Magnesium Protects Against Cisplatin-Induced Acute Kidney Injury Without Compromising Cisplatin-Mediated Killing of an Ovarian Tumor Xenograft in Mice

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Running Head: Mg Regulates Cisplatin-Induced AKI in Tumor-Bearing Mice

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Abstract

Cisplatin, a commonly used chemotherapeutic for ovarian and other cancers, leads to hypomagnesemia in most patients and causes acute kidney injury (AKI) in 25-30% of patients. Previously, we showed that magnesium deficiency worsens cisplatin-induced AKI and magnesium replacement during cisplatin treatment protects against cisplatin-mediated AKI in non-tumor-bearing mice. This study investigates the role of magnesium in cisplatin-induced AKI using a human ovarian tumor (A2780) xenograft model in mice and the effect of magnesium status on tumor growth and the chemotherapeutic efficacy of cisplatin in vivo. Tumor progression was unaffected by magnesium status in saline-treated mice. Cisplatin treatment reduced tumor growth in all mice, irrespective of magnesium status. In fact, cisplatin-treated magnesium-supplemented mice had reduced tumor growth after 3 weeks compared to cisplatin-treated controls. While Mg status did not interfere with tumor killing by cisplatin, it significantly affected renal function following cisplatin. Cisplatin-induced AKI was enhanced by magnesium deficiency, as evidenced by increased blood urea nitrogen (BUN), creatinine and other markers of renal damage. This was accompanied by reduced renal mRNA expression of the cisplatin efflux transporter Abcc6. These effects were significantly reversed by magnesium replacement. On the contrary, magnesium status did not affect the mRNA expression of cisplatin uptake or efflux transporters by the tumors in vivo. Finally, magnesium deficiency enhanced platinum accumulation in the kidneys and renal epithelial cells, but not in the A2780 tumor cells. These findings demonstrate the renoprotective role of magnesium during cisplatin AKI, without compromising the chemotherapeutic efficacy of cisplatin in an ovarian tumor-bearing mouse model.

Keywords: hypomagnesemia, nephrotoxicity, renal protection
Introduction

The Food and Drug Administration (FDA) approved cisplatin or cis-diamineplatinum (II) dichloride for the treatment of both testicular and ovarian cancer in 1978. Today, cisplatin is commonly used to treat numerous cancers including, bladder, lung, and head and neck cancers, as well as testicular and ovarian cancers (6,21,28,34). Ovarian cancer is the fifth leading cause of cancer death among women in US (4) and the most lethal gynecologic malignancy (3). Each year, approximately 20,000 new cases of ovarian cancer are identified and in 2011, there were more than 14,000 deaths from ovarian cancer (4). Today, ovarian cancer is commonly treated with a combination of surgery, radiation and chemotherapy with cisplatin or carboplatin and paclitaxel (induction therapy) depending on the stage and grade of the tumor (25,26).

Despite its success as an antineoplastic agent, cisplatin causes dose-limiting nephrotoxicity in approximately one third of patients (21,28,34). Females are more likely to experience cisplatin-induced acute kidney injury (AKI) than males (11,21,34). Cisplatin invariably causes hypomagnesemia in approximately 90% of treated patients, which may persist even after treatment discontinuation (17). Hypomagnesemia has been linked to the nephrotoxic effects of cisplatin (17).

As an essential nutrient, magnesium (Mg) is required in our daily diet. However, less than 50% of the population in the US consumes the recommended amount (300-400 mg/day) of magnesium (13,32). Mg is required for various critical cellular processes and enzymatic reactions; it is the co-factor required for ATP activity and is critical for cell growth and the synthesis of DNA, RNA, and protein, as well as mitochondrial respiration (1,23,31). Hence, Mg has been proposed to play a role in tumor
growth and metastasis (38). Recently, we reported that Mg deficiency enhances renal damage mediated
by cisplatin in a non-tumor-bearing mouse model of cisplatin-induced AKI and that Mg replacement
after deficiency offers significant renoprotection (35). In the same study, we showed that Mg
supplementation protected against cisplatin-AKI by regulating renal platinum accumulation with
changes in cisplatin efflux transporter expression by the kidneys. These observations are consistent
with recent retrospective and prospective clinical studies showing the renoprotective effects of Mg
supplementation in cancer patients taking cisplatin (2,16,22,37,43). Together, these studies clearly
support maintaining Mg homeostasis during cisplatin treatment. However, none of the studies
published to date have examined whether Mg status modulates tumor growth and/or tumor killing by
cisplatin in vivo. In this study, we extended our initial studies to investigate the role of host Mg status
on cisplatin’s chemotherapeutic efficacy using a human ovarian tumor xenograft model and cisplatin-
induced AKI in mice.
Methods

Animals and cell lines.

All animal studies were submitted to and approved by The Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research (IACUC #2012-009).

*Foxn1nu/Foxn1nu* female athymic nude mice (Harlan, Indianapolis, IN, USA) were acclimatized under normal environmental conditions and allowed free access to standard chow and water for 1 wk before experimentation. The LLC-PK₁ porcine renal epithelial cell line was purchased from ATCC (Manassas, VA, USA). The A2780 human ovarian carcinoma cell line was obtained from T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA, USA).

Model of ovarian tumor xenograft combined with cisplatin-induced AKI.

