

EFFECTS OF HYPERBILIRUBINEMIA ON CISPLATIN NEPHROTOXICITY

By

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Abstract of Thesis Presented to the Graduate School
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Cisplatin is a powerful chemotherapeutic agent used in a variety of malignancies in many species. Systemic dose-related toxicities associated with cisplatin have precluded its use in many species. Of these toxicities, nephrotoxicity is the most frequent and clinically significant toxicity. No specific compound has yet ameliorated cisplatin nephrotoxicity. Recent research has shown that the heme oxygenase-1 (HO-1) enzyme provides beneficial effects in mitigating cisplatin nephrotoxicity in a rodent model. HO-1 is normally induced in response to cellular stress and converts the heme molecule into equimolar quantities of biliverdin (BV), carbon monoxide (CO), and iron. Biliverdin is then converted to bilirubin (BR) by the enzyme biliverdin reductase. Many previous studies suggest that HO and its products are important endogenous mechanisms for cytoprotection. These products were once considered to be toxic metabolites but have been shown to have dose-dependent vasodilatory, anti-oxidant, and anti-inflammatory properties that are desirable for tissue protection during a toxic insult. Our first objective was to review the literature regarding cisplatin and, in particular, nephrotoxicity associated with cisplatin administration and current methods to prevent nephrotoxicity. Our second objective was to determine if hyperbilirubinemia would ameliorate cisplatin

nephrotoxicity in a rat model. Our third objective was to determine if bilirubin would prevent cisplatin's ability to kill neoplastic cells *in vitro*.

The *in vivo* cisplatin model involved 3 groups of rats (n=6 rats/group): homozygous Gunn rats (j/j), heterozygous Gunn rats (j/+) and congenic Wistar (+/+) rats. Homozygous Gunn rats lack the UDPGT enzyme needed to conjugate bilirubin resulting in an unconjugated hyperbilirubinemia while heterozygous Gunn rats lack normal levels of UDGPT, but can conjugate bilirubin to a certain degree. On Day 0, all rats were anesthetized and administered 4 mg/kg cisplatin IP. Blood was sampled on Day 0, 3 and 5 for comparison of serum BR, creatinine (Cr), and BUN. On Day 5, kidney tissue samples were obtained prior to euthanasia.

Cell culture studies were then performed using 4 canine osteosarcoma cell lines (POS, HMPOS, COS, D17) incubated with the average concentrations of BR for j/j rats at Day 0 and 3. We added BR to all cell lines (alone and with cisplatin) and cell viability was assessed using the CellTiter Blue™ assay.

Serum BR levels were $72 \pm 16 \mu\text{M/L}$ in homozygous Gunn rats, $7 \pm 3 \mu\text{M/L}$ in heterozygous Gunn rats, and $0 \pm 0 \mu\text{M/L}$ in Wistar rats at Day 0. The BR provided a dose-dependent nephroprotective effect, with significantly lower BUN ($24 \pm 5 \text{ mg/dL}$) and Cr ($0.35 \pm 0.05 \text{ mg/dL}$) in homozygous Gunn rats when compared to Wistar rats (BUN- $79 \pm 17 \text{ mg/dL}$, Cr- $1.4 \pm 0.4 \text{ mg/dL}$) at Day 5 ($P < 0.05$). An intermediate level of nephroprotection was noted in the heterozygous Gunn rats, although BUN ($38 \pm 10 \text{ mg/dL}$) and Cr ($0.4 \pm 0.06 \text{ mg/dL}$) remained significantly lower than Wistar rats at Day 5 ($P < 0.05$). Histological grading demonstrated preservation of the S3 segment in homozygous Gunn rats when compared to heterozygous Gunn rats and Wistar rats ($P < 0.05$). The BR had no significant effect on the

antineoplastic effect of cisplatin at either concentration in the 4 osteosarcoma cell lines (P <0.001).

Hyperbilirubinemia in the homozygous Gunn rat provided nearly complete preservation of renal function and pathology in this model of cisplatin nephrotoxicity. Addition of exogenous BR did not interfere with the antineoplastic activity of this chemotherapy agent in cell culture.

CHAPTER 1 CISPLATIN: A REVIEW

Introduction

Cisplatin is one of the most potent chemotherapy agents used in human and veterinary medicine. Its use in veterinary medicine began more than 2 decades ago and was prompted by the success of cisplatin in treating human malignancies. Unfortunately, cisplatin administration was associated with numerous adverse side effects including: nephrotoxicity, severe nausea and vomiting, myelosuppression, ototoxicity, and neurotoxicity. Of these, the most clinically significant and common toxicity is nephrotoxicity. Despite the nephrotoxicity, many veterinary oncologists are of the opinion that cisplatin is more potent than its other platinum counterparts with regard to its anti-neoplastic activity. This has resulted in its continued use throughout veterinary hospitals around the world for malignancies such as osteosarcoma (OSA), transitional cell carcinoma (TCC), intralesional therapy, and radiation sensitization.

Since cisplatin's development, research has centered on mitigating nephrotoxicity to allow cisplatin to be delivered at therapeutic doses without adversely affecting the kidneys. Recent studies have discovered new protocols, compounds, enzymes, and molecular alterations that reduce the nephrotoxicity of cisplatin.

This review will focus on cisplatin's chemistry, pharmacokinetics, pharmacodynamics, mechanisms of resistance, toxicity, prevention of nephrotoxicity, and use in animals. Also included is a section on the other platinum drugs that have been used in veterinary medicine to date.

Chemistry

Cisplatin was inadvertently discovered while studying the growth characteristics of *Escherichia coli*. (1) Initially, researchers observed that platinum compounds exhibited

antibacterial properties and subsequently it was discovered that they also possessed anti-neoplastic properties. (1, 2) The molecular structure of cisplatin comprises a central platinum atom surrounded by two chlorine atoms and two ammonia groups in a *cis* configuration. (3) Other platinum compounds have the same core platinum compound, and *cis* configuration, however, their leaving groups are different (Figure 1-1). (4) The bond angles for the platinum core are fixed resulting in DNA bending to accommodate the structure of the drug. (4)

Pharmacokinetics

Plasma platinum has been shown to be highly protein bound. (5) Most cisplatin present in the cell is found in the cytosol and is not protein bound. (6) Cisplatin's clearance in the dog is biphasic in nature with a rapid phase half-life of 22 minutes and a slow phase half-life of 5 days. (7) Significant amounts of platinum are still detectable in plasma 12 days after intravenous injection. (7) A study performed in humans demonstrated that plasma platinum levels corresponded with nephrotoxicity. (8) Plasma platinum levels may serve as a marker for risk of nephrotoxicity in certain patients.

Urinary levels of platinum in the dog elevate rapidly after administration with 60% of the dose recovered in the urine within the first 4 hours and 76% of the administered dose recovered by 48 hours after treatment. (7, 9) Only small amounts of platinum were detected in bile suggesting minimal fecal excretion. (7) Free platinum clearance has been shown to exceed the creatinine clearance by 156% suggesting that in addition to excretion by filtration, cisplatin or a metabolite is secreted by the kidney. (5) It is thought that secretion involves active accumulation of secreted substances in the renal cells and passive transport into the tubule of the lumen. (10) The pars recta of the proximal tubule is the most active site of secretion and also the most damaged site of the kidney during cisplatin nephrotoxicity. (5) Renal accumulation of platinum is dependent on the presence of normal oxygen utilization and the organic base transport

system. (11) Thus, concurrent administration of drugs known to be transported by this system can significantly reduce cisplatin uptake in the kidney. (11)

Cisplatin is initially distributed to all tissues, however, in the first hour, it tends to accumulate in the kidney, liver, muscle, and skin. (7) The localization in the kidney and liver is protracted, with high renal tissue concentrations present as long as 12 days after treatment in the dog. (7) The highest tissue platinum concentrations occur in those tissues where the drug exerts its most potent antineoplastic activity, such as the ovary and uterus. (7) It is thought that the presence of tumor may alter the toxicity and pharmacokinetics of drugs. (12) One study in tumor bearing rats showed that the distribution half-time was longer for the tumor bearing rats than their controls, while the terminal elimination half-time was the same for both groups. (12) Based on this study, it is unlikely that tumor presence would alter toxicity or other distribution-dependent drug parameters. (12)

Pharmacodynamics

Cisplatin is activated by an aquation reaction involving the exchange of the two chloride leaving groups with water or hydroxyl ligands. (13) When a high concentration of chloride is present, as in isotonic saline or extracellular fluid, the aquation reaction does not occur and the drug remains neutral. (13) The neutral form is believed to be biologically inactive. (14) Intracellular fluid has approximately one-thirteenth the chloride concentration of extracellular fluid and it is under these conditions that the aquation reaction proceeds, leading to eventual DNA damage. (13) The primary effect produced by cisplatin in cancerous cells is inhibition of DNA synthesis. (15, 16) The ability to inhibit DNA synthesis occurs at much lower doses than that necessary to inhibit RNA and protein synthesis. (15) DNA damage induced by cisplatin is similar to that caused by alkylating agents. (17) With aquation of the platinum compound the two chloride groups are replaced with water and will bind to two sites in DNA. (4) Generally, if

the two sites are on the same DNA strand, the lesion is referred to as a DNA adduct and if the sites are on different strands, the lesion is referred to as a DNA cross-link. (4) Cisplatin has been noted to bind to all DNA bases but has a preference for the N-7 positions of adenine and guanine due to the high nucleophilicity of the N-7 sites of these purine bases (Figure 1-2). (4, 13, 18) Cisplatin forms bifunctional adducts >90% of the time with crosslinks being < 2% of the lesions formed. (4) These adducts and crosslinks inhibit DNA template replication in mammalian cells. (19) DNA crosslinks and adducts increase with time after the drug is removed and are repaired slowly. (20) In vitro studies have also indicated that interaction between the cisplatin molecule and DNA may contribute to the generation of superoxide radicals, causing further toxicity to cancer cells. (21, 22)

Mechanisms of Resistance

Four generic pathways for cisplatin resistance have been uncovered. They include: altered cellular accumulation, cytosolic inactivation of cisplatin, DNA repair, and altered apoptosis (Figure 1-3). (4) *In vitro* studies have described active efflux particularly as mediated by Cu^{H} transporters, ATP7A and ATP7B, and other less well-defined systems. (4) Covalent binding of proteins or peptides with increased levels of sulfhydryl groups to cisplatin may confer cellular resistance. (4) These compounds include glutathione (GSH) and metallothionein. (23-26) MRP2 (multidrug resistance-associated protein 2) may also play a role in cisplatin resistance by removing the cisplatin-GSH complex from the cells. (27) Platinum-DNA repair occurs by the nucleotide-excision repair (NER) and NER is increased in cisplatin-resistant cells. (4, 28, 29) Mismatch repair (MMR) mediates apoptosis in response to cisplatin. (30-32) Defects in MMR result in altered cell sensitivity to cisplatin, most likely resulting in greater resistance. (4) Reports indicate that where alterations in MMR exist, concurrent enhancement of the activity of NER exists. (4)

