Hyperbilirubinemia's protective effect against cisplatin nephrotoxicity in the Gunn rat

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Gunn rats, deficient in the enzyme uridine diphosphate glucuronyl transferase, were used to investigate the effects of unconjugated hyperbilirubinemia in cisplatin nephrotoxicity. The effect of bilirubin on the antineoplastic activity of cisplatin in osteosarcoma cell lines was also determined. The *in vivo* model involved three groups of rats ($n=6$ rats/group): homozygous Gunn rats (j/j), heterozygous Gunn rats (j/+), and congenic Wistar rats. On day 0, all rats were given 4 mg/kg cisplatin intraperitoneally. Blood was sampled on days 0, 3, and 5 for bilirubin, BUN, and creatinine and kidneys were taken on day 5. Cell culture was performed in four canine osteosarcoma cell lines using the average concentrations of bilirubin for homozygous Gunn rats at day 0 and 3. Bilirubin was added to cell lines alone and with cisplatin. Cell viability was assessed using the CellTiter Blue assay. Serum bilirubin levels were highly elevated in Gunn j/j, moderately elevated in Gunn j/+, and undetectable in Wistar rats at day 0. Bilirubin provided a nephroprotective effect, with significantly lower BUN and creatinine in Gunn j/j when compared with Wistar rats at day 5. Histological grading demonstrated preservation of the S3 segment in Gunn j/j when compared with Wistar rats ($P<0.05$). Bilirubin had no significant effect on the antineoplastic effect of cisplatin at either concentration in the four cell lines ($P<0.001$). Hyperbilirubinemia in the Gunn rat provided marked preservation of renal function and histology in a cisplatin nephrotoxicity model. Exogenous bilirubin did not interfere with the antineoplastic activity of cisplatin *in vitro*. *Anti-Cancer Drugs* 19:495–502 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Cisplatin is one of the most commonly used antineoplastic agents in human patients. It 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 lung, and esophageal cancers. Cisplatin is also used as a rescue agent in the treatment of other solid tumors [1–7].

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 [8–12]. One theory is that reactive oxygen species and mitochondria play a role in the apoptotic cascade involved in cisplatin nephrotoxicity [13]. 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 [14].

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 prooxidant heme molecule into equimolar quantities of biliverdin, carbon monoxide (CO), and iron [15]. Biliverdin is converted to unconjugated bilirubin (BR) through biliverdin reductase [16]. Unconjugated BR is then converted to conjugated BR by the hepatic microsomal enzyme, uridine diphosphate glucuronyl transferase (UDPGT) [17]. These molecules of heme degradation were once considered to be toxic metabolites, but have recently been shown to have dose-dependent vasodilatory, antioxidant, and anti-inflammatory properties that may be useful in protection of various organ systems from toxic insult [18].

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 [19]. With regard to cisplatin-mediated nephrotoxicity, depletion of the HO-1 enzyme resulted in more

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significant renal failure and renal injury in one study [20] and the administration of CO along with cisplatin ameliorated signs of renal failure in another study [21]. The objectives of this study were to investigate the protective effect of hyperbilirubinemia *in vivo* in the rat model of cisplatin-induced nephrotoxicity. Hyperbilirubinemia *in vivo* was achieved using both the homozygous and heterozygous Gunn rats. The homozygous Gunn rat is unable to induce UDPGT as a result of an autosomal recessive deficiency in UDPGT [22,23]. The lack of induction of UDPGT results from an alteration in the coding region of the mRNA that results in an instability of the mRNA and a synthesis of a truncated, functionally inactive UDPGT [23]. In addition, an intermediate level of BR can be obtained using heterozygous Gunn rats that have varying degrees of functional UDPGT. We also investigated the effect of BR on the antineoplastic activity of cisplatin using four established canine osteosarcoma cell lines (POS, HMPOS, COS31, and 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, and heterozygous Gunn rats weighing 200–400 g were purchased (Harlan Sprague Dawley Inc., Indianapolis, Indiana, USA) and maintained in a temperature-controlled room with alternating 12 h light/12 h 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: (i) Wistar, (ii) homozygous Gunn, and (iii) heterozygous Gunn. Rats were weighed and observed daily 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. Before blood sampling on days 0 and 3, the rat's tail was soaked in 40–42°C water for 3–5 min 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 (i.p.) injection of cisplatin (American Pharmaceutical Partners Inc., Schaumburg, Illinois, USA) at 4 mg/kg. Once the i.p. 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 subcutaneously for pain associated with the i.p. injection. Blood was sampled on days 0, 3, and 5 for evaluation of BUN, serum creatinine, and serum BR 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, Iowa, USA).

