS31/rCDDP cells as compared to equivalent cisplatin concentrations from bicarbonate-containing media controls. Removing bicarbonate from the media has been shown to reduce the activity of Na^+-dependent HCO_3^-/Cl^- exchangers.[5] Therefore, an increase in Cl^- concentration resulting from cisplatin exposure would not be buffered by the action of Na^+-dependent HCO_3^-/Cl^- exchangers and an increase in cell survival should be observed. One possible hypothesis for the observations in this study is that the absence of HCO_3^- resulted in a decreased pHi that

can not be recovered from the use of other ion exchangers (e.g. Na^+/H^+) resulting in cell death independent of cisplatin concentration.

For COS31 and COS31/rCDDP cells cultured in bicarbonate-free media, cell survival was significantly increased when SITS was added. SITS inhibits the Na^+-dependent HCO_3^-/Cl^- exchange. In the absence of HCO_3^- in the media, other possible HCO_3^-/Cl^- ion exchangers are also inhibited. Thus, after cisplatin undergoes the aquation reaction, high intracellular Cl^- concentration is maintained, and cell survival is increased because the equilibrium for further aquation of cisplatin is shifted away from the hydrolyzed/toxic form. This results in an intracellular Cl^- concentration sufficient to prevent further cisplatin aquation and maintain cell survivability, even in an acidic pHi.

In this study, Cl^- concentration was significantly increased in COS31 cells upon addition of 0.05mM SITS. It is hypothesized by others that increased intracellular Cl^- concentration is a result of cisplatin losing Cl^- during the intracellular aquation reaction,[5,20,21] If SITS prohibits Cl^- transport out of the cell, intracellular Cl^- levels should rise. As intracellular Cl^- levels rise, the aquation reaction is shifted away from completion, thus less cisplatin aquires the active cytotoxic form resulting in drug resistance.

CHAPTER VI

CONCLUSION AND SUMMARY

This study has shown that cell survival can be influenced by the inhibition of the Na^+-dependent HCO_3^-/Cl^- exchanger using SITS. The addition of SITS increases the intracellular Cl^- concentration in canine osteosarcoma cells cultured in a bicarbonate-containing media. In a bicarbonate-free media, the addition of SITS results in a decrease in the cytotoxic action of cisplatin. This study has also shown that it is likely that other HCO_3^-/Cl^- exchange mechanisms exist because when Na^+-dependent HCO_3^-/Cl^- exchangers are inhibited in cells which are cultured in bicarbonate-containing media, cisplatin resistance does not develop. Since these observations were obtained from two canine osteosarcoma cell lines with different profiles of cisplatin sensitivity, it is hypothesized that similar responses would be observed in other cell lines of differing drug sensitivity.

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BIBLIOGRAPHY

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APPENDIX

Figure 1.

Cisplatin dose-response curves for COS31 cells exposed to pH 7.4 and 6.4. All points are an average of three replicates with +/- standard error of the mean shown.

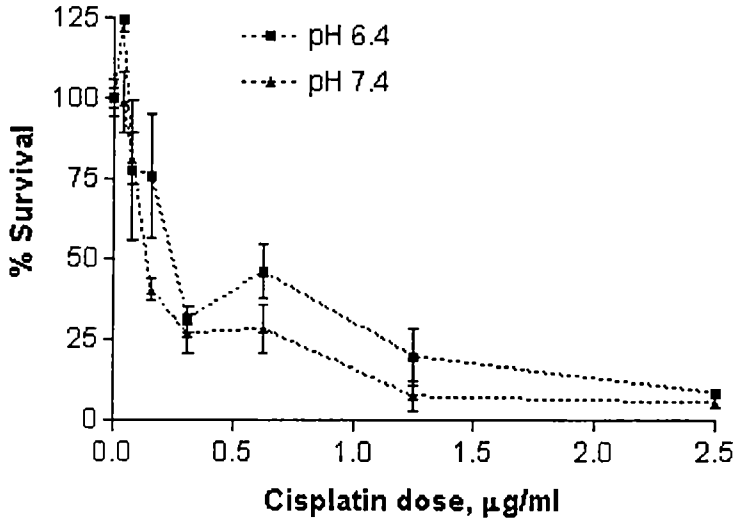

125
100
75
50
25
0
% Survival
pH 6.4
pH 7.4
0.0
0.5
1.0
1.5
2.0
2.5
Cisplatin dose, µg/ml

Figure 2.