Female athymic nude mice (*n* = 7-8 per group, 6-8 wks old) were randomized to receive either (a) control diet (normal chow containing 100% of the recommended Mg) or (b) a magnesium-deficient (MgD) diet (containing 10% of the recommended amount of Mg (prepared by Teklad/Harlan, Madison, WI, USA) on day 1, to produce mild-moderate Mg deficiency as previously described (35). A2780 human ovarian carcinoma cells, grown in RPMI 1640 media containing 10% fetal bovine serum (FBS), penicillin (P), streptomycin (S), and glutamine (Q), were washed, resuspended (5x10⁶ cells in 100µl saline), and injected subcutaneously (s.c.) in the right flank on day 2, as previously described (27). Saline or cisplatin (10mg/kg, i.p.) was injected on days 12, 19 and 21 before euthanasia on day 22. In addition, one group of mice (*n* = 8, ‘Mg replacement or supplemented’ group) received the MgD diet starting on day 1 through day 15 followed by the control (100%Mg) diet along with 0.3%MgCl₂ (w/v) in their drinking water until day 22. This group also received MgSO₄ (100mg/kg/day, s.c.) twice daily from day 19 (after 2nd dose of cisplatin) until euthanasia by CO₂
asphyxiation/exsanguination on day 22. All mice were weighed during the entire study and tumor sizes/volumes were recorded on days 13, 15, 19, 21 and 22. This model is similar to Pabla et al (27), except (1) the duration between cisplatin treatments was shorter in our model and mice were euthanized 24 hours after the last dose of cisplatin (compared to weekly cisplatin treatment for 4 weeks, with euthanasia 7 days post-cisplatin treatment used by (27)) and (2) Mg status was assessed in our model. As predicted from our previous study (35), consumption of a Mg-deficient diet (MgD) by tumor-bearing athymic nude mice for 22 days reduced plasma Mg levels by 30% in the presence of cisplatin (cisplatin: 1.58 ± 0.14 mg/dL vs. MgD+cisplatin: 1.1 ± 0.05 mg/dL, mean±SD, p<0.05); this decline was significantly reversed by Mg supplementation (MgD MgS+cisplatin: 2.26 ± 0.18mg/dL, p<0.0001). Heparinized blood was collected by cardiac puncture; isolated plasma was frozen at -80°C until analysis. Kidneys were collected and either flash frozen in liquid N₂ (outer medulla and cortex only) or fixed in 10% formalin. Tumors were collected, flash frozen, and stored at -80°C until analysis.

Antibodies and reagents.

MRP4 (ABCC4, rabbit anti-human) and GAPDH (rabbit anti-human) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). CTR1 (rabbit anti-human), MRP2 (rabbit anti-human) and MRP6 (rabbit anti-human) antibodies were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). Cisplatin (cis-diamineplatinum (II) dichloride) was purchased from Acros Organics (Pittsburgh, PA, USA). MgCl₂-6H₂O and MgSO₄ (anhydrous) were purchased from Thermo Scientific (Waltham, MA, USA).
Determination of plasma Mg, blood urea nitrogen and creatinine levels.

Quantichrom Magnesium, Urea and Creatinine Assay kits were used to determine plasma Mg\(^{2+}\) (mg/dL), blood urea nitrogen (BUN, mg/dL) and creatinine (mg/dL) levels, respectively, according to manufacturer’s instructions (BioAssay Systems, Hayward, CA, USA).

Real-time quantitative PCR (qPCR)

qPCR reactions were performed using high quality RNA isolated from frozen kidneys and A2780 tumors (in vivo) using the RNeasy Universal Plus Mini kit (Qiagen, Valencia, CA, USA), as previously described (35). RNA preps with OD 260/280 and OD 260/230 ratios ≥1.9 were used. The qPCR reactions were performed in duplicate/triplicate using specific primers (Tables 1 and 2), as previously described (35). Relative changes in gene expression were calculated as fold-changes using the comparative Ct (ΔΔCt) method (8). Mouse Gapdh and human GAPDH were used as housekeeping genes for normalizing transcript levels in mouse kidneys and human tumors, respectively.

Histological assessment of renal cell apoptosis and renal injury.

Formalin-fixed kidneys were embedded in paraffin and sectioned (5µm). Renal apoptosis (in the cortex and outer medulla regions) was measured by TUNEL using the ApopTag kit (Millipore, Temacula, CA, USA). The slides were scored by a reviewer blinded to experimental conditions by counting the number of densely stained apoptotic cells per high power field (using > 5 random fields/section, 4-5 mice per group). AKI-associated tubular injury (tubular epithelial cell loss, necrosis, tubular epithelial simplification, intratubular debris and casts) was scored by a pathologist (IR) blinded to the experimental groups. Tubule injury scores (ranging between 0 and 4) were based on the percentage of
tubules affected as follows: 0 = <10%, 1 = 10–25%, 2 = 26–50%, 3 = 51–75%, and 4 = >75%, as previously described (35).

Assessment of cisplatin transporter expression by A2780 cells.

A2780 (human ovarian tumor) cells grown in RPMI 1640 media containing 10%FBS and PSQ at 60% confluence; media was replaced with complete MEM media as described above containing either 100%Mg (4.9mg/dL) or 10%Mg (0.49mg/dL). After 3 days, one set of A2780 cells maintained in 10%Mg media were supplemented with 90%Mg (final=100% Mg) approximately 36hrs prior to cisplatin treatment. MEM or cisplatin (diluted in MEM, 25μM final) was added incubated for 4hrs and the cells were collected, washed and lysed in RIPA buffer. Lysates were western blotted using primary antibodies (1:1000 for Cell Signaling antibodies [MRP4 and GAPDH] and 1:300 for Santa Cruz antibodies [CTR1, MRP2, MRP6]) and appropriate near-infrared-fluorescently labeled secondary antibodies (1:15,000, LI-COR, Lincoln, NE, USA), as previously described (35). Band densities, determined using Image J Software (NIH), were normalized using GAPDH as a loading control. Representative blots from n = 4-7 experiments are shown in the figures.

Quantification of platinum (Pt) accumulation in the kidneys.

Renal tissue platinum (Pt) levels were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using the Agilent 7500cx. All assays were performed at the University of North Carolina at Chapel Hill, as previously described (35). Renal Pt levels were normalized to kidney weight in milligrams.
**Determination of cellular Pt accumulation in LLC-PK₁ and A2780 cells.**

LLC-PK₁ (renal epithelial) cells were grown in M199 media containing 5%FBS, PSQ in T75-flasks until 60% confluent. Thereafter, the media was replaced with ‘complete’ minimum essential media (MEM) media containing 5%FBS, Ca (2.7g/dL), non-essential amino acids, PSQ, and either 100%Mg (9.63mg/dL for M199) or 10%Mg (0.963mg/dL), supplied as MgSO₄ and the cells were incubated for 4 days. One set of the cells maintained in 10%Mg media were supplemented with 90%Mg (final=100% Mg) approximately 36hrs prior to cisplatin treatment. For assessment of platinum (Pt) uptake, MEM or cisplatin (diluted in MEM, 50μM final) was added to the cultures. The dose and time point were based on prior experiments. After 6.5hrs the cells were collected, washed, and cellular Pt levels were measured by ICP-MS using the Agilent 7500cx, as described above. Cellular Pt levels were normalized to protein concentration using the micro BCA™ Protein Assay (Thermo Scientific).

