Sex-dependent effects of CYP2E1 on the kidney and the protective potential of 4MP against cisplatin-induced nephrotoxicity

Akakpo JY[1], Abbott E[5], Woolbright BL[2], Schnellmann RG[3], Wallace DP[4], and Taylor JA[2,5]

[1]Department of Pharmacology, Toxicology & Therapeutics, University of Kansas Medical Center, Kansas City, KS

[2]Department of Cancer Biology, University of Kansas Medical Center, Kansas City, KS

[3]Department of Pharmacology and Toxicology, R. Ken Coit College of Pharmacy, University of Arizona, Tucson, Arizona, USA; Coit Center for Longevity and Neurotherapeutics, R. Ken Coit College of Pharmacy, University of Arizona, Tucson, Arizona, USA; BIO5 Institute, College of Medicine, University of Arizona, Tucson, Arizona, USA.

[4]Jared Grantham Kidney Institute, University of Kansas Medical Center, Kansas City, KS

[5]Department of Urology, University of Kansas Medical Center, Kansas City, KS

For Correspondence: John A. Taylor (JAT) III MD, MS, John W. Weigel Endowed Professor of Urology & Cancer Biology, Department of Urology, University of Kansas Medical Center, 3901 Rainbow Blvd, MS 1018, Kansas City, KS, 66160 USA, Phone: +1 913 574 0517, jtaylor27@kumc.edu

Abstract

Cisplatin is the most effective chemotherapeutic agent used to treat various solid tumors and is the single most active chemotherapeutic in the management of bladder cancer (BCa). However, the most common adverse effect limiting cisplatin use is nephrotoxicity. Despite extensive mechanistic studies of the pathophysiology of cisplatin-induced nephrotoxicity (CIN), there are currently no clinical antidotes to treat CIN or second-line chemotherapy agents to treat BCa. Previous research demonstrated that CIN develops primarily in the proximal tubules where cisplatin accumulates and induces renal cell death after the formation of a highly reactive cisplatin-thiol metabolite. Recent evidence shows that the drug-metabolizing enzyme CYP2E1 is also involved in CIN, and its inhibition protects against nephrotoxicity. However, whether therapeutic strategies targeted at CYP2E1 may be beneficial in reducing CIN in the clinic has never been explored. Our group previously demonstrated that the clinically available drug, 4-methylpyrazole (4MP), blocks CYP2E1 activity. Thus, the objective of the current study was to examine the protective effect of 4MP in preclinical mouse models and translational human kidney and BCa cell models. C57BL/6J mice were co-treated with an acute (20 mg/kg for 3 days), or chronic (9 mg/kg for 4 weeks) cisplatin regimen, with or without 50 mg/kg 4MP. We show that severe kidney injury induced by cisplatin 3 days after the acute treatment, as indicated by elevated plasma BUN and creatinine, dilated tubules, tubular necrosis, and cast formation in male mice, was absent in female mice, which lack renal CYP2E1 expression. Importantly, cisplatin induced the activity of CYP2E1 in vitro, and inhibition of this activity by 4MP treatment almost completely eliminated CIN in male mice. These findings are relevant to humans because we show that patients do not express CYP2E1 in BCa cells, unlike kidney cells. 4MP also prevented cisplatin-induced cell death in normal human kidney cells without interfering with

its anti-cancer effects in human BCa HTB9 cells. In conclusion, our study highlights the critical role of CYP2E1 in mediating sex-specific differences in CIN, and 4MP treatment appears to be a potential prophylactic therapeutic option to prevent CIN in the clinic.

Keywords: Cisplatin induced nephrotoxicity; acute and chronic kidney injury; bladder cancer, cytochrome P450 2E1, 4-methylpyrazole (4MP)

INTRODUCTION

Bladder cancer (BCa) is the sixth most common cancer in the US and has high morbidity and mortality rates. Cisplatin is the single most actively used drug in the management of bladder cancer (BCa)[1-5]. However, clinical use of cisplatin is limited by cisplatin-induced nephrotoxicity (CIN), and there are limited alternate treatments for BCa[5-7]. Moreover, despite the substantial increase in knowledge of the complex pathophysiology of CIN, there are currently no clinically approved therapeutic agents to prevent cisplatin-associated kidney injury[8-13]. This lack of treatment options is likely due to the fact that the various pharmacological, molecular, and genetic approaches identified as potential renoprotective strategies for CIN target pathways that are, at the same time, crucially involved in the cytotoxic effect of cisplatin on tumor cells[3,11,14,15]. Thus, the advent of new therapeutic strategies to reduce CIN without affecting cisplatin's anti-cancer effect is a critical unmet medical need.