Cisplatin dose-response curves for COS31 sensitive and COS31/rCDDP resistant cell lines. +/- standard error of the mean is shown for each cisplatin concentration.

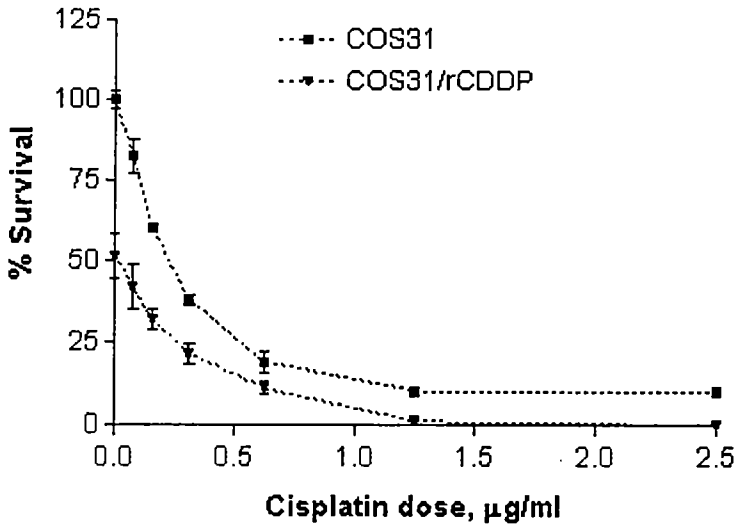

125
100
75
50
25
0
% Survival
COS31
COS31/rCDDP
0.0
0.5
1.0
1.5
2.0
2.5
Cisplatin dose, µg/ml

Figure 3

NHMA dose response curve for COS31 cells. +/- standard error of the mean is included for each dose

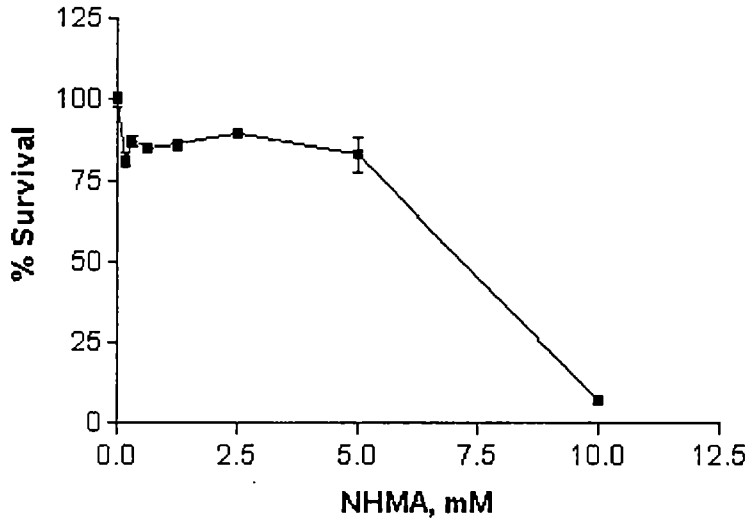

125
100
75
50
25
0
% Survival
0.0
2.5
5.0
7.5
10.0
12.5
NHMA, mM

Figure 4.

Cisplatin dose-response curve for COS31 cells with 2.5μM of NHMA present. All points include +/- standard error of the mean.