Toxicities

Nephrotoxicity

Cisplatin is associated with several systemic toxicities, but is most frequently associated with nephrotoxicity. Cisplatin induced nephrotoxicity occurs in a number of species including mice, rats, dogs, and humans. An estimated 28 to 36% of human patients receiving an initial dose of 50-100 mg/m² of cisplatin develop acute renal failure. (33, 34) Cisplatin nephrotoxicity is dose and duration of treatment dependent and is enhanced by the use of other nephrotoxins such as aminoglycosides. (35, 36) Most patients who develop some degree of renal dysfunction never fully recover. (37) However, one study evaluating the long-term renal effects of cisplatin in human patients showed that renal dysfunction may not be progressive provided further insult is avoided. In this study, an initial increase in creatinine and a decrease in GFR and renal plasma flow were noted directly after treatment, but these levels remained stable for up to 12 to 24 months after discontinuing cisplatin treatment. (38)

The morphologic alterations in the kidney attributed to cisplatin administration occur in the pars recta of the proximal tubule situated in the outer stripe of the medulla. (39) Histological changes are consistent with both apoptosis and necrosis. (40) One of the earliest histopathological changes noted is the swelling of mitochondria. (14) Most of the pathological changes start 3 days after cisplatin administration, including clumped nuclear chromatin, increased number of cytoplasmic vesicles, focal loss of microvillus brush border, and completely necrotic cells sloughed in the tubular lumen. (39) The most severe damage is seen 5 days after cisplatin administration and consists of widespread tubular necrosis in the pars recta, desquamation of necrotic epithelia cells resulting in a denuded basement membrane, necrotic cells and debris in the tubular lumen, and the changes seen in 3 days after cisplatin

administration for non-necrotic cells. (39) By 7 days after cisplatin administration, extensive regeneration in the pars recta is noted with necrotic cells still present. (39)

Research into the mechanism of cisplatin nephrotoxicity is an important step in developing methods for renal protection. One theory involves DNA crosslinks and the position in the cell cycle. It was thought that cisplatin-DNA crosslinks could be the cause of cytotoxicity in the renal cell, but the proximal tubule cells selectively killed by cisplatin are relatively quiescent and therefore should not be as sensitive to the toxicity of DNA damaging agents. (41) However, there is a fall in DNA turnover that precedes necrosis in the proximal tubule and the later increase in DNA turnover in those cells coincides with the timeline of regeneration. (42) Both the outer cortex and outer stripe of the outer medulla (pars recta) have decreased DNA synthesis 1 day after cisplatin administration yet only the cells in the pars recta undergo necrosis. (42) Three possibilities have been considered regarding this theory including: inhibition of DNA synthesis is irrelevant to cytotoxicity in the kidney; cells in the pars recta cannot repair the damage as cells elsewhere can; or the levels of DNA adducts in the pars recta are lethal whereas those produced in other segments are not. (42, 43) Interestingly, recent studies have related cisplatin administration to alterations in the cell cycle. Cells in the kidney enter the cell cycle after cisplatin administration and genes for the p21 and 14-3-3 σ cell cycle inhibitors are simultaneously upregulated. (44-46) Mice with a deleted p21 gene were more sensitive to cisplatin injury. (46) Price *et al.* showed that the addition of p21 adenovirus and the pharmacological inhibitor of cyclin dependent kinases, roscovitine both protected kidney cells from cisplatin induced nephrotoxicity in vitro. (47) p21 inhibits caspase activation which is discussed later as a mechanism of apoptosis in the renal tubule. (47)

Platinum drugs are similar to other heavy metals such as mercury. Another possible mechanism for nephrotoxicity incorporates the known mechanism of nephrotoxicity in these other heavy metals. (48) This toxicity occurs due to the binding of cisplatin to sulfhydryl (SH) groups in the kidney which are necessary for enzyme function and depletion of intracellular glutathione. (48, 49) The decrease in SH groups occurred before the rise in BUN and creatinine and was not seen with acute renal failure caused by glycerol, another potent nephrotoxin. (49)

Proximal tubular cell death was initially believed to occur mostly by necrosis. However, another mechanism of proximal tubule cell death is apoptosis. A previous study showed that the type of cell death was concentration dependent with high concentrations of cisplatin leading to necrosis and low concentrations causing apoptosis. (50) Reactive oxygen species (ROS) and mitochondria are thought to play a role in the apoptotic cascade. (51) Mitochondrial dysfunction occurs early in cisplatin-induced renal tubular toxicity and is potentially mediated by ROS. (52, 53) It was shown in a previous study that overexpression of manganese superoxide dismutase, an antioxidant enzyme found in mitochondria, protected renal epithelial cells *in vitro* from cisplatin toxicity. (54) *In vitro* studies performed on renal proximal tubule cells examined the role of caspases and p53 in apoptosis related to cisplatin. Lau *et al.* showed that caspase 3 was activated *in vitro* in response to cisplatin but the initiators of the activation were not found. (55) The tumor suppressor gene p53 is activated in response to DNA damage, alterations in the cell cycle, and hypoxia. (56) Another study found that 50% of cisplatin induced renal proximal tubular cell apoptosis was mediated by p53 and that p53 activates caspase 3 independent of other caspases or mitochondrial dysfunction. (57) The other 50% is mediated by additional mechanisms independent of p53 and caspases 3, 8, and 9. (57) Interestingly, a different *in vitro*

study performed by Xiao *et al.* showed that when a caspase inhibitor that has no effect on p53 was applied to renal tubular cells, cisplatin-induced apoptosis did not occur. (58)

Recent studies in rats and mice have shown that the nephrotoxicity of cisplatin can be blocked by inhibiting either of two enzymes expressed in proximal tubules, gamma-glutamyl transpeptidase (GGT) or cysteine-S-conjugate beta-lyase. (59-61) This suggested that metabolic activation of cisplatin to a nephrotoxin occurred in the kidney. (41) For this reaction to occur, cisplatin must form a conjugate with glutathione which has been shown to occur spontaneously in solution. (62, 63) Selective inhibition of each enzyme resulted in a decrease in toxicity, *in vitro*. (41) Interestingly, conjugation of cisplatin with glutathione reduces cisplatin crosslinks with DNA resulting in decreased toxicity to dividing cells, suggesting decreased antitumor activity. (64) Also, GGT expression in tumors has been shown to decrease the antitumor activity of cisplatin. (65) These conflicting reports show that there are many areas regarding mechanisms of cisplatin-induced apoptosis and necrosis that still need to be investigated.

The physiologic alterations seen with cisplatin are relatively consistent. Renal failure is gradual and usually occurs 3 to 5 days after administration. (66) Polyuria may be due to a reduction in normal cortical-papillary solute gradient in association with a failure to recycle urea. (67) Whole kidney GFR, single nephron GFR, and renal plasma flow are all decreased after cisplatin administration. (38, 38, 42, 68) Initially, it was thought that the renin-angiotensin system may play a role in cisplatin-induced acute renal failure, although experimental studies failed to confirm this hypothesis. (42, 69) In rats, decreases in GFR are related to afferent vasoconstriction and possibly an altered ultrafiltration coefficient, both of which occur before evidence of tubular obstruction. (70) It should be restated that histopathologically, the glomerulus is minimally affected by cisplatin.

Hypomagnesemia and Hypocalcemia

Other physiologic changes related to cisplatin administration include hypomagnesemia and hypocalcemia. Hypomagnesemia has been reported to occur in more than half of human patients receiving cisplatin chemotherapy. (71) The persistent excretion of magnesium in the presence of declining magnesium levels suggests that the hypomagnesemia is due to a renal defect in magnesium reabsorption. (71) This is not necessarily associated with overt renal insufficiency and may be a more common manifestation than renal failure. (71) The mechanism for this is still slightly unclear but studies in rats suggest that abnormal magnesium excretion may be due to a defect in magnesium transport in juxtamedullary nephrons or collecting ducts. (72) When clinical manifestations of hypomagnesemia such as neuromuscular, CNS, and cardiac function abnormalities occur usually seen with serum levels less than 1 mEq/L, parenteral replacement of magnesium sulfate should be administered. (34)

Hypomagnesemia is usually complicated by hypocalcemia that is probably secondary to diminished PTH release and/or end-organ resistance to parathyroid hormone induced by hypomagnesemia. (73, 74) Hypocalcemia resolves when magnesium is replaced and is unresponsive to calcium replacement alone. (34)

Gastrointestinal and Myelosuppression

Gastrointestinal toxicity and myelosuppression appear to be associated with the death of the rapidly dividing cells in the lining of the gastrointestinal tract and in the bone marrow. Cisplatin also activates the chemoreceptor trigger zone to induce vomiting. (3) The use of antiemetics such as metoclopramide, butorphanol, dolasetron, ondansetron, and chlorpromazine can be given prior to, during, and after cisplatin infusion to decrease nausea and vomiting. (75, 76)

Ototoxicity

Ototoxicity has been reported more commonly in human patients than the dog. Ototoxicity has been observed in 7-90% of human patients receiving doses of up to 120 mg/m² per course. (77, 78) Hearing loss is in the high-frequency range and is dose-related, cumulative, and frequently irreversible. (79, 80) Concurrent cranial irradiation enhances the ototoxicity. (81) In one study with high-dose cisplatin, in spite of significantly lower hearing levels, no human patient suffered a disabling hearing loss requiring a hearing aid and the use of hypertonic saline and vigorous hydration was not found to minimize ototoxicity. (82) While humans manifest this toxicity in hearing a high-pitched tinnitus (ringing in ears) and hearing sounds differently, small animals express this as an inappropriate response or an unusually strong response to an auditory stimulus, such as hyperactivity or excessive barking. (83) The mechanism for this toxicity is unclear but may involve spontaneously recruiting adjacent neurons, aberrant cochlear fluid currents, or undermodulation of membrane movement. (83)

Neurotoxicity

Neurotoxicity is described as a peripheral neuropathy and usually develops in human patients that receive a cumulative dose of 400 mg/m² or higher. (84) In studies using high dose cisplatin with protective measures, mild to moderate paresthesias have occurred in some human patients. (81, 85)

Syndrome of Inappropriate Secretion of Antidiuretic Hormone (SIADH)

SIADH has been reported in the human literature with the administration of cisplatin and other cytotoxic drugs. (86) SIADH is characterized by hyponatremia with concurrent hypoosmolality of the serum, continued renal excretion of sodium, no clinical evidence of volume depletion, urine osmolality greater than that appropriate for concurrent osmolality of serum, and normal function of the kidneys, suprarenal glands, and thyroid glands. (86)

Protective Measures for Nephrotoxicity

Saline Diuresis

The most common protocol for administering cisplatin consists of pre- and post-hydration with concurrent saline diuresis. The maintenance of adequate hydration is important for decreasing nephrotoxicity, but the mechanism of protection is unknown.