Three groups of male rats ($n = 4$ rats/group) were used as sham control rats. The groups consisted of (i) Wistar, (ii) homozygous Gunn, and (iii) heterozygous Gunn rats. These groups were treated as described above for the cisplatin-induced acute renal failure groups, but instead of receiving 4-mg/kg cisplatin, the rats received the equivalent amount of 0.9% sodium chloride i.p.

Assays

BUN and serum BR concentrations were determined using an automated chemistry analyzer (Hitachi 911 Chemistry Analyzer, Hitachi, Boehringer Mannheim, Indianapolis, Indiana, USA). Serum creatinine (Cr) concentrations were determined using a dry chemistry analyzer (Johnson & Johnson Vitros DT6011, Ortho-Clinical Diagnostics, Rochester, New York, USA) to prevent the false color artifacts due to hyperbilirubinemia seen with the standard Jaffe methodology for measurement of Cr [24].

Histological grading

Both kidneys were placed in 10% buffered formalin for at least 24 h before processing. Transverse sections of the left kidney for all rats were processed using hematoxylin and eosin staining and periodic-acid-Schiff staining. Histological examination was performed by a renal pathologist who was blinded with respect to the treatment groups. Renal tissue was divided into four 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. Renal injury was graded in seven different categories: normal, cellular swelling/vacuolization, brush border loss, nuclear condensation, karyolysis/apoptosis/necrosis (most severe form of injury), regeneration, and peritubular capillary injury. Apoptosis was defined as cells with dense, eosinophilic, shrunken cytoplasm with nuclei showing condensed chromatin at the nuclear periphery with or without fragmentation. Necrosis was identified as cells with swollen cytoplasm and karyolytic nuclei or other nuclear changes with homogeneously shrunken, wrinkled nuclei, or karyorrhexis. Nuclear condensation was defined as pyknoses with intact cell cytoplasm (neither shrunken nor swollen). Regeneration was defined as tubules without brush border on low or flattened epithelial cells with large, activated nuclei and nucleoli. Peritubular capillary changes were defined as margination of leukocytes with or without intact or activated endothelium. Each feature was assigned a semiquantitative score in

CPT, proximal tubules of outer stripe of outer medulla, medullary thick ascending limb of inner stripe of outer medulla and collecting duct of inner medulla. These criteria have previously been used by us [25,26].

Each category was assigned a numerical score: 0 = none, 1 = $\leq$ 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 commonly treated with cisplatin. Therefore, four canine osteosarcoma cell lines, which have been well characterized in our laboratory, were used in this study. The POS (parent osteosarcoma) cell line was originally developed from a primary osteosarcoma affecting the left proximal femur of a 1.5-year-old male mongrel dog (Dr Tsuyoshi Kadosawa, University of Sapporo, Japan) [27]. The HMPOS (highly metastatic parent osteosarcoma) cell line is a pulmonary metastatic derivative of POS cell line (Dr Tsuyoshi Kadosawa, University of Sapporo, Japan) [28]. D17 is another established canine osteosarcoma cell line (American Type Tissue Culture Collection, Manassas, Virginia, USA). The COS31 cell line was established from a dog with spontaneously occurring osteosarcoma (Dr Ahmed Shoieb, University of Tennessee, College of Veterinary Medicine, Knoxville, Tennessee, USA). Cells were cultured at 37°C under 5% CO_2 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, nonessential 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 phosphate-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, Wisconsin, USA). 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% CO_2 and 95% room air for 24 h. The IC_{50} for each cell line with cisplatin was determined in pilot studies and were as follows: 0.5 µmol/l for POS, 1 µmol/l for HMPOS, 5 µmol/l for COS31, and 20 µmol/l for D17. All cells were treated with 50 µl of BR at concentrations of 71 and 128 µmol/l alone and combined with each cell type's IC_{50} concentration of cisplatin. BR was dissolved in the respective media to achieve appropriate concentrations. The micromolar concentrations of BR used in this study were equivalent to the average serum BR levels on days 0 and 3 that provided functional nephroprotection of the homozygous Gunn rats receiving cisplatin. After incubation for 72 h under the conditions described previously, 20 µl 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. Plates were placed on a low-speed shaker for 10 s and then incubated for 4 h. 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, Illinois, USA). Data were 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 nonparametric data. Differences between groups were identified using (*post hoc*) pairwise multiple comparison procedures (Holm–Sidak method or Dunn's method). Parametric data are reported as mean ± SD and nonparametric data as median with an interquartile range (25–75%). *P* value of less than 0.05 was considered significant.