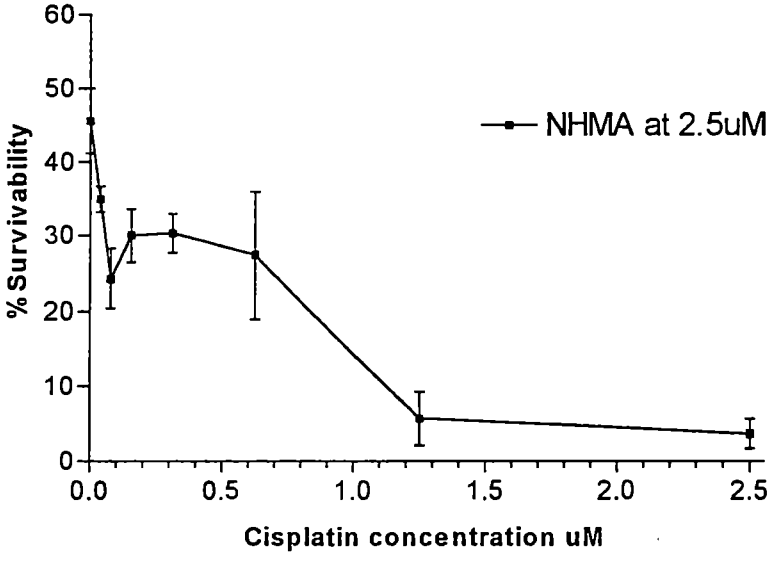

60
50
40
30
20
10
0
% Survivability
NHMA at 2.5uM
0.0
0.5
1.0
1.5
2.0
2.5
Cisplatin concentration uM

Figure 5.

SITS dose-response curve for COS31 cells. +/- standard error of the mean for each concentration is shown.

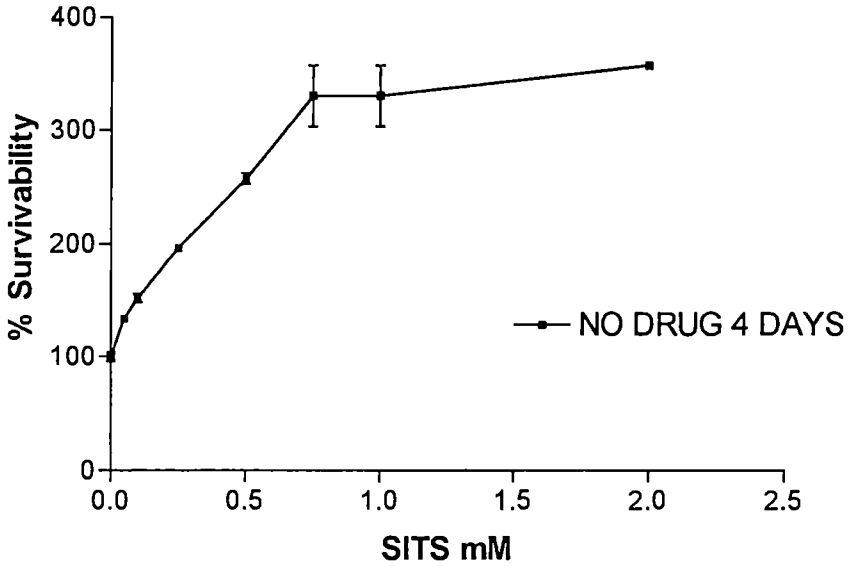

400
300
200
100
0
% Survivability
0.0
0.5
1.0
1.5
2.0
2.5
SITS mM
NO DRUG 4 DAYS

Figure 6.

Cisplatin dose-response curves for COS31 cells in the presence and absence of 0.5mM SITS. +/- standard error of the mean is shown for each drug concentration.