Diuretics

Other common methods for decreasing the nephrotoxicity of cisplatin include mannitol or furosemide administration. The exact mechanism behind diuretics ameliorating cisplatin toxicity is unknown, but postulated mechanisms include: accelerating the passage of cisplatin through the renal tubules by increased urinary excretion, reversing the osmotic gradient in tubules by mannitol, and blocking of sodium and water reabsorption by furosemide. (87) It has also been suggested that these diuretics may also attenuate cisplatin nephrotoxicity reducing the concentration of platinum in the urine. (9, 88) However, in the study by Pera *et al.* it was noted that, while diuretic administration significantly improved renal function, some degree of tubular necrosis was still present. (88) Another study showed neither mannitol nor furosemide was superior to the other in reducing nephrotoxicity in the human patients involved. (87) However, there have been conflicting studies comparing hydration with or without mannitol. In one study, mannitol ameliorated nephrotoxicity better than hydration alone. (89) The conflicting study used the same dose of cisplatin but stated there was no difference between groups receiving mannitol and hydration or hydration alone. (90) In spite of these conflicting reports, administration of mannitol or furosemide along with continuous saline diuresis has become standard practice when using cisplatin chemotherapy in human cancer patients.

Hypertonic Saline

Protection from nephrotoxicity was also seen when cisplatin was dissolved in a hypertonic NaCl solution (4.5%) relative to distilled water with no effect on the antitumor action of cisplatin. (91) It is postulated that the presence of the high concentration of NaCl in the vehicle was great enough to force the aquation reaction far to the left thus favoring the presence of the parent *cis* molecule decreasing binding to plasma proteins and tissue binding sites. (91)

Pharmaceuticals

Additional drugs have been administered in conjunction with cisplatin to reduce nephrotoxicity. Amifostine (WR-2721) is a SH-containing compound that when injected before cisplatin in rats, decreased nephrotoxicity by a factor of 1.7 without inhibiting its antitumor effect. (92-94) Diethyldithiocarbamate (DDTC) is a chelating agent that potentially removes platinum bound to renal tubules. (95) However, several side effects (hypertension, agitation, flushing, and diaphoresis) that required patients to receive sedation during administration and the failure of the drug to ameliorate gastrointestinal side effects and ototoxicity, have limited the clinical application of this drug. (81) Probenecid is thought to partially inhibit platinum renal secretion and subsequently decreases the platinum concentration in the renal tubules, decreasing nephrotoxicity without affecting cisplatin's antitumor activity. (85) This drug is nontoxic, inexpensive, and readily available, but failed to protect against the other side effects of cisplatin such as myelosuppression, gastrointestinal toxicity, and ototoxicity. (85) Sodium thiosulfate is an antioxidant in the thiol family. (96) It is used most commonly in conjunction with intracavitary cisplatin to reduce toxicity and allows the dose of cisplatin to be delivered to be as high as 270 mg/m². (97) In a recent study performed on a rat model, sodium thiosulfate was found to provide protection from cisplatin ototoxicity when delivered at 4 to 8 hours after cisplatin, but was not consistently protective against nephrotoxicity. (98) Procainamide, an

antiarrhythmic agent, has also been shown to protect against cisplatin nephrotoxicity without altering its antitumor effects. (99) Procainamide and cisplatin form a complex that increases the amount of platinum bound to DNA and may prevent metabolism of cisplatin to a nephrotoxin by GGT through the formation of a cisplatin-glutathione complex. (41, 100) Methimazole, an antithyroid drug, was given intraperitoneally 30 minutes prior and 4 hours after cisplatin infusion without saline prehydration to normal dogs and was found to significantly decrease nephrotoxicity. (101) Methimazole is thought to exert an antioxidative effect to protect the kidney, but it is unknown whether this compound affects cisplatin tumoricidal activity. (101) Liposome-encapsulation of cisplatin has also been proven to allow an increase in dose of cisplatin that can be safely administered without increasing the nephrotoxicity in dogs and cats. (102-104)

Enzymatic and Molecular Alterations

Reduction of nephrotoxicity has also been associated with some enzymes or agents that control or prevent the formation of free radicals. One study demonstrated a decrease in cisplatin nephrotoxicity in rats treated with a superoxide dismutase mimetic, orgotein. (105) Another rat model study used N-acetylcysteine, an antioxidant, delivered at 400 mg/kg IV 15 minutes prior to cisplatin injection and found that treated rats had normal BUN, creatinine, and histologically normal kidneys 3 days after injection. (98) Many studies investigating the mechanism of cisplatin nephrotoxicity have been using agents such as caspase inhibitors and antioxidants to view their role in apoptosis of renal tubular cells. (47, 54, 58) Also, upregulation of the genes p21 and manganese superoxide dismutase is considered as a potential future therapy, as studies done *in vitro* have demonstrated that upregulation of these two genes resulted in protection against cisplatin nephrotoxicity. (47, 54) Unfortunately, many of these studies have been *in vitro*

on kidney tubule cells and these genes' effect on cisplatin's *in vivo* effects in higher mammals and antitumor effect is still unknown.

Another recent development has been with the endogenous enzyme heme oxygenase-1 (HO-1). HO-1 is an inducible enzyme that degrades heme and produces carbon monoxide (CO), iron, and biliverdin. Biliverdin is reduced to bilirubin *in vivo* and is a powerful antioxidant. (106, 107) Carbon monoxide possesses vasodilatory, anti-inflammatory, and anti-apoptotic properties. (108-111) HO-1 upregulation occurs after cisplatin administration and upregulation protects against cisplatin nephrotoxicity. (112) Tayem *et al.* recently showed that a water-soluble carbon monoxide releasing molecule protected renal tubular cells from cisplatin injury *in vitro* and *in vivo* in rats. (113) Again, as with other recent studies mentioned previously, it is unknown whether HO-1 interferes with cisplatin's antitumor effect. Recently, in our laboratory, we have reported that hyperbilirubinemia ameliorates nephrotoxicity in rats receiving cisplatin. (114)

Cisplatin Use in Animals

Dogs

Cisplatin has been used as a systemic or local chemotherapy agent in dogs via intravenous, intraarterial, intramedullary, intralesional and intracavitary routes. Two short-term diuresis protocols have been utilized in the dog (Table 1-1). The incidence of nephrotoxicity was similar between the studies and survival times for the dogs developing nephrotoxicosis were similar to those that did not develop nephrotoxicosis in both studies. (115, 116) In human patients, the cisplatin dose can be divided over 5 days with the patient receiving concurrent NaCl diuresis during cisplatin administration, adequate pre- and posthydration, and possible use of the diuretics mannitol and furosemide. (117) Administering cisplatin over many days under constant diuresis is not utilized in veterinary medicine because it is not cost effective in animals.

Cisplatin is most commonly used as a single agent or as part of combination chemotherapy with doxorubicin to treat osteosarcoma. Median survival times in dogs treated with cisplatin as the sole chemotherapeutic agent range from 262 to 413 days. (118-122) Various combination protocols have been investigated. Doxorubicin (30 mg/m² IV on day 1) and cisplatin (60 mg/m² IV on day 21) repeated for 2 treatment cycles resulted in median survival times of 300 days. (123) A more recent study investigated the use of doxorubicin and cisplatin administered within 24 hours of each other. (124) Cisplatin (50 mg/m²) was administered IV followed by doxorubicin (15 mg/m²) 24 hours later with the intent of completing 4 treatment cycles. (124) Median survival times were equivalent to studies where cisplatin and doxorubicin were administered 3 weeks apart at 300 days, but significant toxicity was encountered with this protocol. (124) Renal toxicity was present in 11% of patients, which is higher than the reported incidence in the diuresis studies utilizing cisplatin as a sole treatment agent. (124) Gastrointestinal toxicity and myelosuppression were comparable to larger studies utilizing only cisplatin. (124-126) The use of STEALTH liposome-encapsulated cisplatin versus carboplatin has found that while the use of the STEALTH cisplatin allowed safe administration of five times the maximally tolerated dose of free cisplatin, this did not translate into prolonged disease-free or overall survival. (127)

Other methods of administration of cisplatin for the treatment of osteosarcoma that have been investigated include intralesional (with implants or injection), intraarterial, and intramedullary administration. Amputation is usually recommended for dogs that have appendicular osteosarcoma to achieve local tumor control and palliation, but concurrent orthopedic or neurological disease may make amputation less feasible in certain dogs. When this occurs, limb-spare procedures and palliative radiation have been utilized as acceptable

alternatives. Cisplatin-containing implants have been utilized in limb-spare procedures for additional local control. (128) Dogs receiving cisplatin implants were 53.5% less likely to develop local recurrence than the control groups, although this effect did not reach statistical significance. (128)

Percent tumor necrosis was found to be statistically significant when predicting local tumor control. (129) Significantly greater tumor necrosis is observed after intraarterial cisplatin administration when compared to systemic cisplatin administration using an intravenous route. (129) The use of radiation therapy in addition to intraarterial cisplatin further increased percent of tumor necrosis. (129) One study investigated the use of intraarterial cisplatin and radiation in dogs for local tumor control. (130) Radiation was administered in 10 equal fractions, 3 days a week while cisplatin (70 mg/m^2) was given intraarterial in the affected leg on the first and last treatment days over 2 hours with appropriate diuresis. (130) Eighty-nine percent of dogs had an improvement in limb function with no toxicity noted as a result of therapy. (130) Intraarterial cisplatin did not improve survival times by preventing metastasis as intravenous cisplatin does. (130) Intramedullary cisplatin (60 mg/m^2 over 20 minutes) administered with a Jamshidi biopsy needle inserted into the tumor provided effective local control in 50% (2/4) of dogs unable to undergo amputation or a limb-spare procedure. (131)

Cisplatin has also been utilized for transitional cell carcinoma (TCC) and squamous cell carcinomas (SCC). At a dose of 50 mg/m^2 given IV every 28 days, cisplatin was found to have a palliative effect for dogs with SCC and TCC. (118) No complete responses were observed in this study, but partial remission and stable disease were observed in the majority of dogs without significant toxicity noted. (118) In another study in which cisplatin was given at 60 mg/m^2 IV every 3 weeks to dogs with a variety of malignant tumors that included TCC and SCC, an overall

response rate (complete or partial remission) of 19% was observed. (126) Those dogs demonstrating progressive disease did so by 42 days after the start of treatment, suggesting that dogs should be evaluated at this time to determine response to treatment. (126)

The use of cisplatin concurrently with radiation therapy is a source of interest as cisplatin reportedly enhances radiation-induced cell kill. (132-136) A study performed on dogs with nasosinus carcinomas compared cobalt radiation alone with radiation in addition to cisplatin at 7.5mg/m² IV bolus before every other radiation treatment. (137) The mean and median tumor control and survival times were not significantly different between treatment groups; however, there was a trend toward longer tumor control and survival times in the cisplatin treatment group. (137) Another study regarding nasal tumors utilized an OPLA-Pt implant concurrently with radiation and found that the implant was clinically tolerable and yielded comparable or perhaps improved survival times when compared to other published protocols. (138) The use of radiation with cisplatin has also been researched with regard to oral melanomas. One study gave cisplatin 10-30 mg/m² IV with diuresis or carboplatin 90 mg/m² IV prior to 6 weekly 6-Gy radiation fractions. (139) The use of low dose chemotherapy resulted in a median survival of 363 days which was longer than the previously reported survival times for surgery or radiation therapy alone. (139) It should be noted that the dogs in this study had small initial tumor size and no lymph node or lung metastasis at the start possibly positively affecting survival times. (139)

Intralesional cisplatin has also become a possibility for local cisplatin administration. Implants containing cisplatin, viscous gel, and a vasoactive modifier have been used as primary treatment for melanomas. (140) Implants were injected until tumor saturation was visualized and these treatments occurred weekly until complete tumor resolution was observed. (140) Seventy

percent of dogs had a >50% decrease in volume and 55% of these dogs had a complete response. (140) Tumors that responded received a mean of 2.6 treatments. (140) The most common side effect was local necrosis seen in 85% of patients and was associated with tumor response. (140) Systemic toxicosis was minimal with no dog exhibiting renal toxicosis. (140) Patient survival was comparable to other forms of local treatment such as radiation and surgery. (140) Although there are no current published reports in dogs, intralesional cisplatin is also being utilized for other tumors using sesame oil instead of the viscous gel described above.