Results

Body weights

The homozygous and heterozygous Gunn rats had a lower mean body weight than the Wistar rats throughout all days during the experiment. The mean weight for rats receiving cisplatin on day 0 for Wistar rats was 304 ± 1.2 g, for the homozygous Gunn rat was 216 ± 1.2 g, and for the heterozygous Gunn rat was 249 ± 1.2 g. A significant difference was observed in the body weights between days 0 and 5 for the Wistar rats and heterozygous Gunn rats receiving cisplatin. Both of these groups of rats lost weight by day 5 (mean Wistar weight 288 ± 1.2 g, mean heterozygous Gunn weight 236 ± 1.2 g). The homozygous Gunn rats, however, maintained their mean body weight by day 5 at 216 ± 1.2 g.

Renal functional parameters

The homozygous and heterozygous Gunn rats were protected from the nephrotoxic effects of cisplatin based on functional kidney parameters. BR levels were significantly higher for all days in the homozygous Gunn rats when compared with the heterozygous Gunn rat and the Wistar rat (Table 1). Statistically, BR levels were not significantly higher in the heterozygous Gunn rat compared with the Wistar rat at any day. BR levels were, however, generally undetectable in the Wistar rats, whereas the heterozygous Gunn rats had a mean of

0.4–0.1 mg/dl, suggesting that a mild degree of hyperbilirubinemia existed in the heterozygous Gunn rats.

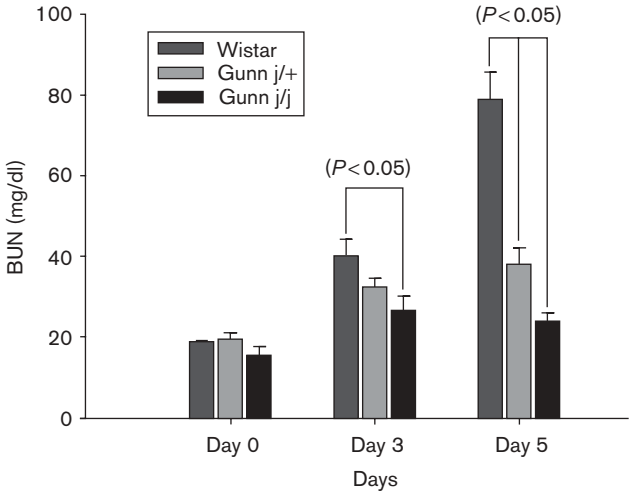
No significant difference was observed in BUN or Cr on day 0 between any of the groups of rats given cisplatin (Fig. 1a and b) or controls (Fig. 2a and b). Importantly, in the face of a nephrotoxic dose of cisplatin, the homozygous Gunn rat was protected compared with the heterozygous Gunn rat and the Wistar rat (Fig. 1) and although BUN and Cr values increased slightly in homozygous rats they remained within the normal range. Some protection was found in the heterozygous Gunn rat compared with the Wistar rat, but 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 days 3 and 5 with regard to Cr.

When comparing the homozygous Gunn rat to the heterozygous Gunn rat there was no significant difference in BUN on day 3 and Cr on days 3 and 5 (Fig. 1a and b). 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.