A similar experimental protocol was followed for the A2780 human ovarian cancer cells, except they were grown in RPMI 1640 medium containing 10%FBS and PSQ and after 60% confluent, media was replaced with complete MEM media containing either 100%Mg (4.9mg/dL MgSO₄ for RPMI media) or 10%Mg (0.49mg/dL MgSO₄) (±90%MgSO₄, as Mg supplement). The cisplatin dose used for the A2780 cells was 25μM, which was based on preliminary experiments testing the cytotoxicity of cisplatin.
Statistical analyses.

Experiments were performed at least twice and data are expressed as mean±SEM (or ±SD), as indicated. Analysis of Variance (ANOVA) followed by Bonferroni post-hoc testing using GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used to examine those factors that were measured at a single time point. For analyses of tumor growth over time, data were log transformed and analyzed using repeated measures ANOVA (RMANOVA). A “Bonferroni-like” adjustment, such that p<0.01 was considered significant, was used for pre-specified pairwise comparisons. These analyses were carried out using SAS Version 9.3 (Sas Institute Inc., Cary, NC). For all omnibus tests, p<0.05 was considered significant.
Results

Mg status does not affect tumor growth in vivo and Mg supplementation does not impair cisplatin-mediated tumor killing in vivo

In the absence of cisplatin administration, A2780 tumor cells implanted in control (100%Mg) and Mg-deficient mice grew slowly over the 3 wk period (Fig. 1, A and B). No significant differences were found in A2780 tumor growth over the course of the study in untreated mice, irrespective of their Mg status (Fig. 1, A and B). As expected, we observed reduced ovarian xenograft tumor growth in all cisplatin-treated mice (±Mg deficiency ±Mg supplementation) when compared to saline-treated tumor-bearing mice (±Mg deficiency ±Mg supplementation), with significantly reduced tumor growth on day 22 in cisplatin alone mice compared to saline-treated controls (Fig. 1, A and B, p<0.01). However, there was no significant effect of Mg-deficiency on ovarian xenograft tumor growth in cisplatin-treated mice when compared to 100%Mg cisplatin-treated mice (Fig. 1A). Tumor growth in Mg supplemented mice treated with cisplatin did not significantly differ from the cisplatin control mice until after day 21, when the tumors in the cisplatin-treated Mg-supplemented mice showed considerably less growth when measured on day 22 (p<0.01) when compared to tumors grown in cisplatin-treated 100%Mg-fed controls on day 22 (Fig. 1A). Similarly, tumor growth in cisplatin-treated Mg supplemented mice was significantly lower than that observed in the cisplatin-treated Mg-deficient mice when measured on day 22 (Fig. 1A, p<0.01).

Mg status modulates cisplatin-induced AKI in a human ovarian tumor xenograft model

Cisplatin alone (10mg/kg: 3 doses given on days 12, 19 and 21 after starting the Mg-deficient or control diet) did not enhance plasma BUN or creatinine levels in tumor-bearing female mice compared to saline-treated controls (Fig. 2, A and B). However, when combined with Mg deficiency, the same
regimen of cisplatin significantly enhanced BUN (Fig. 2A, p<0.001) and creatinine levels (Fig. 2B, p<0.05) compared to cisplatin alone. As shown in Fig. 2, A and B, Mg replacement during cisplatin treatment significantly reduced plasma BUN and creatinine levels compared to Mg-deficient mice treated with cisplatin (Fig. 2, A and B, respectively, p<0.01).

Assessment of histological kidney damage revealed that cisplatin-treated Mg-deficient mice showed significantly increased renal tubular injury compared to cisplatin alone (Fig. 2, C, D and E), while Mg replacement was associated with significantly reduced renal tubular injury compared to kidneys obtained from Mg-deficient mice treated with cisplatin (Fig. 2, C, D and E). Renal inflammation has been implicated in cisplatin-mediated kidney damage (21,30,34). Cisplatin alone and MgD alone did not affect the renal expression of inflammatory genes including, Cxcl2 (Fig. 2F) and Cxcl10 (Fig. 2G) compared to untreated control mice bearing A2780 ovarian tumors. However, cisplatin-treated Mg-deficient tumor-bearing mice had significantly enhanced Cxcl2 (Fig. 2F, p<0.0001, ~2.5 fold over control) and Cxcl10 mRNA expression in their kidneys (Fig. 2G, p<0.0001, ~3 fold over control) when compared to cisplatin-treated controls. Mg replacement during cisplatin treatment significantly reduced renal Cxcl2 and Cxcl10 mRNA expression when compared to cisplatin-treated Mg-deficient mice (Fig. 2, F and G, respectively, p<0.01).

Cisplatin-mediated renal cell apoptosis in tumor-bearing mice is regulated by Mg status

Renal Bak mRNA expression in tumor-bearing mice was not affected by either cisplatin alone or Mg deficiency alone when compared to untreated controls (Fig. 3A). However, when Mg deficiency was combined with cisplatin, renal Bak mRNA expression was increased by ~3 fold when compared to
kidneys obtained from tumor-bearing mice treated with cisplatin alone or Mg deficiency alone (Fig. 3A, p<0.0001). This effect was significantly reversed by Mg replacement during cisplatin treatment when compared to Mg-deficient mice treated with cisplatin (Fig. 3A, p<0.01).

Based on TUNEL staining, significant renal cell apoptosis was not observed in control athymic tumor-bearing mice in the absence of cisplatin or in untreated tumor-bearing Mg-deficient mice (Fig. 3, B and C). Cisplatin treatment of normal-fed mice slightly enhanced renal cell apoptosis (although not significantly) and this was further and significantly increased when cisplatin was accompanied by Mg deficiency (Fig. 3, B and C, p<0.0001). The kidneys of Mg-supplemented tumor-bearing mice treated with cisplatin showed significantly less apoptosis compared to cisplatin-treated Mg-deficient tumor-bearing mice (Fig. 3, B and C, p<0.001).