Intracavitary cisplatin is the last well-known use of cisplatin in dogs. In one study, 50 mg/m² of cisplatin was administered every 28 days for a median of 2.5 treatments to dogs with mesothelioma and carcinomatosis. (141) When using intracavitary chemotherapy, the tumor is exposed by the capillary blood supply to a concentration equivalent to that achieved by IV administration, and the surface cell layers are exposed to a concentration that is 1-3 logs higher. (142, 143) Although intracavitary cisplatin administration is a local method of chemotherapy delivery, significant systemic absorption does occur and the dose limiting toxicity for intracavitary cisplatin is renal toxicity.(141) Animals in this study underwent diuresis and 66.7% of dogs received additional treatment with sodium thiosulfate preventing toxicity during the study. (141) This method of cisplatin delivery was associated with palliation and control of malignant pleural and/or abdominal effusion in 5 of 6 dogs and this palliation lasted 129 to greater than 807 days. (141) Although results for the previous study were promising, a more recent study found that the use of intracavitary carboplatin or mitoxantrone was just as effective for dogs with similar diseases. (144) The ease of administration for carboplatin and mitoxantrone is superior to cisplatin since diuresis is not required and minimal side effects were

noted with the former two chemotherapy agents, suggesting that the use of cisplatin in intracavitary infusions will become obsolete in veterinary medicine. (144)

Cats

Cisplatin is unable to be administered to cats due to its acute drug toxicity in this species. Cats receiving 60 mg/m² of cisplatin became dyspneic and died 48-96 hours after administration. (145) Postmortem findings included severe hydrothorax, pulmonary edema, and mediastinal edema. (145) A group of cats undergoing equivalent saline diuresis as the cisplatin group did not show these signs, causing the belief that cisplatin had induced the changes mentioned. (145) Lowering the dose of cisplatin to 40 mg/m² resulted in similar but less severe pulmonary changes, while decreasing the dose to 20 mg/m² showed no pulmonary changes. (145) The tumoricidal activity of cisplatin alone at such doses is not known. Using repetitive low dosing (10 mg/m² 3 times a week for 10 txt), cisplatin use resulted in reversible pulmonary edema and renal insufficiency. (146) The use of liposome encapsulated cisplatin has been researched in cats. Studies with this form of cisplatin showed no renal or pulmonary toxicity but all cats had transient pyrexia and/or lethargy, vomiting, inappetence, and an acute infusion reaction prevented by administering atropine-diphenhydramine. (102, 103) When this formulation was looked at in cats with squamous cell carcinoma, the liposome encapsulated cisplatin was found to be an ineffective treatment since none of the cats had complete or partial remissions. (147)

Horses

Intratumoral cisplatin in oily emulsion has proven efficacious in treatment of cutaneous tumors in horses such as squamous cell carcinoma and sarcoids. (148, 149) Intralesional cisplatin can be used alone for treatment of small tumors or in combination with surgery for larger tumors. (150) Treatments are given at 2 week intervals at a dose of 1 mg cisplatin for each

cm³ of tissue in the target field. (151) When surgery is performed, cisplatin should be administered perioperatively or early in the postoperative period. (151)

Other Platinum Drugs

The most commonly used alternative platinum drug to cisplatin is carboplatin. Carboplatin has been shown to be less nephrotoxic than cisplatin and can be administered without saline diuresis. Similarly, nausea and vomiting are common side effects with carboplatin as they are with cisplatin. Carboplatin alone or in combination with doxorubicin has been utilized to treat dogs with osteosarcoma with survival times at 321 days and 320 days respectively which are similar to survival times achieved to cisplatin alone or in combination with doxorubicin. (152, 153) However, in regards to treating TCC, carboplatin was not as effective as cisplatin in producing a clinical response. (154) Other tumors in which carboplatin's use has been researched include: malignant melanoma, nasal tumors, and anal sac adenocarcinoma. As mentioned previously, carboplatin has additional uses as an intracavitary chemotherapeutic and as a radiosensitizer. (139, 144)

Lobaplatin, another platinum analog, was found to result in a one-year survival fraction of 31.8% when administered every 3 weeks to dogs with appendicular osteosarcoma. (155) Clinical signs related to toxicosis were uncommon and usually were vomiting and depression. (155) Unlike cisplatin, lobaplatin did not require pretreatment infusions.

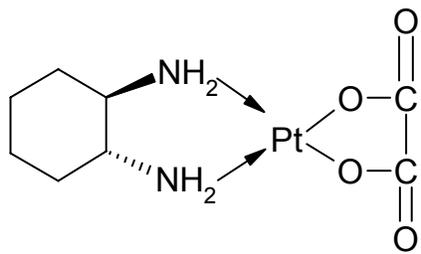
Summary

Cisplatin has long been utilized in both human and veterinary medicine. Cisplatin is still used widely in many protocols in human patients affected with head and neck, lung, and germ cell tumors, as well as OSA. The use of cisplatin in veterinary medicine is not as widespread which is most likely due to cisplatin's severe adverse side effects. In addition, carboplatin, a platinum analog, has shown fewer severe side effects and has recently become a more cost

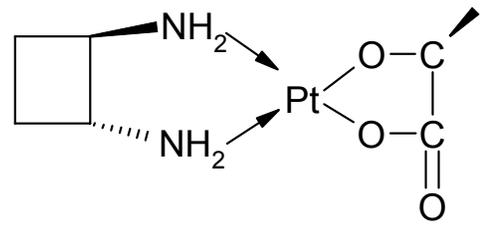
effective alternative to cisplatin. It is still likely that cisplatin will continue to be utilized in veterinary medicine for treatment of OSA, intralesional chemotherapy for a variety of tumors, and as a sensitizer prior to radiation therapy.

Table 1-1: Canine diuresis protocols when administering cisplatin. These protocols utilized 70 mg/m² given with 0.9% NaCl IV over 20 minutes.

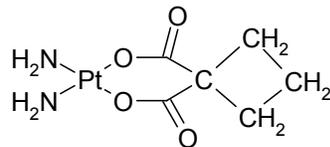
| Duration of diuresis | Fluid rate (ml/kg/hr) | Length of diuresis before cisplatin | Length of diuresis after cisplatin | Incidence of nephrotoxicity | Reference |
|----------------------|-----------------------|-------------------------------------|------------------------------------|-----------------------------|-----------|
| 4 hours | 25 | 3 hours | 1 hour | 7.8% | (116) |
| 6 hours | 18.3 | 4 hours | 2 hours | 6.6% | (115) |



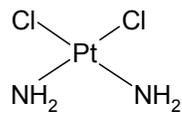
Oxaliplatin



Lobaplatin



Carboplatin



Cisplatin

Figure 1-1: Two-dimensional structures for cisplatin, carboplatin, oxaliplatin, and lobaplatin. While the core structure (Pt) is the same for each drug, the leaving groups are different for each compound.

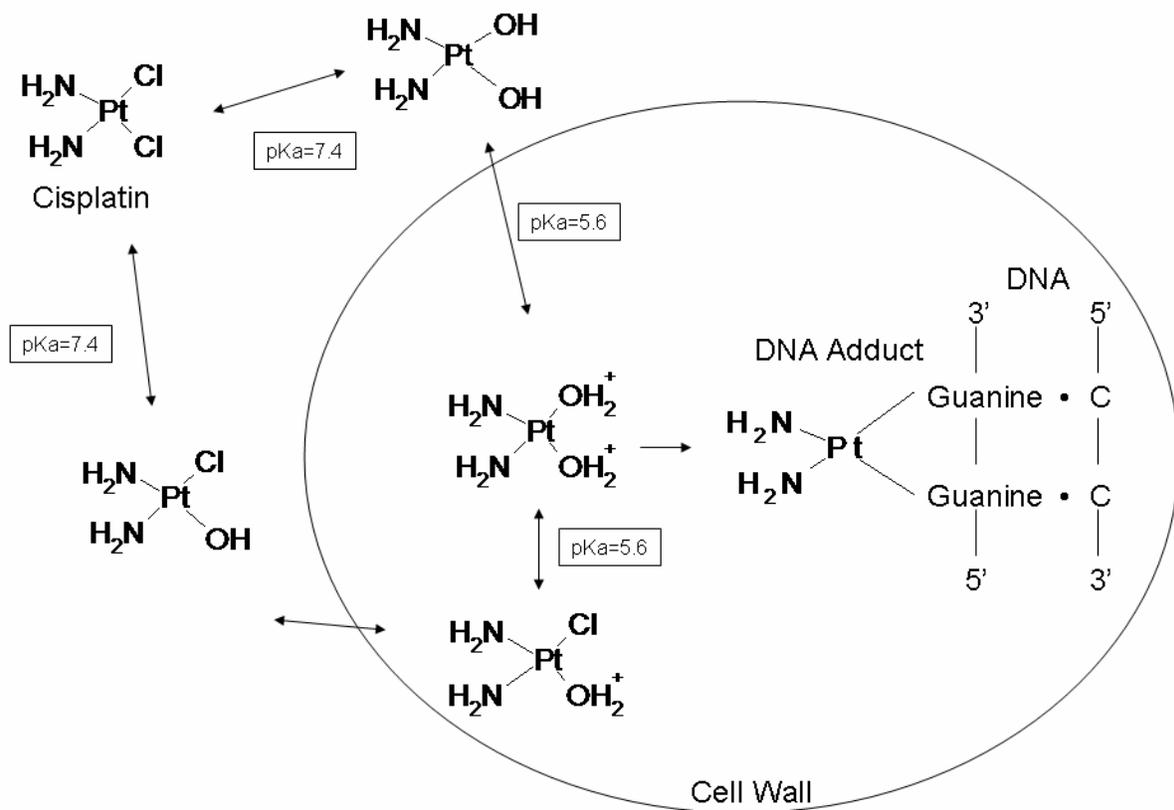


Figure 1-2: Aquation reaction and adduct formation at the N-7 position of guanine on 2 sites of DNA. These adducts result in DNA damage resulting in cell kill.