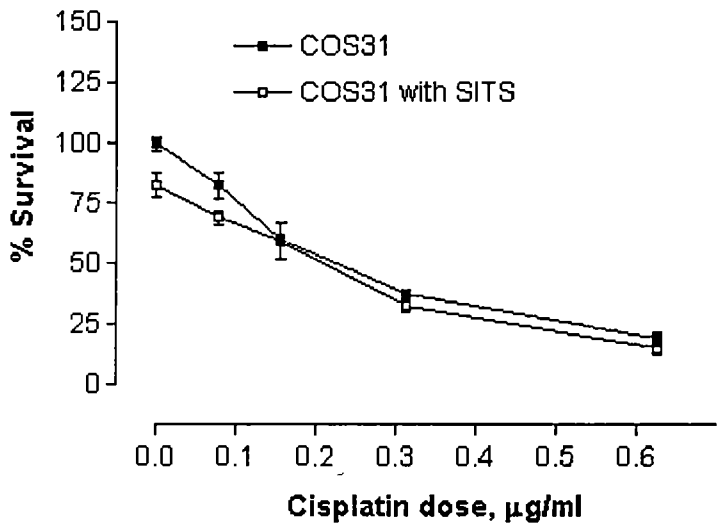

150
125
100
75
50
25
0
% Survival
COS31
COS31 with SITS
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Cisplatin dose, µg/ml

Figure 7.

Cisplatin dose-response curves for COS31/rCDDP resistant cells in the presence and absence of 0.5mM SITS. +/- standard error of the mean is shown for each cisplatin dose.

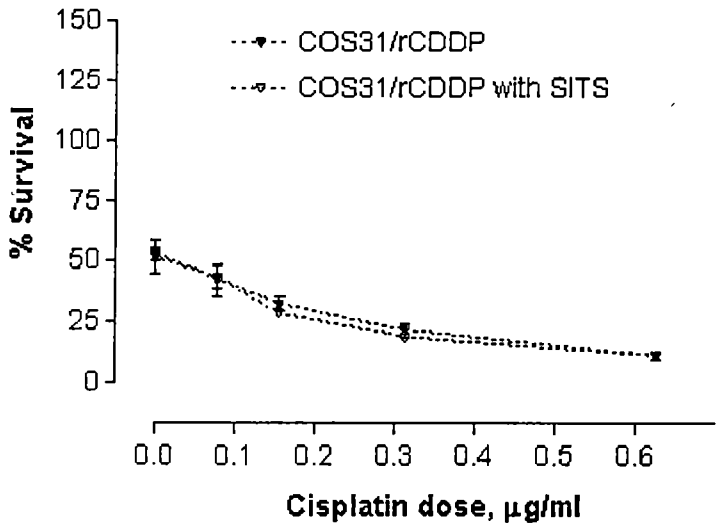

150
125
100
75
50
25
0
% Survival
COS31/rCDDP
COS31/rCDDP with SITS
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Cisplatin dose, µg/ml

Figure 8.

Cisplatin dose-response curves for COS31 cells when bicarbonate is present and absent.

+/- standard error of the mean is shown for each drug concentration value.

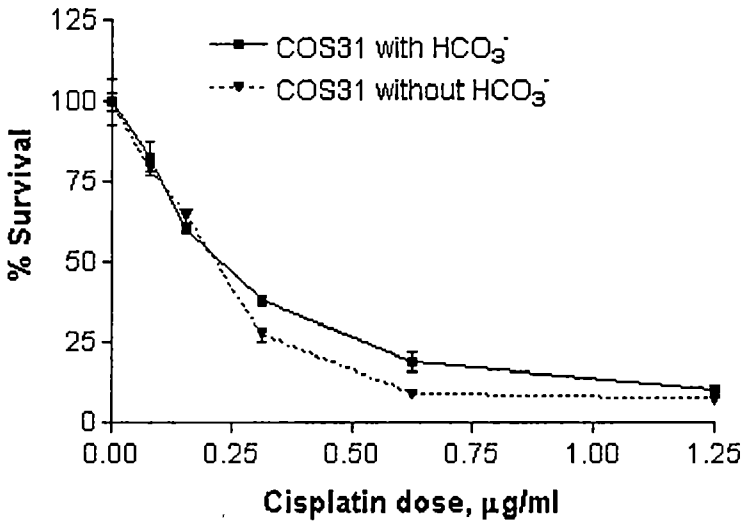

125
100
75
50
25
0
% Survival
COS31 with HCO_3^-
COS31 without HCO_3^-
0.00
0.25
0.50
0.75
1.00
1.25
Cisplatin dose, µg/ml

Figure 9.