**Mg status regulates gene expression of renal cisplatin uptake and efflux transporters**

Next, we investigated the role of Mg status on the expression of several well-established cisplatin uptake transporters by the kidneys. In ovarian tumor-bearing female mice, Mg deficiency alone did not significantly affect renal *Oct1* (Fig. 4A), *Oct2* (Fig. 4B), or *Ctr1* (Fig. 4C) mRNA expression compared to untreated controls. However, cisplatin alone significantly reduced renal *Oct2* (Fig. 4B, p<0.01) and *Ctr1* mRNA expression (Fig. 4C, p<0.05) when compared to untreated controls. When cisplatin was combined with Mg deficiency in tumor-bearing mice, renal *Oct1* (Fig. 4A, p<0.05) and *Oct2* (Fig. 4B, p<0.0001) mRNA expression was significantly reduced when compared to cisplatin treatment alone. Surprisingly, Mg replacement during cisplatin treatment significantly enhanced renal *Oct2* mRNA expression (Fig. 4B, p<0.05) and slightly increased *Oct1* mRNA expression (Fig. 4A, not significant).
when compared to cisplatin-treated Mg-deficient tumor-bearing mice. By contrast, renal Ctr1 mRNA expression in cisplatin-treated mice was not significantly altered by Mg status (Fig. 4C).

When we examined the kidneys for mRNA expression of cisplatin efflux transporters, including Abcc2 (which encodes MRP2), Abcc4v1 and Abcc4v3 (variants 1 and 3 which encode MRP4), as well as Abcc6 (which encodes MRP6) in tumor-bearing mice following cisplatin (± Mg deficiency and supplementation), we found that only renal Abcc2 mRNA expression was significantly enhanced by cisplatin alone compared to controls (Fig. 4D, p<0.001) and this was not affected by Mg status (Fig. 4D). Renal Abcc4v1 mRNA expression was similar in all the groups of tumor-bearing mice, irrespective of Mg status or cisplatin treatment (Fig. 4E). Cisplatin alone significantly reduced renal Abcc4v3 mRNA expression (Fig. 4F, p<0.05) when compared to controls. This decline was further (slightly) reduced by Mg deficiency; however, this decrease was not significant, nor was it reversed by Mg replacement (Fig. 4F). Renal mRNA expression of efflux transporter Abcc6 was not altered by either Mg deficiency alone or cisplatin alone when compared to saline-treated controls, but it was significantly reduced when they were combined (Fig. 4G, p<0.0001). Mg replacement after Mg deficiency and during cisplatin treatment significantly enhanced renal Abcc6 mRNA expression in tumor-bearing mice (Fig. 4G, p<0.05) when compared to cisplatin-treated Mg-deficient tumor-bearing mice.
Mg status does not affect cisplatin uptake or efflux transporter gene expression by A2780 tumors grown in vivo

Next, we investigated the effect of Mg status on the mRNA expression of cisplatin uptake and efflux transporters expressed by human A2780 tumors grown in athymic nude mice. Neither OCT1 nor OCT2 mRNA expression was observed by the tumors (data not shown). By contrast, A2780 tumor tissue expressed CTR1 mRNA and its expression was similar in all the groups of tumor-bearing mice, irrespective of Mg status or cisplatin treatment (Fig. 5A). A2780 tumors obtained from the mice expressed numerous cisplatin efflux transporters, including ABCC2 (which encodes for MRP2, Fig. 5B), ABCC4 (variants 1 and 2 which encode MRP4, Fig. 5, C and D), and ABCC6v2 (which encodes for MRP6, Fig. 5E). With the exception of ABCC6v2 mRNA, which was upregulated by cisplatin (Fig. 5E), tumor efflux transporter mRNA expression was not significantly altered by Mg deficiency, Mg supplementation and/or cisplatin treatment (Fig. 5B-D).

Mg status does not affect cisplatin uptake or efflux transporter protein expression by A2780 cells grown in vitro

Because adequate tumor specimens were not available, particularly following cisplatin treatment, for the analyses of uptake and efflux transporters at the protein level, we examined the effect of Mg status on their expression using untreated and cisplatin-treated A2780 cells grown in 100%Mg and 10%Mg media (+ Mg supplementation) in vitro. Mg deficiency alone and cisplatin treatment alone significantly reduced the protein expression of uptake transporter CTR1 by A2780 cells (grown in 10%Mg) compared to A2780 cells grown in 100%Mg media (Fig. 6, A and B, p<0.001). However, Mg status did not modulate the effect of cisplatin on CTR1 expression by A2780 cells in vitro (Fig. 6, A and B). For MRP2 (Fig. 6, A and C) and MRP6 (Fig. 6, A and D), no significant differences were observed in
protein expression by A2780 cells following exposure to various Mg conditions or cisplatin treatment
in vitro. Similar to that observed with CTR1, MRP4 efflux protein expression by A2780 cells was
significantly reduced by Mg deficiency alone and cisplatin alone compared to control cells (100%Mg-
media) (Fig. 6, A and E, p<0.0001), whereas Mg status (Mg deficiency + Mg supplementation) did not
alter the effect of cisplatin on MPR4 expression (Fig. 6, A and E).

**Renal Pt accumulation in A2780 tumor-bearing mice and cellular Pt accumulation in LLC-PK1**
renal epithelial cells is increased by Mg deficiency but Pt accumulation in A2780 tumor cells is not

Our recent study in non-tumor-bearing older female mice showed enhanced renal platinum (Pt)
accumulation following Mg deficiency (35). Herein, we confirmed that renal Pt accumulation was
significantly enhanced in Mg-deficient nude mice treated with cisplatin (Fig. 7A, p<0.001) when
compared to control-cisplatin treated nude mice. Mg replacement of Mg-deficient mice during
cisplatin treatment significantly reduced renal Pt accumulation compared to cisplatin-treated Mg-
deficient mice (Fig. 7A, p<0.05).

Similarly, we confirmed the effect of Mg status on renal epithelial cell Pt accumulation in vitro using
untreated and cisplatin-treated LLC-PK1 renal epithelial cells grown in either 100%Mg or 10%Mg
media (+ Mg supplementation). As expected, cisplatin treatment increased cellular accumulation of Pt
when compared to untreated LLC-PK1 cells (Fig. 7B, p<0.0001). Similar to our in vivo results, we
observed that LLC-PK1 renal epithelial cells grown in 10%Mg media had significantly higher cellular
Pt concentration compared to LLC-PK1 cells grown in 100%Mg media (Fig. 7B, p<0.001). Whereas
Mg supplementation of LLC-PK₁ cells modestly but significantly reduced the Pt accumulation when compared to Mg-deficient LLC-PK₁ cells treated with cisplatin (Fig. 7B, p<0.01).