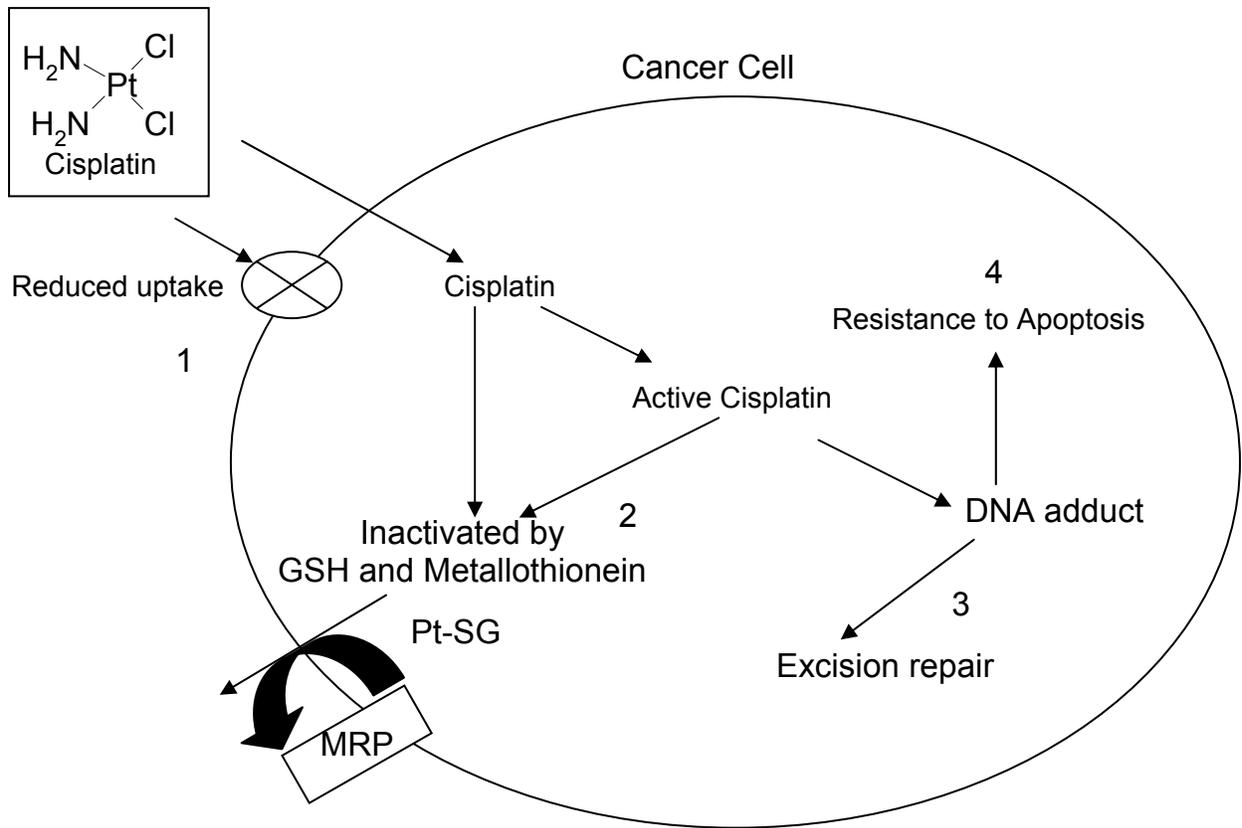


Figure 1-3: Four pathways exist for cisplatin resistance. They include: (1) decreased cellular accumulation, (2) inactivation of the drug, (3) DNA repair, and (4) prevention of apoptosis.

CHAPTER 2 HYPERBILIRUBINEMIA PROTECTS AGAINST CISPLATIN NEPHROTOXICITY IN THE GUNN RAT

Introduction

Cisplatin is one of the most commonly used antineoplastic agents in human patients. Cisplatin is currently a front line drug used in chemotherapy protocols to treat a wide variety of tumors including ovarian, cervical, testicular, head and neck tumors, transitional cell carcinomas, osteosarcomas, small cell lung, and esophageal cancers. Cisplatin is also utilized as a rescue agent in the treatment of other solid tumors. (4, 156-161)

Systemic dose-related toxicities associated with cisplatin are well-documented and have precluded its use in many patients. Of these, nephrotoxicity is the most frequently observed and most clinically significant toxicity. The mechanism for cisplatin nephrotoxicity has not been completely elucidated; however, many theories have been developed. (42, 43, 48-50) One theory is that reactive oxygen species (ROS) and mitochondria play a role in the apoptotic cascade involved in cisplatin nephrotoxicity. (51) The morphologic alterations in the kidney ascribed to cisplatin occur in the pars recta of the proximal tubule situated in the outer stripe of the medulla and the maximum damage is seen by day 5 after administration. (39) *In vitro* studies have shown that necrosis and apoptosis can occur, with the form of cell death being dependent on the concentration of cisplatin the cells are exposed to. (50)

Recently, the endogenous enzyme heme-oxygenase 1 (HO-1) has been investigated for its role in protecting organ systems from various insults. HO-1 is induced in response to cellular stress, and converts the pro-oxidant heme molecule into equimolar quantities of biliverdin (BV), carbon monoxide (CO), and iron. (162) BV is converted to unconjugated bilirubin (BR) via bilirubin reductase. (163) Unconjugated BR is then converted to conjugated BR by the hepatic microsomal enzyme, uridine diphosphate glucuronyltransferase (UDPGT). (164) These

molecules of heme degradation were once considered to be toxic metabolites, but have recently been shown to have dose-dependent vasodilatory, anti-oxidant, and anti-inflammatory properties that may be useful in protection of various organ systems from toxic insult. (165)

Specifically, the HO-1 enzyme and its products have been studied in association with toxic acute renal failure. Hyperbilirubinemia has been shown to result in protection against acute renal failure caused by the nephrotoxin glycerol. (166) With regards to cisplatin mediated nephrotoxicity, depletion of the HO-1 enzyme resulted in more significant renal failure and renal injury in one study (112) and the administration of CO along with cisplatin ameliorated signs of renal failure in another study. (113) However, upregulation of HO-1 or its products may affect cisplatin's antineoplastic activity.

The objectives of this study were to investigate the protective effect of hyperbilirubinemia *in vivo* in the rat model against cisplatin-induced nephrotoxicity. Hyperbilirubinemia *in vivo* was achieved using both the homozygous and heterozygous Gunn rat. The homozygous Gunn rat is unable to induce UDPGT as a result of an autosomal recessive deficiency in this enzyme. (167, 168) The lack of induction of UDPGT results from an alteration in the coding region of the mRNA which results in an instability of the mRNA and a synthesis of a truncated, functionally inactive UDPGT. (168) In addition, an intermediate level of bilirubin can be obtained using heterozygous Gunn rats that have varying degrees of functional UDPGT. We also investigated the effect of bilirubin on the antineoplastic activity of cisplatin using four established canine osteosarcoma cell lines (POS, HMPOS, COS31, D17).

Materials and Methods

Animals

This study was approved by the University of Florida Institutional Animal Care and Use Committee and was performed in accordance with the Institute for Lab Animal Research Guide

for the Care and Use of Laboratory Animals. Male Wistar, homozygous Gunn (j/j) and heterozygous Gunn (j/+) rats weighing 200-400 g were purchased from Harlan Sprague Dawley, Inc (Indianapolis, IN) and maintained in a temperature controlled room with alternating 12 hour light/12 hour dark cycles in an animal facility at the University of Florida. Animals were fed standard rat chow and allowed free access to water.

Cisplatin-Induced Acute Renal Failure

Three groups of male rats (n=6 rats/group) were used: (1) Wistar (2) homozygous Gunn (3) heterozygous Gunn. Rats were weighed and observed for changes in attitude during the course of the experiment. On Days 0, 3, and 5, rats were anesthetized using 5% inhalant isoflurane in 100% oxygen and maintained with 2-3% isoflurane by mask. Animals were placed on a warm water heating pad to maintain normal body temperature. Prior to blood sampling on Days 0 and 3, the rat's tail was soaked in 40-42°C water for 3-5 minutes to facilitate vasodilation. One milliliter of blood was sampled from the tail veins or the lateral saphenous veins. After blood sampling was complete on Day 0, all rats were given an intraperitoneal injection of cisplatin (American Pharmaceutical Partners Inc, Schaumburg, IL) at 4 mg/kg. Once the IP injection was complete, all rats were recovered. Upon recovery, an injection of 0.01 mg/kg buprenorphine hydrochloride (Reckitt Benckiser Healthcare (UK) Ltd, Hull, England) was given SQ. Blood was sampled on Days 0, 3, and 5 for evaluation of BUN, serum creatinine, and serum bilirubin concentrations. On Day 5, once the rats were anesthetized a midline incision was performed and blood was sampled from the caudal vena cava. Both kidneys were isolated and harvested and the rats were euthanized by an overdose of sodium pentobarbital (Euthasol, Diamond Animal Health, Inc., Des Moines, IA).

Three groups of male rats (n=4 rats/group) were used as sham control rats. The groups consisted of: (1) Wistar (2) homozygous Gunn (3) heterozygous Gunn. These groups were

treated as described above for the cisplatin induced acute renal failure groups; however, instead of receiving 4 mg/kg cisplatin, the rats received the equivalent amount of 0.9% sodium chloride IP. The sham control rats for each group were intended to prove that equivalent times of anesthesia would have no effect on the kidney functional parameters and histopathology.

Assays

BUN and serum bilirubin (BR) concentrations were determined using an automated chemistry analyzer (Hitachi 911 Chemistry Analyzer). Serum creatinine (Cr) concentrations were determined using a dry chemistry analyzer (Johnson & Johnson Vitros DT6011) due to the potential effect of icterus on the standard Jaffe methodology for measurement of Cr. (169)

Histologic Grading

Both kidneys were placed in 10% buffered formalin for at least 24 hours before processing. Transverse sections of the left kidney for all rats were processed using hematoxylin and eosin (H&E) staining and periodic-acid-Schiff (PAS) staining. Histological examination was performed by a renal pathologist who was blinded with respect to the treatment groups. Renal tissue was divided into 4 regions for analysis: cortical proximal tubules (CPT), S3 segment of the outer stripe of the outer medullary proximal tubule (OSOMPT), medullary thick limb in the inner stripe (ISOM mTAL), and collecting ducts (CD). Renal injury was graded in 7 different categories: normal, cellular swelling/vacuolization, brush border loss, nuclear condensation, karyolysis/apoptosis/necrosis (most severe form of injury), regeneration, and capillaritis. Each category was assigned a numerical score: 0= none, 1 =<10%, 2= 10-25%, 3= 25-50%, 4= 50-75%, 5= 75-100% based on the percentage of cells in each region displaying the described injury.