Table 1 **Mean ± standard deviations serum bilirubin concentrations (mg/dl) in rats receiving cisplatin**

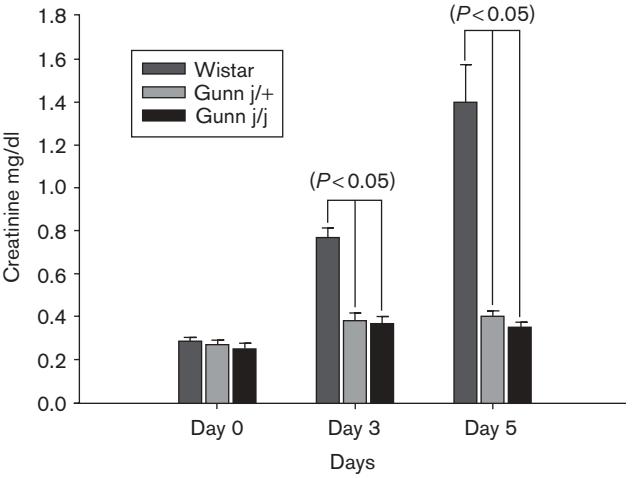
	Wistar	Gunn j/+	Gunn j/j
Day 0	0 ± 0	0.41 ± 0.2	4.2 ± 0.9
Day 3	0.07 ± 0	0.1 ± 0	7.5 ± 1.2
Day 5	0 ± 0	0.12 ± 0	5.5 ± 1.1

Fig. 1



Serum BUN concentrations on days 0, 3, and 5. No significant difference between any groups on day 0. The most significant difference in BUN occurred when comparing the Wistar rat to both the Gunn j/j and Gunn j/+ on day 5. The Wistar rats had a significantly higher BUN.

Fig. 2



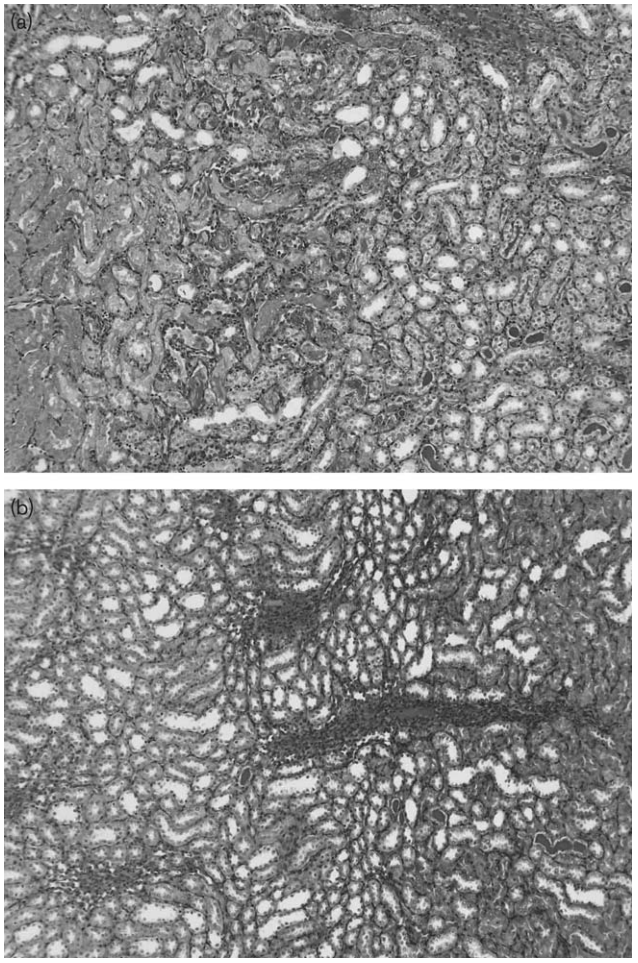
Serum creatinine concentrations on days 0, 3, and 5. No significant differences were found between any groups on day 0. The most significant difference in creatinine occurred when comparing the Wistar rat to both the Gunn j/j and Gunn j/+ on days 3 and 5. The Wistar rats had a significantly elevated creatinine.