Inadequate tumor specimens were available for analyzing Pt accumulation in the A2780 ovarian xenograft tumors in mice because cisplatin treatment dramatically reduced tumor growth in vivo. Therefore, we examined the effect of Mg status on Pt accumulation in vitro using untreated and cisplatin-treated A2780 human ovarian cancer cells, as described for the LLC-PK₁ cell line. As expected, cisplatin treatment of A2780 cells led to cellular Pt accumulation when compared to untreated A2780 cells (Fig. 7C, p<0.0001). However, we did not observe any significant difference in the cellular Pt accumulation by cisplatin-exposed A2780 human ovarian cancer cells grown in 100%Mg vs. 10%Mg (± Mg supplementation) (Fig. 7C).
Discussion

The current study aimed to explore the effects of Mg status (Mg deficiency ± Mg supplementation) on tumor growth in vivo and the chemotherapeutic efficacy of cisplatin in a physiologically relevant tumor-bearing mouse model. Using a previously established human ovarian tumor xenograft model in mice combined with cisplatin-induced AKI (with a reduced time between the last dose of cisplatin and euthanasia, as described in the methods) (27), we show for the first time that Mg status does not affect tumor growth in vivo and that Mg supplementation does not compromise the chemotherapeutic efficacy of cisplatin using A2780-tumor-bearing mice (Fig. 1). These results complement our previous observations revealing the beneficial role of Mg supplementation in cisplatin-induced AKI in non-tumor-bearing physiologically relevant older C57BL/6 female mice in vivo (35), as well as our in vitro data showing that Mg supplementation did not impair cisplatin killing of the A2780 human ovarian, MCF-7 human breast or H460 human lung tumor cell lines (35).

Although little is known regarding Mg deficiency (± Mg supplementation) and cancer, previous studies have connected a high serum Ca/Mg ratio with an increased risk of breast cancer (33) and prostate cancer (9). Furthermore, a recent and extensive meta-analysis reported that higher intakes of Mg were associated with a decreased risk of colon cancer (5). Similarly, higher Mg levels in drinking water correlate with lower risks of gastric cancer (40), prostate cancer (41), esophageal cancer (42), decreased morbidity associated with liver cancer (36) and a decreased risk of death from ovarian cancer (7). Together, these data suggest that Mg deficiency is associated with an increased cancer risk and sufficient Mg intakes correlate with tumor-protective effects.
Several studies also report the inhibition of primary tumor growth in Mg-deficient animals \(^{(24,44)}\). Mg is required for the activity of NM23-H1, a metastasis suppressor gene \(^{(19)}\), and while decreasing primary tumor growth, Mg deficiency was also associated with increased metastasis and higher mortality \(^{(24)}\). However, the A2780 human ovarian tumor xenograft model is not a metastatic tumor model. Using the A2780 human ovarian tumor xenograft model, we observed that Mg deficiency had no effect on tumor growth in the presence or absence of cisplatin when compared to appropriate control mice. There are several differences between our model and previous models investigating the effect of Mg deficiency on tumor growth \(^{in vivo}\) \(^{(24,44)}\) including, the origin, type, and site of tumor, the species (mouse vs. rat models), the mouse strain (athymic nude mice vs. other strains), and the severity of Mg deficiency (our model has mild-moderate Mg deficiency which was considerably less severe). Furthermore, unlike previous studies which only investigated the effect of Mg deficiency on tumor growth \(^{(24,39,44)}\), we also examined the effect of Mg deficiency (+Mg supplementation) on the chemotherapeutic efficacy of cisplatin and cisplatin-induced AKI. In our model, tumor growth was not significantly affected by Mg status in saline-treated mice (Fig.1, A and B). Similarly, cisplatin-induced A2780 ovarian tumor growth inhibition \(^{in vivo}\) was not affected by Mg deficiency (Fig. 1, A and B). In fact, in Mg-supplemented mice, A2780 ovarian xenograft tumors grew significantly slower after day 21 compared to the control cisplatin-treated and the cisplatin-treated Mg-deficient mice (Fig. 1A).

Consistent with these observations, we have previously shown that the efficacy of cisplatin-mediated killing of A2780 ovarian tumor cells \(^{in vitro}\) was not impaired by either Mg deficiency or Mg supplementation \(^{(35)}\).

Athymic nude mice are commonly used in studies investigating human tumor growth \(^{in vivo}\) because they have a paucity of functional T cells and therefore, do not exhibit acute graft vs. host rejection and thus, allow the growth of human tumors. However, the absence of T cells in these mice complicates the
complete understanding of the mechanisms and effects which involve T cell functionality, including cisplatin-mediated AKI. Mice lacking T cells exhibit significantly less cisplatin-mediated AKI than other immunocompetent mouse strains (18), emphasizing the importance of T cells in cisplatin-induced nephrotoxicity. This may explain why cisplatin treatment (administered in multiple doses) alone did not significantly affect kidney function in tumor-bearing female athymic nude mice in this study and Pabla et al (27), while cisplatin at a similar single dose did induce AKI in female C57BL/6 mice (with functional T cells) (35). However, other strain-related differences may account for this effect.

In addition, this is the first report to reveal the augmentation of cisplatin-mediated kidney injury by Mg deficiency in tumor-bearing mice, and the improvement of cisplatin-induced AKI provided by Mg supplementation (Fig. 2, A-E). Following a similar cisplatin dosing strategy, as previously described for the A2780 ovarian tumor xenograft model in athymic nude mice (27), we found that cisplatin administered in 3 doses (10mg/kg, i.p., on days 12, 19 and 21 after start of the Mg-deficient and control diets) to tumor-bearing athymic nude female mice did not significantly affect kidney function, as determined by BUN (Fig. 2A) and creatinine levels (Fig. 2B), as well as renal tubular damage (Fig. 2, C, D and E), when compared to saline-treated tumor-bearing controls (when measured one day after the 3rd dose of cisplatin). These results are consistent with a previous study where cisplatin-induced AKI in athymic nude mice was not observed until 7 days after the 3rd dose of cisplatin (27). However, when combined with Mg deficiency, cisplatin treatment caused significant kidney damage in tumor-bearing athymic nude mice, as evidenced by enhanced plasma BUN and creatinine levels (Fig. 2, A and B, respectively). Mg deficiency has been linked to increased inflammation, as well as increased inflammatory cytokines in rodent models, including cisplatin-induced kidney inflammation (35).