Cispaltin dose-response curves for COS31/rCDDP resistant cells in the presence and absence of bicarbonate. +/- standard error of the mean is shown for each drug concentration.

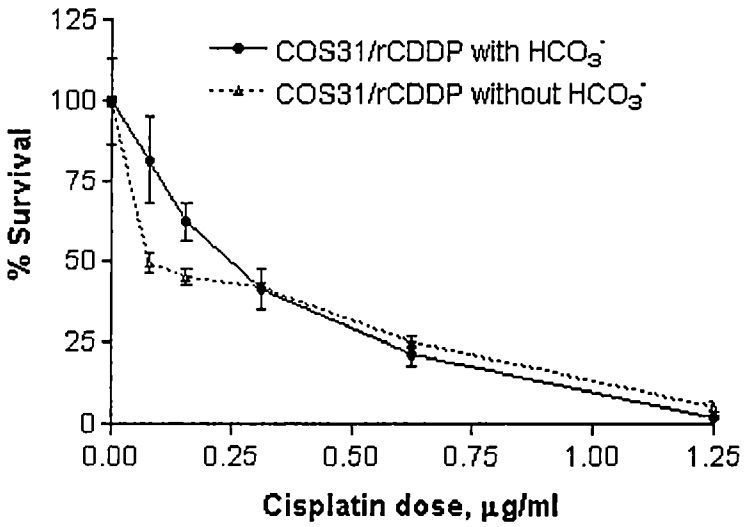

125
100
75
50
25
0
% Survival
COS31/rCDDP with HCO_3^-
COS31/rCDDP without HCO_3^-
0.00
0.25
0.50
0.75
1.00
1.25
Cisplatin dose, µg/ml

Figure 10.

Cisplatin dose-response curves for COS31 cells in bicarbonate free media in the presence or absence of 0.05mM SITS. +/- standard error of the mean is shown for each drug concentration used.

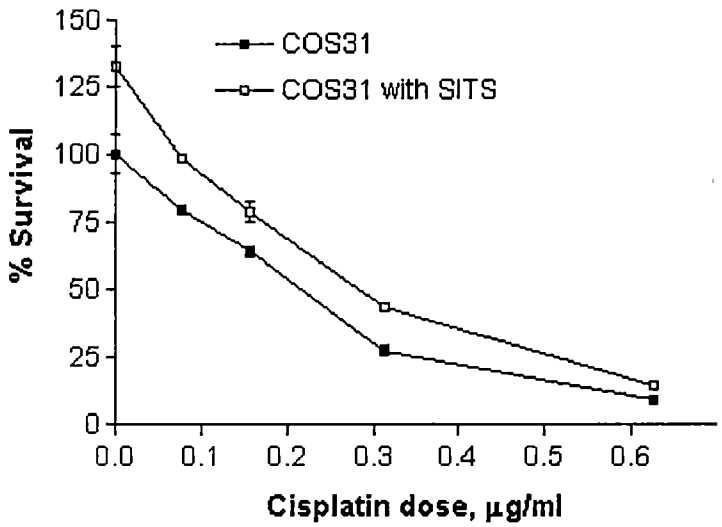

150
125
100
75
50
25
0
% Survival
COS31
COS31 with SITS
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Cisplatin dose, µg/ml

Figure 11.

Cisplatin dose-response curves for COS31/rCDDP resistant cells in bicarbonate free media in the presence or absence of 0.05mM SITS. +/- standard error of the mean is shown for each drug concentration used.