When sham rats (homozygous Gunn, heterozygous Gunn, and Wistar rats) were compared with the respective group receiving cisplatin, there were no significant differences in BUN or Cr on day 0 (Figs 1 and 2). Significant differences were found between sham Wistars and Wistars given cisplatin in BUN and Cr for days 3 and 5. Between the sham groups, there were no statistically significant differences in BUN and Cr at days 0, 3, or 5. Importantly, there was also no difference between the homozygous Gunn rat receiving cisplatin and any of the sham groups in BUN and Cr at days 0, 3, or 5.

Light microscopy

For all treatment groups, the CPT and collecting ducts were graded as 75–100% of cells being normal. The area of most interest in regard to cisplatin nephrotoxicity is the S3 segment of the OSOMPT. Cisplatin-treated homozygous Gunn rats showed significantly decreased karyolysis/apoptosis/necrosis than the heterozygous Gunn rats and the Wistar rats in the OSOMPT (Fig. 3a and b). No significant difference in karyolysis/apoptosis/necrosis between heterozygous Gunn rats and Wistar rats in the OSOMPT was observed. 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 (Table 2).

Fig. 3



Renal histology of rats treated with cisplatin. Histological grading showed significant preservation of the outer stripe of the outer medullary proximal tubule in homozygous Gunn rats when compared with heterozygous Gunn and Wistar rats given cisplatin (Table 2). (a) Note the outer stripe of the outer medulla (left and center) with necrotic and sloughed tubular epithelial cells coupled with regeneration in the Wistar rats. ISOM (right side of field) is better preserved with protein casts in tubular lumens [periodic-acid-Schiff (PAS) stain, 50 ×]. (b) This is compared with the remarkably well-preserved tubules at the junction between the inner and outer stripes of the outer medulla (center of the field) with occasional apoptotic and sloughed cells which can only be seen well at a higher magnification in the homozygous Gunn rats (PAS stain, 50 ×).

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 histological 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 with the sham groups. In addition, significantly more cellular swelling, brush border loss, and regeneration in the OSOMPT in the homozygous Gunn rats receiving cisplatin versus the sham groups was observed. A similar difference was 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, BR had mild cytotoxic effect on COS31 cells at 72 and 128 μmol/l and D17 cells at 128 μmol/l versus control cells. No significant cytotoxic effects of BR were seen with HMPOS and POS cells at either concentration or D17 cells at 72 μmol/l. BR had no significant effect on the antineoplastic effect of cisplatin at either concentration in any of the four canine osteosarcoma cell lines (Fig. 4).

Discussion

Although the toxic properties of BR are well documented, particularly kernicterus and neonatal hyperbilirubinemia, the therapeutic properties of BR are just recently being discovered [29–32]. This study suggests that the hyperbilirubinemia in the Gunn rat has a nephroprotective effect when the nephrotoxic, antineoplastic agent cisplatin was administered, as evidenced by the maintenance of normal renal function and markedly reduced tubular necrosis.

Protective effects of BR in regard to cell injury have been studied previously but never in the cisplatin model of nephrotoxicity. Leung *et al.* [19] showed that hyperbilirubinemia produced by ligation of the common bile duct effectively protected against glycerol-induced acute renal failure in the rat. Our laboratory has also reported protection from ischemia-reperfusion injury with exogenous BR when delivered into the isolated, perfused rat kidney [25]. We subsequently reported that protection was, however, not seen with exogenous BR in ischemia-reperfusion injury *in vivo* [18]. This may mean that the protective effects of BR only occur in specific, nephrotoxic models or perhaps the dose of exogenous BR required to protect the kidney from various insults is variable. BR has also been shown to exert a protective effect with other organ systems such as the liver [33,34], intestine [35], and neural tissue [36]. In fact, clinically, the incidence of coronary and ischemic heart disease is lower in humans with hyperbilirubinemia [37,38].