Similar to our previous study (35), Mg-deficiency in ovarian tumor-bearing female mice was
accompanied by higher expression of both pro-inflammatory (Cxcl2 and Cxcl10 mRNA) (Fig. 2, F-G) and pro-apoptotic markers (Bak mRNA) and apoptosis (Fig. 3, A-C) in the kidneys following cisplatin administration when compared to cisplatin-treated control (100%Mg) ovarian tumor-bearing female mice; these effects were reversed by Mg replacement.

The beneficial effects of Mg supplementation on kidney damage induced by cisplatin in patients with testicular, ovarian, lung, thoracic and head/neck cancer have been shown by three small clinical trials (2,16,37) and retrospective studies (22,43). In all of these studies, cancer patients received cisplatin as a part of their chemotherapeutic regimen with or without Mg supplementation. Significantly better kidney function was observed in the Mg-supplemented patients compared to controls (2,16,22,37,43). Overt consequences of Mg supplementation on overall long-term survival, tumor progression, and metastases were not assessed in these studies. We have confirmed the nephroprotective role of Mg in cisplatin-induced AKI, as previously shown in non-tumor-bearing mice (35), in an ovarian tumor xenograft model in mice where Mg supplementation does not interfere with the anti-tumor killing capacity of cisplatin. Thus, our results are limited to the A2780 human ovarian tumor model and additional tumor models are required to better understand the role of Mg status on tumor cell growth and cisplatin-mediated tumor killing. Likewise, additional studies using animal models of Mg deficiency (±Mg supplementation) are needed to better understand the role of Mg in tumor initiation, development, progression, and metastases.

Cisplatin causes kidney damage owing to its accumulation in the proximal and distal kidney tubules (19,21,28,34). In this model, we observed significantly more Pt accumulation in the kidneys in vivo (Fig. 7A) and LLC-PK1 renal epithelial cells in vitro (Fig. 7B) following Mg deficiency and this was
significantly reduced by Mg replacement (Fig. 7, A and B). Accumulation of cisplatin is balanced by its uptake and efflux. Renal uptake has been attributed to passive diffusion (15), along with active transporter-mediated uptake (34). Organic cation transporter 2 (OCT2) and CTR1, a high affinity copper transporter, have been implicated in renal and tumor cisplatin uptake (12,14,29). Similar to our previous study in non-tumor-bearing older female mice, we found that renal Mg deficiency led to significantly reduced Oct1 and Oct2 mRNA expression in the kidneys of cisplatin-treated Mg-deficient tumor bearing mice (Fig. 4A and B), which was reversed by Mg supplementation (Fig. 4A and B). However, the potential effect of Mg on cell membrane lipid content and fluidity (20) and further, on additional renal cisplatin uptake mechanisms, including diffusion cannot be eliminated. This may explain why increased Pt accumulation is observed despite reduced expression of uptake transporters.

Removal of cellular cisplatin via various efflux transporters also contributes to cisplatin levels in the kidneys. Renal mRNA expression of Abcc6 (which encodes MRP6) was significantly reduced in cisplatin-treated Mg-deficient mice and this was reversed by Mg supplementation (Fig. 4G). These results are consistent with the increased renal cisplatin accumulation observed in Mg-deficient mice and decreased renal cisplatin accumulation observed in Mg-supplemented mice. The expression of other renal efflux transporters was not significantly altered in Mg-deficient mice following cisplatin treatment (Fig. 4). Again, the effect of Mg status on additional cisplatin efflux transporters in the kidneys cannot be ruled out.

Cisplatin uptake and efflux transporters are differentially expressed in tumors and they are modulated to reduce cisplatin uptake, enhance cisplatin efflux, and in extreme cases promote cisplatin resistance (Reviewed in (10)). Consistent with reports describing CTR1 as the main cisplatin influx transporter
expressed by human tumors, we observed constitutive \textit{CTR1} mRNA expression by A2780 tumors grown \textit{in vivo} (Fig 5A) in the absence of \textit{OCT1} and \textit{OCT2} mRNA expression (data not shown). \textit{CTR1} mRNA expression by the A2780 tumor was not significantly altered by either cisplatin treatment or Mg status (Fig. 5A). Likewise, no significant differences in efflux transporter expression were observed at the mRNA level in the A2780 tumors obtained from the various groups of mice, except for \textit{ABCC6v2} mRNA (which encodes MRP6) expression and this was enhanced by cisplatin alone (Fig 5, B-E). \textit{ABCC6v1} mRNA expression was not consistent across tumors obtained from the same group of mice (and therefore, not included in the analysis). These results in combination with our tumor growth data suggest that Mg deficiency and/or supplementation does not affect gene expression of uptake or efflux transporters in the A2780 tumors, nor does Mg status affect A2780 tumor progression.

Unfortunately, due to the success of tumor killing by cisplatin one of the limitations of this study is the lack of adequate tumor tissues available to examine the protein expression of cisplatin uptake and efflux transporters, as well as accumulation of tumor Pt, particularly in cisplatin-treated mice where final tumor sizes were quite small. In addition, Pt accumulation within the tumors on day 22 would be difficult to assess following cisplatin-induced killing due to loss of membrane integrity and intracellular release. Therefore, we investigated the effect of Mg status on protein expression of the uptake and efflux transporters using A2780 cell line \textit{in vitro}. Untreated A2780 cells maintained in 100%Mg-containing media had significantly higher CTR1 (Fig. 6, A and B) and MRP4 (Fig. 6, A and E) expression than untreated A2780 cells maintained in Mg-deficient media or cisplatin-treated A2780 cells maintained in 100%Mg-containing media. Overall, among cisplatin-treated A2780 tumor cells grown \textit{in vitro}, no differences were observed in the protein expression of CTR1, MRP2, MRP4 and MRP6, irrespective of Mg levels in the media (control-100%Mg, Mg deficiency-10%Mg \[\pm\] Mg supplementation]) (Fig. 6A-E). Similarly, we did not observe any significant differences in the Pt accumulation/levels in A2780 human ovarian cancer cells grown \textit{in vitro}, irrespective of their
maintenance in either 100% Mg, Mg deficient (10% Mg) or Mg supplemented (10% /100% Mg) media (Fig. 7C). Together, these results suggest that Mg status regulates Pt accumulation in renal epithelial cells in vivo (Fig. 7A) and cisplatin-treated LLC-PK1 renal epithelial cells in vitro (Fig. 7B). However, A2780 human ovarian cancer cells are less sensitive to changes in extracellular Mg concentrations, as shown by unaltered uptake and efflux transporter expression and similar cellular platinum accumulation in Mg-deficient (± Mg supplemented) cisplatin-treated cells (Fig. 7C).