Cell Lines

Osteosarcoma is a highly-aggressive tumor in dogs and affected dogs are commonly treated with cisplatin. Four canine osteosarcoma cell lines which have been well characterized in our laboratory were utilized in this study. The POS (parent osteosarcoma) cell line was originally developed from a primary osteosarcoma affecting the left proximal femur of a 1 and a half year-old male mongrel dog (Dr. Tsuyoshi Kadosawa, University of Sapporo, Japan). (170) The HMPOS (highly metastatic parent osteosarcoma) cell line is a pulmonary metastatic derivative of POS cell line (Dr. Tsuyoshi Kadosawa, University of Sapporo, Japan). (171) D17 is another established canine osteosarcoma cell line (American Type Tissue Culture Collection, Manassas, Virginia). The COS31 cell line was established from a dog with spontaneously occurring osteosarcoma (Dr. Ahmed Shoieb, University of Tennessee, College of Veterinary Medicine, Knoxville, TN). Cells were cultured at 37°C under 5% CO₂ and 95% room air with their respective media. POS and HMPOS media consisted of RMPI 1640 media supplemented with 10 % heat inactivated fetal calf serum, vitamins, sodium pyruvate, non-essential amino acids, L-glutamine, and antibiotics (penicillin (0.0625 g/L) and streptomycin (0.1 g/L)). D17 and COS31 media consisted of Dulbecco's Modified Eagle's medium with 10% heat inactivated fetal calf serum, L-glutamine, and antibiotics (penicillin (0.0625 g/L) and streptomycin (0.1 g/L)). The cells were grown to confluence, washed with physiological buffered saline, and detached from the flasks with trypsin. Cells were stained with Trypan blue and counted with a hemacytometer.

Cell Viability Assay

An assessment of cell viability was performed with the CellTiter Blue™ Cell Viability Assay (Promega Corporation, Madison, WI). Assays were performed in 96-well flat-bottomed black microtiter plates. All cell lines were seeded at 10,000 cells/well with 50 µL of media and

placed in the incubator at 37°C under 5% CO₂ and 95% room air for 24 hours. The IC₅₀ for each cell line with cisplatin was determined prior to treating the cells with bilirubin and cisplatin. All cells were treated with 50 µL of bilirubin at concentrations of 71 µM and 128µM alone and combined with each cell type's IC₅₀ concentration of cisplatin. Bilirubin was dissolved in the respective media to achieve appropriate concentrations. The micromolar concentrations of bilirubin used in this study were taken from the average serum bilirubin levels on Day 0 and 3 that provided functional nephroprotection of the homozygous Gunn rats receiving cisplatin. After incubation for 72 hours under the conditions described previously, 20µL of CellTiter Blue™ reagent, resazurin, was added to each well. Viable cells retain the ability to reduce resazurin into resorufin, which is pink and highly fluorescent. (172) Plates were placed on a low-speed shaker for 10 seconds and then incubated for 4 hours. The amount of fluorescence was recorded with a fluorescence plate reader at 530/590 nm.

Statistical Analysis

Statistical calculations were performed using a computer software program (SigmaStat for Windows, version 3.00, and SigmaPlot for Windows, version 8.02, SPSS Inc, Chicago, Ill.) Data was tested for normality and equal variance using the Kolmogorov-Smirnov test. The comparisons between groups used ANOVA for parametric data and ANOVA on Rank's for non-parametric data. Differences between groups were identified using (post-hoc) pair wise multiple comparison procedures (Holm-Sidak method or Dunn's Method). Parametric data is reported as mean ± SD and nonparametric data as median with an inter-quartile range ([IQR], 25% to 75%). P value < 0.05 was considered significant.

Results

Renal Functional Parameters

Bilirubin levels were significantly higher for all days in the homozygous Gunn rats when compared to the heterozygous Gunn rat and the Wistar rat (Table 2-1). Statistically, bilirubin levels were not significantly higher in the heterozygous Gunn rat compared to the Wistar rat at any day. However, the Wistar rats had a mean of 0 mg/dL and the heterozygous rats had a mean of 0.4 to 0.1 mg/dL, indicating that a mild degree of hyperbilirubinemia existed in the heterozygous Gunn rats.

The homozygous and heterozygous Gunn rats were protected from the nephrotoxic effects of cisplatin based on functional kidney parameters. There was no significant difference in BUN or Cr on Day 0 between any of the groups of rats (Figures 2-1 and 2-2). Importantly, in the face of a nephrotoxic dose of cisplatin, the hyperbilirubinemia present in the homozygous Gunn rat resulted in a significant nephroprotective effect when compared with the heterozygous Gunn rat and the Wistar rat (means BUN homozygous: Day 5- 23.83 ± 5.49 mg/dL, $p < 0.05$; means Cr homozygous: Day 5- 0.35 ± 0.05 mg/dL). The protection was clinically significant as the homozygous rats' BUN and Cr values remained within the normal range. While, the intermediate level of hyperbilirubinemia provided by the heterozygous Gunn rat still provided nephroprotective effects when compared to the Wistar rat, the protection was not as effective as for the homozygous Gunn rat. When comparing heterozygous Gunn rat and Wistar rat BUN and Cr levels with each other, the differences in BUN were not significant until Day 5 but were present on Day 3 and 5 with regard to Cr (means BUN heterozygous: Day 5- 38.17 ± 10.34 mg/dL; means Cr heterozygous: Day 5- 0.40 ± 0.06 mg/dL; means BUN Wistar: Day 5- 78.67 ± 16.92 mg/dL; means Cr Wistar: Day 5- 1.4 ± 0.43 mg/dL)

When comparing the homozygous Gunn rat to the heterozygous Gunn rat in regards to functional kidney parameters, it was found that there was no significant difference in BUN on Day 3 and Cr on Day 3 and 5 (Figure 2-1 and 2-2). However, there was a statistically significant difference in BUN between the groups on Day 5, with the homozygous Gunn rats showing a lower BUN than the heterozygous rats.

When all groups of sham rats (homozygous Gunn, heterozygous Gunn, Wistar rats) were compared to the control group of rats receiving cisplatin, it was found that there was no significant difference in BUN or Cr on Day 0. There were significant differences for the sham groups when compared to the control Wistar group in BUN and Cr for Day 3 and 5. Between the sham groups, there were no statistically significant differences in BUN and Cr at Day 0, 3, or 5. Importantly, there was also no significant difference between the homozygous Gunn rat receiving cisplatin and any of the sham groups in BUN and Cr at Day 0, 3, or 5. With regard to bilirubin levels, there was a significant difference between the control Wistar rats and the sham homozygous Gunn rats, but no significant difference between the control Wistar rats and the sham heterozygous Gunn rats. This is equivalent to what was reported for the groups receiving cisplatin.

Light Microscopy

For all treatment groups, the cortical proximal tubules and collecting ducts were graded as 75-100% of cells being normal. The area of most interest with regard to cisplatin nephrotoxicity is the S3 segment of the outer stripe of the outer medullary proximal tubule (OSOMPT). Homozygous Gunn rats had significantly decreased karyolysis/apoptosis/necrosis than the heterozygous Gunn rats and the Wistar rats in the OSOMPT (Figure 2-3A and 2-3B). There was no significant difference in karyolysis/apoptosis/necrosis between heterozygous Gunn rats and Wistar rats in the OSOMPT. The homozygous Gunn rats had a significantly greater proportion

of normal cells and cells without cellular swelling in the medullary thick limb in the inner stripe (ISOM mTAL) than the Wistar rats.

The sham groups had identical normal histological scores for all segments of the kidney and were thus grouped together for statistical analysis. Even though the homozygous Gunn rats receiving cisplatin had significantly decreased histologic damage to the OSOMPT than the other groups receiving cisplatin, there was significantly greater degree of injury in the homozygous Gunn rats receiving cisplatin with regard to karyolysis/apoptosis/necrosis in the OSOMPT when compared to the sham groups. There was also a significant difference in cellular swelling, brush border loss, and regeneration in the OSOMPT between the homozygous Gunn rats receiving cisplatin and the sham groups. A significant difference was also noted between the heterozygous Gunn rats receiving cisplatin and the sham groups in cellular swelling, brush border loss, and karyolysis/apoptosis/necrosis in the OSOMPT.

Cell Culture Studies

In vitro, bilirubin had mild cytotoxic effect on COS31 cells at 72 and 128 μM and D17 cells at 128 μM versus control cells. No significant cytotoxic effects of bilirubin were seen with HMPOS and POS cells at either concentration or D17 cells at 72 μM . Bilirubin had no significant effect on the antineoplastic effect of cisplatin at either concentration in any of the four canine osteosarcoma cell lines (Figure 2-4).

Discussion

While the toxic properties of bilirubin, particularly kernicterus and neonatal hyperbilirubinemia are well documented, the therapeutic properties of bilirubin are just recently being discovered. (173-176) This study demonstrated that hyperbilirubinemia found in the Gunn rat had a nephroprotective effect when the nephrotoxic, anti-neoplastic agent cisplatin was

administered, as evidenced by the maintenance of normal renal function values and markedly less histologic evidence of tubular necrosis.

Protective effects of bilirubin against cellular injury have been studied previously but never in the cisplatin model of nephrotoxicity. Leung *et al.* showed that ligation of the common bile duct effectively protected against glycerol induced acute renal failure in the rat model. (166) Results of our study and the one performed by Leung suggest that sustained hyperbilirubinemia protects the kidney from known potent nephrotoxins. (166) Our lab has also reported protection from ischemia-reperfusion injury with exogenous bilirubin when delivered into the isolated, perfused rat kidney. (177) The same effects, however, were not noted when the equivalent *in vivo* rat study was performed. (165) This suggests that the protective effects of bilirubin may only be applicable to nephrotoxins or perhaps the dose of exogenous bilirubin required to protect the kidney from various insults has yet to be ascertained.

Bilirubin has also shown a protective effect in other organ systems such as the liver (178, 179), intestine (180), and neural tissue (181). Clinical trials have shown that the incidence of coronary and ischemic heart disease is lower in humans with hyperbilirubinemia. (182, 183)

Bilirubin is a product of HO-1. The beneficial effects of HO-1 induction have been well established in multiple organ systems. (106, 166, 178, 184, 185) In fact, Shirashi *et al* demonstrated that mice deficient in HO-1 (-/-) developed more severe renal failure and renal injury than wild type mice (+/+) when cisplatin was administered. (112) These findings prompted a look at the individual agents produced by HO-1 to determine if bilirubin or CO alone could mimic this effect. A study by Tayem *et al.* showed that treatment with a water-soluble carbon monoxide-releasing molecule protected the kidney function and improved histology of rats treated with cisplatin. (113) The results of our study indicate that hyperbilirubinemia in the

homozygous Gunn rat was completely nephroprotective with regards to functional renal parameters and partially protective histologically. Partial protection of renal functional parameters were also provided to the heterozygous Gunn rat, whose bilirubin levels were not statistically higher than the Wistar rat at any day, although histologically there was no significant difference in the amount of most severe kidney damage to the S3 segment. An improvement in functional kidney parameters is still important and a trend was present at day 0 for higher bilirubin levels in the heterozygous Gunn rat than the Wistar rat. This suggests that the protective effects of bilirubin may be exerted on the day the nephrotoxin is administered (Day 0).

The exact concentration of bilirubin in serum needed to prevent severe nephrotoxicity due to cisplatin remains in question. Higher average serum bilirubin values present in the homozygous Gunn rat resulted in significantly less kidney injury than the heterozygous Gunn rat suggesting that the value of bilirubin required for any histological protection may lie somewhere in between those two groups. For full histological protection, serum bilirubin values may need to be higher than those seen in the homozygous Gunn rat. Unfortunately, a higher dose may result in bilirubin toxicity. Most likely, individual variation in serum bilirubin levels exist even between the homozygous and heterozygous Gunn rats due to a variation in the levels of the enzyme UDPGT. Homozygous Gunn rats have a deficiency of this enzyme needed to conjugate bilirubin but UDPGT may still be present in varying small amounts in these rats.