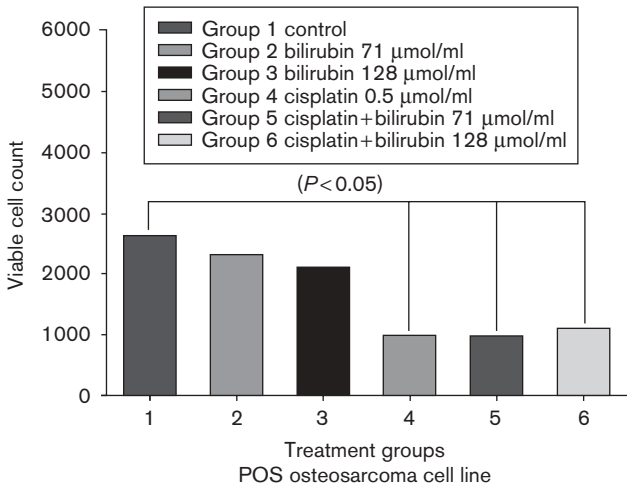
BR is a product of HO-1 and the protective effects of HO-1 induction have been well established in multiple organ systems [19,33,39–41]. Shiraishi *et al.* [20] demonstrated that mice deficient in HO-1 (−/−) developed more severe renal failure and renal injury than wild-type mice (+/+) when cisplatin was administered. This prompted investigation into the individual agents produced by HO-1 to determine if BR or CO alone could mimic this effect. A study by Tayem *et al.* [21] showed

Table 2 Histological scores

Tissue region	Type of rats	Cell swelling	Brush border loss	Nuclear condensation	Karyolysis apoptosis necrosis	Regeneration	Capillaritis	Normal
CPT	Wistar	0	0	0	0	0	0	5 (5.5)
	j/+	0	0	0	0	0	0	5 (5.5)
	j/j	0	0	0	0	0	0	5 (5.5)
OSOMPT	Wistar	3 (0.3)	4 (4.5)	0	3.5 (3.5)	0 (0.1)	0.5 (0.2)	0 (0.0)
	j/+	2.5 (2.5)	3.5 (2.4)	0	2.5 (2.4)	0	1 (0.2)	0 (0.2)
	j/j	3.5 (1.4)	4 (3.4)	0	1 (1.1)	1 (1.1)	0	1 (0.3)
ISOMmTAL	Wistar	4 (3.5)	0	0	0.5 (0.1)	1 (0.1)	0	1.5 (0.4)
	j/+	0 (0.2)	0	0	0	0	0	5 (4.5)
	j/j	0 (0.0)	0	0	0	0.5 (0.1)	0	5 (5.5)
CD	Wistar	0	0	0	0	0	0	5 (5.5)
	j/+	0	0	0	0	0.5 (0.1)	0	5 (5.5)
	j/j	0	0	0	0	0	0	5 (5.5)

Medians with interquartile range (25, 75%) after cisplatin administration in Wistar, heterozygous Gunn rats (j/+), and homozygous Gunn rats (j/j).
CD, collecting ducts; CPT, cortical proximal tubule; ISOMmTAL, medullary thick limb in inner stripe; OSOMPT, S3 segment of outer stripe of outer medullary proximal tubule.
Numerical score based on the percentage of cells in each region displaying the described injury: 0=none, 1= $\leq$ 10%, 2=10–25%, 3=25–50%, 4=50–75%, 5=75–100%. Cellular damage was most evident in the OSOMPT with all groups.

Fig. 4



Viable cell count for one of the canine osteosarcoma cell lines used (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 antineoplastic activity in culture.

that treatment with a water-soluble carbon monoxide-releasing molecule protected the kidney function and improved histology of rats treated with cisplatin. In a recent study reviewing the effects of conjugated BR and cisplatin on human renal proximal tubular epithelial cells *in vitro*, it was demonstrated that conjugated BR significantly decreased the cisplatin sensitivity of the cells [42]. The IC_{50} values (mean ± SD) were reported as 9.5 ± 1.2 µmol/l for cisplatin alone and 39.4 ± 9.4 µmol/l for cisplatin with conjugated BR suggesting BR has a cytoprotective effect for renal proximal tubular epithelial cells exposed to cisplatin *in vitro* [42]. The results of our study indicate that hyperbilirubinemia in the homozygous Gunn rat was markedly nephroprotective with regard to functional and histological measures in the cisplatin-treated rat. Partial protection of renal functional parameters were also provided to the heterozygous Gunn rat, whose BR levels were not statistically higher than the Wistar rat at any day, although histologically there was no difference in the amount of damage to the S3 segment. An improvement in functional kidney parameters is nevertheless important and a trend was present at day 0 for higher BR levels in the heterozygous Gunn rat than the Wistar rat. This suggests that the protective effects of BR may be exerted on the day the nephrotoxin is administered (day 0).