In summary, our results show for the first time enhanced kidney injury following cisplatin in A2780 tumor-bearing Mg-deficient mice (vs. cisplatin-treated controls) and significant renal benefits of Mg supplementation (following Mg deficiency) in preventing the renal injury, inflammation and apoptosis caused by cisplatin in tumor-bearing mice (Fig. 8). We also demonstrate that while the renal uptake and efflux transporter expression is altered by Mg status, the transporter expression in the A2780 tumors grown in nude mice is more tightly regulated and unaltered by Mg deficiency and/or supplementation (Fig. 8). Similarly, Mg status did not affect Pt accumulation in A2780 cells in vitro, nor did it affect uptake or efflux transporter expression in cisplatin-treated A2780 cells. Most importantly, these data indicate that Mg supplementation does not promote tumor growth in vivo and/or compromise cisplatin efficacy in the ovarian tumor-bearing mouse model (Fig. 8) and thus, warrant additional studies in other tumor-bearing mouse models. Finally, future large scale, long-term studies are required to better understand the renoprotective role of Mg in cancer patients receiving cisplatin and to monitor the effects of Mg deficiency vs. the long-term effects of Mg supplementation on tumor-related outcomes, including metastases and survival.
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**Figure 1: Mg supplementation did not promote ovarian tumor growth or protect against cisplatin-mediated tumor killing in vivo**

Control (100%Mg, CTRL), Mg-deficient (MgD), and Mg supplemented mice (MgD MgS) bearing A2780 tumors (as described in the methods section) were treated with saline or cisplatin (CIS, 10mg/kg, i.p.) on days 12, 19, and 21 after starting the diets. A2780 (human ovarian tumor) cells were injected s.c., in the right flank on day 2. Mice were euthanized 24hrs after the last dose of CIS (or saline) on day 21. Tumor sizes/volumes were recorded on days 13, 15, 19, 21 and 22. All mice were euthanized 24 hrs after the last dose of CIS (or saline) given on day 21 and (A) tumor volumes (as geometric means) over time are shown. *** p<0.001 vs. CTRL (Day 22), †† p< 0.01 vs. CIS (Day 22) and vs. CIS/MgD (Day 22). Representative examples of tumors obtained from each group of mice on day of euthanasia (day 22) are shown in (B).

**Figure 2: Severity of cisplatin-induced AKI in a human ovarian tumor xenograft model is modulated by Mg status**

Control (100%Mg, CTRL), Mg-deficient (MgD), and Mg supplemented (MgD MgS) mice bearing A2780 tumors were treated ±cisplatin (CIS) (as described in the methods section and Figure 1). Mice were euthanized 24hrs after the last dose of CIS (or saline) on day 21. Blood urea nitrogen (BUN) (A) and plasma creatinine levels (B) were determined. Data are shown as mean (±SEM) mg/dL. Fixed kidney tissues were stained with H&E and evaluated for histology. Representative images for each group are shown in (C) (x200 magnification) and (D) (x400 magnification). Histological damage scores (mean ±SEM), ranging between 0 and 4) shown in (E) were based on the percentage of tubules affected using x200 magnification (0: <10%; 1: 10-25%; 2: 25-50%; 3: 50-75%; 4: >75%). Scale bar (-) = 50µm (C) and 20µm (D). Renal cortical mRNA expression of inflammatory markers Cxcl2 (F) and Cxcl10 (G) was measured by qPCR. Data are shown as mean (±SEM) fold-change (vs. Gapdh housekeeping gene). † p<0.05 vs. CIS, ††† p<0.001 vs. CIS, †††† p<0.0001 vs. CIS, ‡‡ p<0.01 vs. MgD CIS

**Figure 3: Cisplatin-mediated renal cell apoptosis in tumor-bearing mice is regulated by Mg status**

Control (100%Mg, CTRL), Mg-deficient (MgD), and Mg supplemented (MgD MgS) mice bearing A2780 tumors (as described in the methods section and Figure 1) were euthanized 24hrs after the last dose of cisplatin (CIS) (or saline) given on day 21. Renal cortical mRNA expression of (A) Bak was measured by qPCR. Data are shown as mean (±SEM) fold-change (vs. Gapdh housekeeping gene). Renal cell apoptosis was measured by TUNEL staining and representative photomicrographs are shown (400x magnification) in (B). Scale bar (-) = 20µm. Apoptosis was determined by counting the number of TUNEL positive cells per high power field (HPF) using ≥ 15 random sections and the mean apoptosis scores (±SEM) are shown in (C). Arrows indicate TUNEL positive cells. †††† p<0.0001 vs. CIS, ‡‡ p<0.01 vs. MgD CIS, ‡‡‡ p<0.001 vs. MgD CIS.
Figure 4: Mg status regulates the mRNA expression of renal cisplatin uptake and efflux transporters in vivo

Control (100%Mg, CTRL), Mg-deficient (MgD), and Mg supplemented (MgD MgS) mice bearing A2780 tumors (as described in the methods section) were euthanized 24hrs after the last dose of cisplatin (CIS) (or saline) given on day 21. Renal cortical mRNA expression of cisplatin uptake transporters (A-C): (A) Oct1, (B) Oct2 and (C) Ctr1 and cisplatin efflux transporters (D-G): (D) Abcc2, (E) Abcc4v1, (F) Abcc4v3, and (G) Abcc6 were measured by qPCR. Data are shown as mean (±SEM) fold-change (vs. mouse Gapdh housekeeping gene). * p<0.05 vs. CTRL, ** p<0.01 vs. CTRL, *** p<0.001 vs. CTRL, † p<0.05 vs. CIS, †††† p<0.0001 vs. CIS, ‡ p<0.05 vs. MgD CIS

Figure 5: Mg status does not affect cisplatin uptake or efflux transporter mRNA expression by A2780 tumors grown in vivo

Control (100%Mg, CTRL), Mg-deficient (MgD), and Mg supplemented (MgD MgS) mice with A2780 tumors (as described in the methods section) were euthanized 24hrs after the last dose of cisplatin (CIS) (or saline) given on day 21. Tumor tissues were assessed for mRNA expression of cisplatin uptake transporter CTR1 (A) and efflux transporters: ABCC2 (B), ABCC4V1 (C), ABCC4V2 (D), and ABCC6IV2 (E) by qPCR. Data are shown as mean (±SEM) fold-change (vs. human GAPDH housekeeping gene). **** p<0.001 vs. CTRL