The sham group of rats served as a negative control group in this experiment. The purpose of the sham group was to verify that the anesthesia episodes required for venipuncture had no functional or histological effect on the kidneys that would skew our results. All of the rats in this group maintained normal BUN and Cr and had histologically normal kidneys substantiating that anesthesia did not contribute to renal pathology.

Another aim of our study was to determine if adding cisplatin with bilirubin would have an additive, inhibitory, or no effect on neoplastic cells in culture. For this experiment, we utilized four different canine osteosarcoma cell lines for completeness. Bilirubin did not adversely effect cisplatin's *in vitro* antineoplastic activity in any of the four cell lines. This is a very important aspect to view for clinical application of this study. Agents such as bilirubin that proved nephroprotection cannot interfere with a chemotherapeutic agents efficacy to be useful clinically. Bilirubin's antineoplastic effect needs to be tested in other neoplastic cell populations and in an *in vivo* setting before any definitive conclusions can be drawn. Bilirubin has been shown to have some cytotoxic effects on human colon adenocarcinoma cells and human carcinoma cell lines. (186, 187) While two of the cell lines showed some cytotoxicity caused by bilirubin alone (COS31 at 72 μ M and 128 μ M and D17 at 128 μ M), these effects were minimal and had no additional impact on the cells after cisplatin was added. Bilirubin's cytotoxicity against neoplastic cells may only pertain to certain types of neoplasia as evidenced by our cell culture results.

Future studies would be helpful to determine if the same effects could be duplicated using rats with a sustained conjugated hyperbilirubinemia. It is unknown if protection is afforded by unconjugated or conjugated bilirubin. Future studies are also warranted to determine if administration of exogenous bilirubin can mitigate cisplatin induced nephrotoxicity. Delivering a bilirubin dose high enough to exceed the serum concentrations seen in the homozygous Gunn rat without toxicity with resulting complete histological protection of the S3 segment of the outer medulla may be possible. Another area to research may include concurrent administration of exogenous bilirubin and the water-soluble carbon monoxide releasing molecule to determine if the combination could prevent any histologic nephrotoxicity caused by cisplatin.

In conclusion, hyperbilirubinemia present in the homozygous Gunn rat resulted in complete nephroprotection with functional renal parameters and partial nephroprotection histologically. Only partial functional nephroprotection was seen in heterozygous Gunn rats that did not demonstrate significant hyperbilirubinemia when compared to the control Wistar rats. The anesthetic episodes each rat underwent did not impact functional or histologic kidney parameters. In the four canine osteosarcoma cell lines utilized in our lab, bilirubin did not have an adverse effect on the antineoplastic activity of cisplatin. Our findings suggest that HO-1, more specifically HO-1's products especially bilirubin, may protect the kidney from toxic nephropathy caused by cisplatin.

Table 2-1. Serum bilirubin concentrations were significantly higher for the Gunn j/j when compared to the other groups as noted by the *. No significant difference was noted between Gunn j/+ and Wistar rats but a trend toward higher bilirubin values was noted on D0.

| Means ± standard deviations serum bilirubin concentrations (mg/dL) in rats receiving cisplatin | | | |
|------------------------------------------------------------------------------------------------|----------|------------|------------|
| | Wistar | Gunn j/+ | Gunn j/j |
| Day 0 | 0 ± 0 | 0.41 ± 0.2 | 4.2 ± 0.9* |
| Day 3 | 0.07 ± 0 | 0.1 ± 0 | 7.5 ± 1.2* |
| Day 5 | 0 ± 0 | 0.12 ± 0 | 5.5 ± 1.1* |

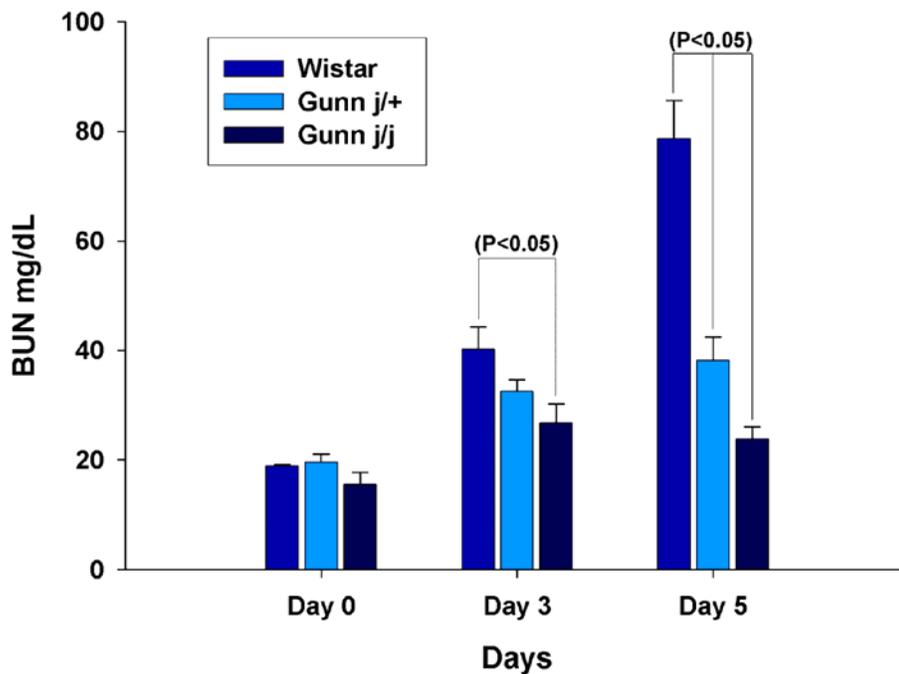


Figure 2-1. Serum BUN concentrations on D0, 3, and 5. No significant difference between any groups on D0. The most significant difference in BUN occurred when comparing the Wistar rat to both the Gunn j/j and Gunn j/+. The Wistar rats had a significantly higher BUN .

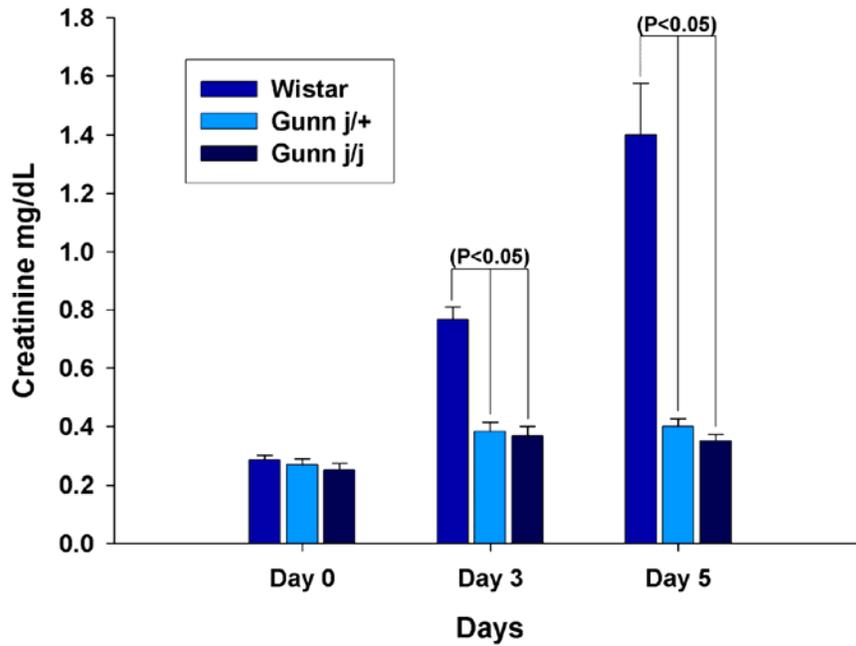


Figure 2-2. Serum creatinine concentrations on D0, 3, and 5. No significant difference between any groups on D0. The most significant difference in creatinine occurred when comparing the Wistar rat to both the Gunn j/j and Gunn j/+. The Wistar rats had a significantly creatinine.