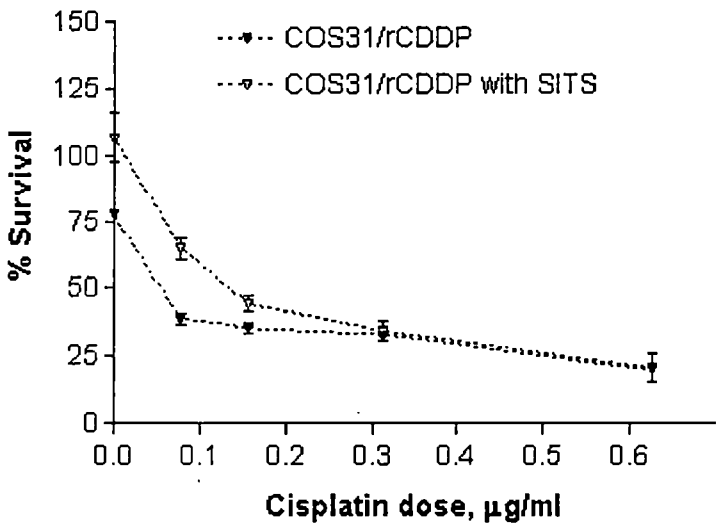
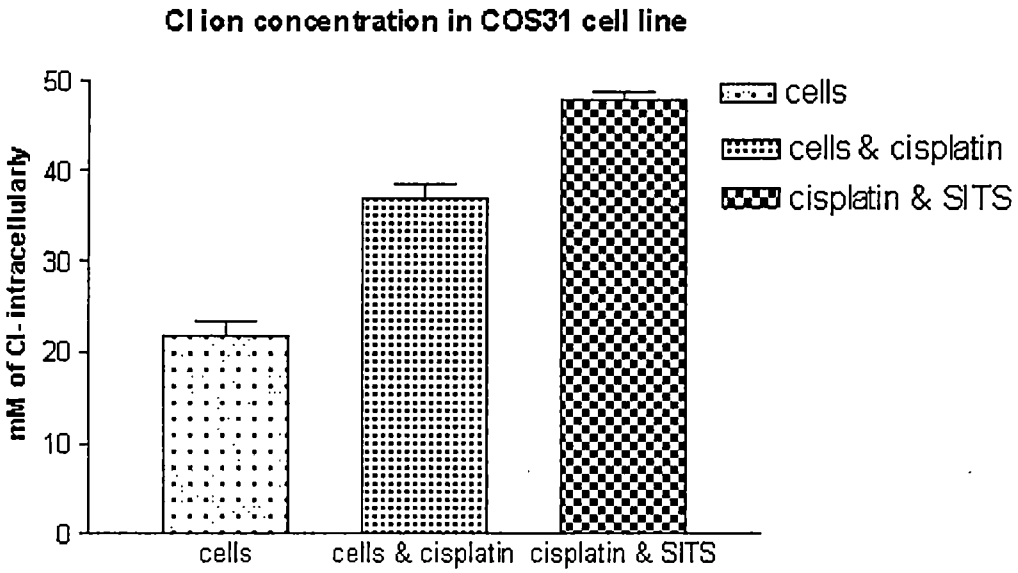

150
125
100
75
50
25
0
% Survival
COS31/rCDDP
COS31/rCDDP with SITS
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Cisplatin dose, µg/ml

Figure 12.

Chloride ion concentration for COS31 cells with and without cisplatin present and with and without SITS


Cl ion concentration in COS31 cell line
mM of Cl- intracellularly
50
40
30
20
10
0
cells
cells & cisplatin
cisplatin & SITS
cells
cells & cisplatin
cisplatin & SITS

VITA

Jason Wayne Yarbrough was born in Chicago, Illinois on September 3rd, 1974. He moved to Bolivar, Tennessee in 1978 with his parents. He attended Toone Elementary School and then Bolivar Central High School were he graduated in May 1992. The following August he enrolled at the University of Tennessee at Martin. While at the University of Tennessee he majored in Animal Science. In May of 1996 he received his Bachelor of Science in Animal Science Biotechnology. In August of 1996 he enrolled in the Comparative and Experimental Medicine Master's program at the University of Tennessee. He will receive his Master of Science degree in May of 1999.