Fig. 6: Mg status does not affect cisplatin uptake or efflux transporter protein expression by A2780 tumor cells grown in vitro

A2780 human ovarian tumor cells were maintained in media containing either 100%Mg or 10% Mg (Mg-deficient), as described in the methods. One group of Mg-deficient cells was switched to 100%Mg media (10%/100% Mg) prior to the addition of cisplatin (CIS, 25µM) for 4 hrs. Cell lysates were analyzed by western blotting for the expression of cisplatin uptake and efflux transporters. Representative western blots for tumor cell CTR1, ABCC2 (MRP2), ABCC6 (MRP6) and ABCC4 (MRP4), as well as housekeeping protein, GAPDH expression are shown in (A). Quantitation of band ratios: (B) CTR1/GAPDH, (C) ABCC2 (MRP2)/GAPDH, (D) ABCC6 (MRP6)/GAPDH and (E) ABCC4 (MRP4)/GAPDH (mean band density ±SEM) are shown. *** p<0.001 vs. 100%Mg, **** p<0.0001 vs. 100%Mg

Fig. 7: Platinum (Pt) accumulation in renal epithelial cells in vivo and in vitro is upregulated by Mg deficiency, but Pt accumulation by A2780 human ovarian cancer cells in vitro is not

Control (100%Mg, CTRL), Mg-deficient (MgD), and Mg supplemented (MgD MgS) nude mice bearing A2780 tumors (as described in the methods section and Figure 1) were euthanized 24hrs after the last dose of cisplatin (CIS) (or saline). Renal platinum (Pt) accumulation in the kidneys was measured by ICP-MS (A). Data are shown as mean±SD (ng/mg kidney tissue weight [wt]). LLC-PK1 renal epithelial cells (B) and A2780 ovarian tumor cells (C) were maintained in media containing either 100%Mg or 10% Mg (Mg-deficient), as described in the methods. One set of Mg-deficient cells was switched to 100%Mg media (10%/100% Mg) prior to addition of cisplatin (CIS). Cellular Pt accumulation was measured 6.5 hrs post CIS by ICP-MS. Data are shown as mean±SD (ng/mg of
protein). ND= not detected, **** p<0.0001 vs. 100%Mg (without cisplatin), ††† p<0.001 vs. 100%Mg CIS, ‡ p<0.05 vs. MgD CIS, ‡‡ p<0.01 vs. 10%Mg CIS

**Fig. 8: Proposed model: Mg supplementation using an ovarian tumor xenograft model ameliorates cisplatin-induced AKI without compromising the anti-tumor efficacy of cisplatin**

Saline treatment of athymic nude mice bearing A2780 human ovarian tumor cells has no effect on renal or tumor outcomes, irrespective of Mg status. Cisplatin treatment of Mg-deficient (MgD) tumor-bearing mice enhances renal platinum (Pt) accumulation, reduces Pt efflux transporter expression by the kidneys, and results in renal damage and AKI. These renal effects are attenuated by Mg supplementation after deficiency (MgD-MgS). By contrast, Mg status does not affect Pt accumulation in A2780 tumor cells *in vitro* or Pt transporter expression in tumors *in vivo* and *in vitro*. Tumor growth (*in vivo*) is unaffected by MgD, while MgD-MgS improves the anti-tumor efficacy of cisplatin.
Figure 2

Graphs showing BUN and Creatinine levels in不同groups:

- **A**: BUN (mg/dL) levels
- **B**: Creatinine (mg/dL) levels

Legend for images:
- **CTRL**
- **MgD**
- **CIS**
- **MgD CIS**

Images C illustrate histological changes:
- **CTRL**
- **CIS**
- **MgD**
- **MgD CIS**

Annotations:
- **☆** = Tubular dilatation
- **★** = Tubular casts
- **↑** = Flattening of epithelium
**Figure 2**

- **E**: Tubular Injury Score
- **F**: Cxcl2 mRNA (Fold Change)
- **G**: Cxcl10 mRNA (Fold Change)

Legend:
- * = Tubular dilatation
- * = Tubular casts
- ↑ = Flattening of epithelium

**Graphs**:
- **CTRL** vs. **MgD**, **CIS**, **MgD CIS**
- **MgD** vs. **MgD CIS**
- **MgD** vs. **MgS CIS**

**Note**: The graphs show statistical significance levels indicated by symbols and arrows.
Figure 3

(A) Bar graph showing Bak mRNA (Fold Change) for CTRL, MgD, CIS, MgD CIS, and MgD CIS.

(B) Images showing TUNEL positive cells in different conditions: CTRL, MgD, CIS, MgD CIS, MgD CIS.

(C) Bar graph showing TUNEL positive cells/HPF for CTRL, MgD, CIS, MgD CIS, and MgD CIS.
Figure 6

A

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- CTR1
- MRP2
- MRP6
- GAPDH
- MRP4
- GAPDH

B

- CTR1/GAPDH

C

- MRP2/GAPDH

D

- MRP6/GAPDH

E

- MRP4/GAPDH
Figure 7

A

Renal Pt Accumulation

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B

LLC-PK₁, Pt Accumulation

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C

A2780 Pt Accumulation

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Nude Mice (±MgD) With Human Tumor Xenograft

In Vivo Tumor Progression

- Cisplatin
  ±MgD
  - Kidney
  - Tumor
  - No renal damage
  - Control vs. MgD
  - No Effect

+ Cisplatin
  ±MgD ±MgS
  - Kidney
  - Tumor

Pt Accumulation
  - ↑ With MgD
  - ↓ With MgD-MgS

Pt Efflux Transporter Expression
  - ↓ With MgD
  - ↑ With MgD-MgS

In vitro Pt Accumulation
  - No Effect of MgD or MgD-MgS

Pt Efflux Transporter Expression
  - No Effect of MgD or MgD-MgS

Inflammation
  - ↑ With MgD
  - ↓ With MgD-MgS

Apoptosis
  - ↑ With MgD
  - ↓ With MgD-MgS

Tissue Damage
  - ↑ With MgD
  - ↓ With MgD-MgS

AKI
  - Tumor Volume
  - MgD: No Effect
  - ↓ With MgD-MgS

Figure 8
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