The exact concentration of BR in serum needed to prevent severe nephrotoxicity due to cisplatin remains in question. Higher average serum values present in the homozygous Gunn rat were associated with significantly less kidney injury than in the heterozygous Gunn rat suggesting that the value of BR required for any histological protection may lie somewhere in between those two groups. Most likely, individual variation in serum BR 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 BR but it may still be present in small amounts in these rats.

Other situations where the Gunn rat is protected from kidney damage are found. Nath *et al.* reported that in the chronic angiotensin infusion model where NADPH-dependent superoxide production contributes to the hypertension and fall in renal function; the Gunn rat was protected compared with Wistar rats [43]. In a nephrotoxic model produced by the agricultural fungicide *N*-(3,5-dichlorophenyl) succinimide Rankin *et al.* [44] also reported considerable functional and structural protection from acute injury. In both cases, the antioxidant properties of the elevated BR levels were implicated in

the protection from injury. The Gunn rat, however, also develops BR deposition in the renal papilla and eventual papillary necrosis and a renal concentrating defect [45,46]. Furthermore, the Gunn rat shows an enhanced susceptibility to nonsteroidal analgesic-induced papillary necrosis [47]. Thus, the presence of hyperbilirubinemia does not confer protection against all forms of renal injury.

Another aim of our study was to determine if adding cisplatin with BR would have an additive, inhibitory, or no effect on neoplastic cells in culture. For this part of the experiment, we used four different canine osteosarcoma cell lines for completeness. We found that cisplatin with BR had no effect on cisplatin's antineoplastic activity in cell culture in any of the four cell lines. This is a very important aspect in terms of clinical application. Although nephroprotection is a pertinent area of research, if these agents that protect the kidneys also decrease cisplatin's ability to kill neoplastic cells then those agents have no clinical use. It may be necessary to assess this effect in other neoplastic cell populations and in an in-vivo setting before any definitive conclusions can be drawn. Although it was noted that two of the cell lines showed some mild cytotoxicity caused by BR alone (COS31 at 72 and 128 μmol/l and D17 at 128 μmol/l), these effects were minimal and had no additional impact on the cells after cisplatin was added. BR has been shown to have some cytotoxic effects on human colon adenocarcinoma cells and human carcinoma cell lines [48,49]. This cytotoxicity effect may only pertain to certain types of neoplasia as evidenced by our cell culture results.

Future studies would be helpful in determining if the same protection against cisplatin nephrotoxicity can be obtained by exogenous administration of BR and if so, at what plasma concentration. It may be possible to deliver a dose high enough to exceed the serum concentrations seen in the homozygous Gunn rat and result in complete histological protection of the S3 segment of the outer medulla. Another avenue could include concurrent administration of exogenous BR and the water-soluble carbon monoxide releasing molecule to determine if the combination could completely prevent any histological nephrotoxicity caused by cisplatin.

In conclusion, hyperbilirubinemia present in the homozygous Gunn rat is associated with marked renal functional and structural protection in cisplatin-treated rats. Only mild functional nephroprotection was seen in heterozygous Gunn rats that did not demonstrate significant hyperbilirubinemia when compared with the control Wistar rats. This suggests that the hyperbilirubinemia of the Gunn rat plays a role in the renal protection in this model. In the four canine osteosarcoma cell lines used in our lab, cisplatin with BR had no effect on the antineoplastic activity of cisplatin. Our findings suggest that HO-1, more specifically HO-1's products especially BR, may protect the kidney from toxic nephropathy caused by cisplatin.

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