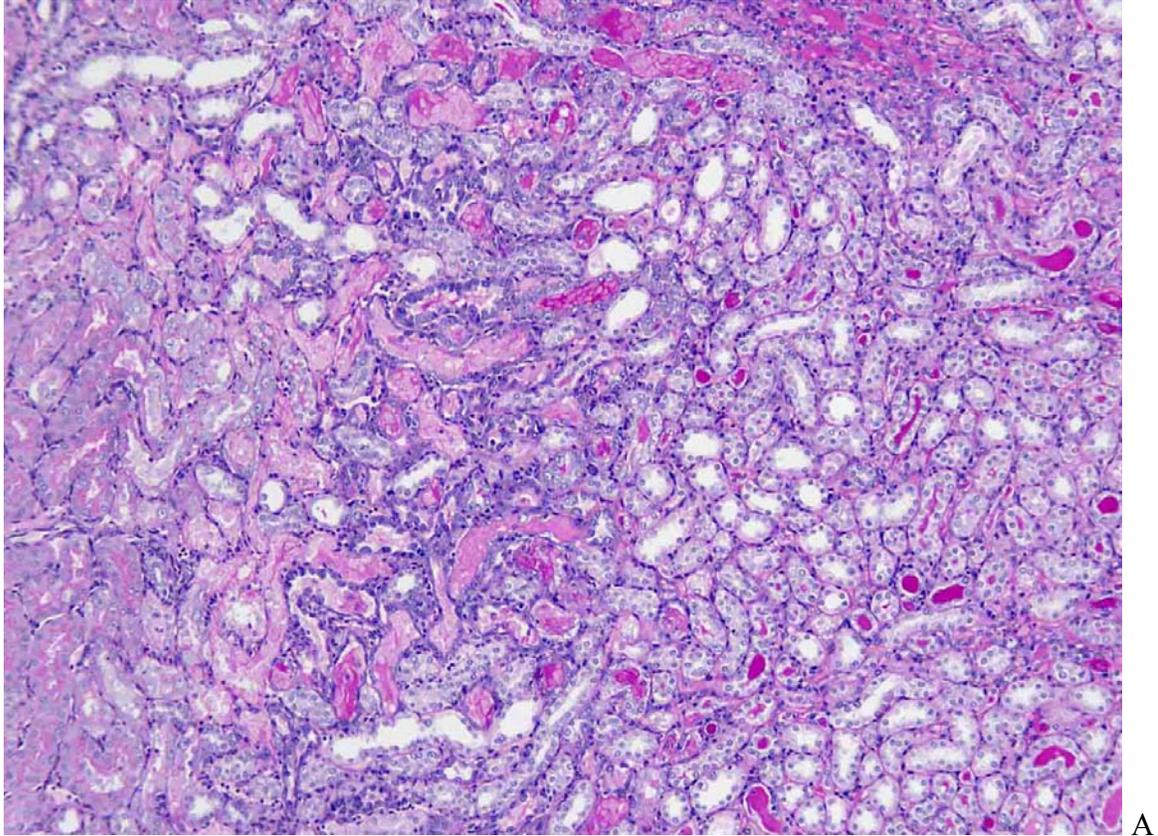
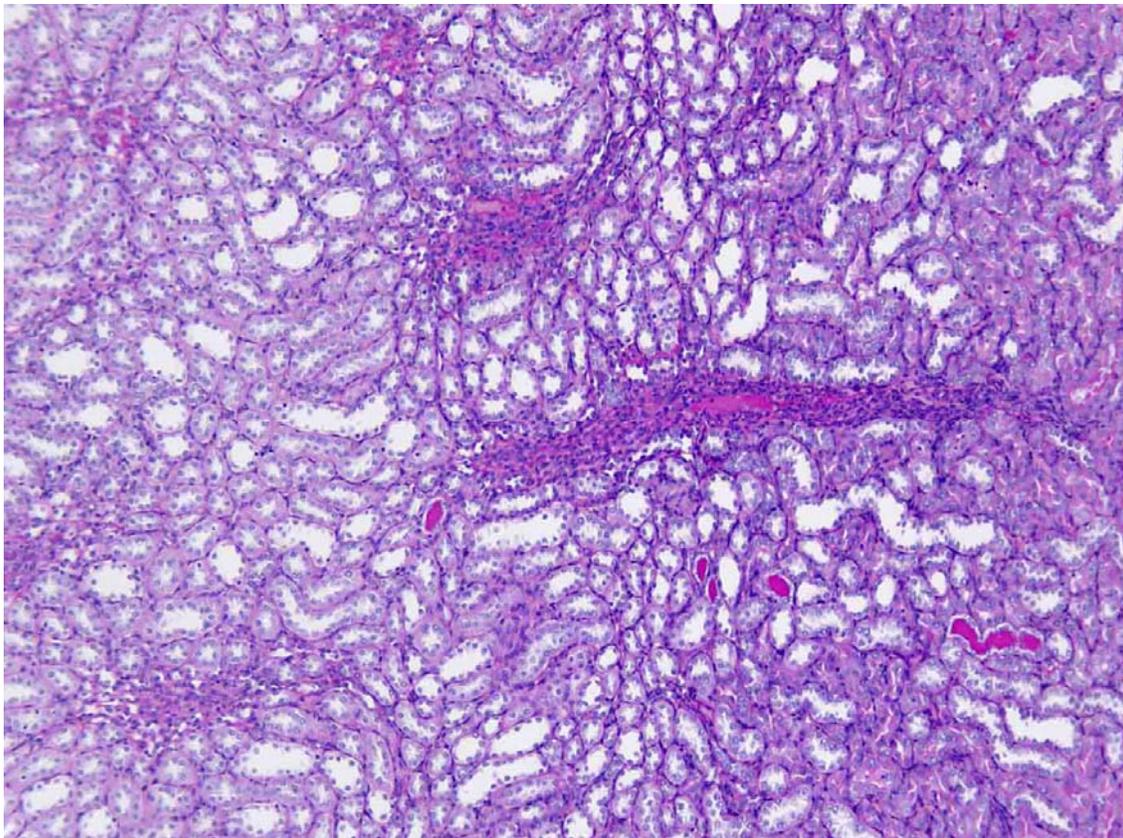


Figure 2-3..Histologic grading showed significant preservation of the OSOMPT in homozygous Gunn rats when compared to heterozygous Gunn rats and Wistar rats given cisplatin. Although functionally heterozygous Gunn rats were significantly different than the Wistar rat, histologically, there was no significant difference. A) Outer stripe of the outer medulla (center) with necrotic and sloughed tubular epithelial cells coupled with regeneration in the Wistar rats (PAS stain, 50X). B) This is compared to the remarkably well-preserved tubules at the junction between the inner and outer stripes of the outer medulla with occasional apoptotic and sloughed cells seen at a higher magnification in the homozygous Gunn rats (PAS stain, 50X).



B

Figure 2-3. continued

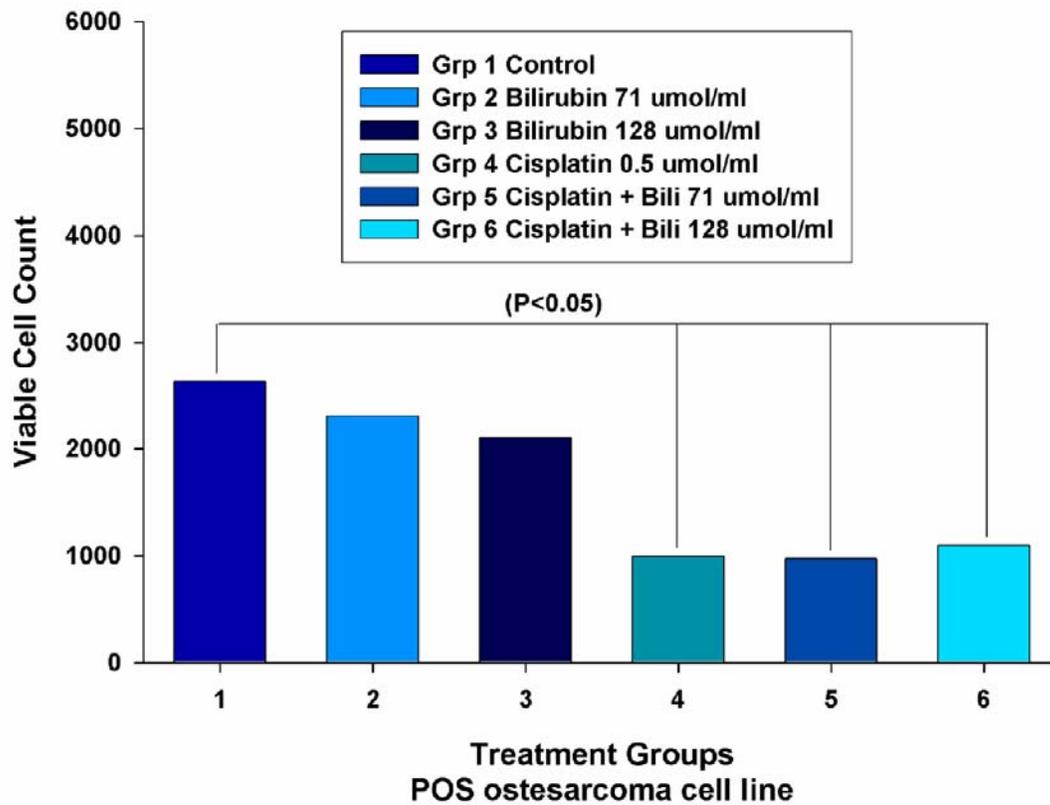


Figure 2-4 Viable cell count for one of the canine osteosarcoma cell lines utilized (POS). With this cell line, no significant difference was noted between cisplatin alone and either concentration of bilirubin alone. As with all cell lines tested, bilirubin had no significant difference on cisplatin's anti-neoplastic activity in culture.

CHAPTER 3 CONCLUSION

The current lifetime risk for a human developing cancer in the United States is approximately 40%. Additionally, our pets are living longer lives making them more prone to developing cancer also. One of the most powerful treatments for cancer in any species is chemotherapy but these agents have countless adverse side effects. Specifically, cisplatin is a chemotherapeutic used in humans and animals to treat a variety of malignancies. The most clinically significant side effect associated with cisplatin is nephrotoxicity.

Chapter 1 discussed the pharmacokinetics, mechanism of anti-tumor action, toxicities (especially nephrotoxicity), current protective measures against nephrotoxicity, and the use of cisplatin in animals. Many theories have been developed regarding the mechanism of cisplatin nephrotoxicity but none have proven to be the sole, underlying cause for this toxicity. Perhaps this is why finding a compound to ameliorate cisplatin nephrotoxicity has been so difficult. Numerous studies have shown that certain compounds and enzyme alterations can protect against cisplatin nephrotoxicity but not without disadvantages of their own, including worsened side effects of the compound, expense, or difficulty in administering the compound or enzyme. While recent studies about HO regarding protection of various organ systems have been performed, no studies utilizing the end-product BR to protect against cisplatin nephrotoxicity exist.

Chapter 2 of this thesis describes an experiment designed to investigate if sustained unconjugated hyperbilirubinemia in the Gunn rat would protect against cisplatin nephrotoxicity. An experiment was also performed to determine if BR had any effect on the ability of cisplatin to kill canine osteosarcoma cell in culture. Homozygous Gunn rats lack the enzyme UDPGT necessary to conjugate bilirubin. Heterozygous Gunn rats lack normal levels of UDPGT but can

still conjugate bilirubin to a certain extent. The homozygous Gunn rats were utilized to achieve a state of unconjugated hyperbilirubinemia. The congenic Wistar rats were the positive control with normal levels of UDPGT. Cisplatin was administered to all rats and functional renal parameters were monitored for 5 days. On day 5, kidneys were harvested for histological evaluation. To ensure that multiple anesthetic episodes had no negative impact on kidney function and histology, sham rats from each group also underwent the same procedures except they did not receive cisplatin.

Cell culture studies were then performed in 4 canine osteosarcoma cell lines using the average concentrations of BR for homozygous Gunn rats at day 0 and 3. BR was added to all cell lines alone and with cisplatin and cell viability was assessed using the CellTiter Blue™ assay.

Results of this study demonstrate complete functional and partial histologic nephroprotection with the homozygous Gunn rats when compared to the heterozygous Gunn rats and Wistar rats. While the heterozygous Gunn rats lacked histologic nephroprotection from cisplatin, they had improved functional parameters when compared to the Wistar rats. The sham rats showed no significant difference in their functional or histologic parameters when compared to one another. This suggests that multiple anesthetic episodes had no negative impact on renal function or histology.

With regard to bilirubin's effect on cisplatin in cell culture, there was no significant difference in the cell viability of any of the four canine osteosarcoma cell lines when bilirubin was added with cisplatin.

Cisplatin nephrotoxicity has provided a difficult obstacle to administering the drug safely to chemotherapy patients of all species. HO and its products have proven to be beneficial in protecting a variety of organ systems from assorted insults. The results obtained from this thesis

have provided directions for future applications regarding the products of HO-1 in cisplatin nephrotoxicity. Future studies using a rodent model with conjugated hyperbilirubinemia and administering exogenous BR are warranted. Potentially, a canine and human model could be developed in the future. Additionally, the administration of BR and CO together may provide complete functional and histological nephroprotection from cisplatin.

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BIOGRAPHICAL SKETCH

Karri Barabas began her collegiate education at the University of Florida in 1996 and received her Bachelor of Science in animal science with highest honors in May 2000. She was accepted to the University of Florida College of Veterinary Medicine and graduated Magna Cum Laude with a Doctor of Veterinary Medicine in May 2003. Karri completed a one-year rotating small animal medicine and surgical internship at Texas A&M University College of Veterinary Medicine immediately after graduation. She then completed an oncology internship at Regional Veterinary Referral Center in Springfield, Virginia after finishing at Texas A&M and returned to the University of Florida to pursue a combined Master of Science and small animal medical oncology residency at the College of Veterinary Medicine. Karri will complete her oncology residency in July 2009.