Acute and chronic doses of cisplatin result in drug accumulation in renal proximal tubular epithelial cells[16], with binding to the intracellular antioxidant glutathione (GSH) in a reaction mediated by glutathione-S-transferase to form Cisplatin-GSH conjugates[17-19]. Extracellular transport of the GSH conjugate and subsequent consecutive reactions with γ–glutamyltranspeptidase (GGT) and aminodipeptidase (DP) result in the formation of cisplatin-cysteinyl-glycine and cisplatin-cys conjugates respectively[20-22]. Pyridoxal 5'-phosphate-containing enzyme, cysteine-s conjugate beta-lyase[23,24], then catalyzes the beta-elimination reactions with cisplatin-cys to generate a highly reactive electrophilic nephrotoxin (thiol-cisplatin)[23,25]. The nephrotoxin binds to cellular macromolecules to ultimately trigger apoptotic and necrotic renal cell death[15,26]. Interestingly, thiol-cisplatin is formed after the extracellular transport and metabolism of a cisplatin GSH conjugate[18,19] (summary figure). However, the

severity of CIN in mice was recently correlated to the intratubular expression of the drug-metabolizing enzyme cytochrome P450, CYP2E1[27,28]. In fact, the genetic deletion of CYP2E1 provided novel protection against CIN in *Cyp2e1*$^{-/-}$ mice[28]. This suggests that CYP2E1 could be a targetable primary event, which could reduce the occurrence of CIN. This warrants further investigation into the influence of CYP2E1 on CIN and its potential impact on the treatment of BCa.

Sex-specific susceptibility to CIN is controversial. Existing studies have demonstrated an increased susceptibility of male patients to CIN [29,30]. Additional reports suggest that female patients are more susceptible to CIN than males depending on the type and stage of malignancy, dosage of cisplatin, drug-drug interactions, or the presence of comorbidities such as diabetes [31]. A Kaplan-Meier survival analysis corroborates this finding and highlights that women have a significantly higher risk of developing CIN [32]. More specifically, the data from stratified patients by age group (childbearing [< 45 years] and postmenopausal [> 55 years]) revealed that peri and postmenopausal women have a risk of developing CIN that is greater than men of the same age [32]. This is consistent with a previous study from Townsend et al. demonstrating that C57BL/6J female mice are less susceptible to CIN [22]. However, the mechanism underlying the sex-dependent susceptibility to CIN in patients is still poorly understood.

Our previous work demonstrated that 4-methylpyrazole (4MP), an FDA-approved drug for clinical treatment of methanol and ethylene glycol poisoning, also inhibits CYP2E1 activity[33]. We have also recently determined that 4MP does not impact the antitumor effect of a severe acetaminophen overdose on commonly used 4T1 breast tumor and Lewis lung carcinoma tumor models [34], suggesting that 4MP is a potential drug to use as a CYP2E1 inhibitor [33,35-38] in CIN. Thus, we hypothesize that 4MP could be a promising drug candidate that can be rapidly

repurposed to treat CIN, improving cisplatin tolerability and use in BCa patients. The purpose of this study was to determine if targeting CYP2E1 with 4MP is a viable therapeutic option to reduce the incidence of CIN. We found that female C57BL/6J mice lack renal CYP2E1 expression and are resistant to CIN, unlike male mice that express CYP2E1. We also found that CYP2E1 expression is significantly reduced in a tissue microarray of male and female BCa patients (Stage I-III) when compared to normal bladder tissue sections on a tissue microarray obtained from TissueArray (Derwood, MD). In contrast, normal kidney tissues of male patients obtained from the Jared Grantham Kidney Institute (KI) at the University of Kansas Medical Center (KUMC) express CYP2E1. Interestingly, 4MP therapy targeting CYP2E1 prevented CIN in the mouse as well as in normal primary human kidney cells also obtained from the Jared Grantham KI without interfering with cisplatin's anti-cancer effects in human BCa cells obtained from the American Type Culture Collection (Manassas, VA). This creates a rationale for the clinical use of 4MP as a feasible treatment to efficiently promote the antineoplastic effect of cisplatin in bladder cancer cells while preventing its toxic side effects on healthy kidney cells.

MATERIALS AND METHODS

Experimental animals, acute and chronic kidney injury model

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Moreover, experiments were performed following the guidelines of the National Research Council for the Care and Use of Laboratory Animals. In this study, 8 to 12-week-old male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were acclimated to a temperature-controlled room with a 12-hour light/dark cycle, food, and water ad libitum for 3–5 days. For the acute kidney injury

model, male and female mice were injected intraperitoneal (IP) with a single acute dose of 20 mg/kg cisplatin (Sigma-Aldrich, St. Louis, MO) or saline and were euthanized 3 days later. Since female studies show that female mice are resistant to CIN, male mice were also treated with a chronic dose of 9 mg/kg cisplatin once a week for 4 weeks before sacrifice. In addition, male mice received a co-treatment of 20 mg/kg cisplatin + 50 mg/kg 4MP or 9 mg/kg cisplatin+ 50 mg/kg 4MP (Sigma-Aldrich, St. Louis, MO). Both cisplatin and 4MP were dissolved in saline. The animals were euthanized by cervical dislocation under isoflurane anesthesia. Blood was drawn from the caudal vena cava into heparinized syringes and was centrifuged at 20,000×g for 3 min at 4°C. Kidney tissue was also collected for analysis.

Histopathological analyses

Collected kidney tissues were fixed in 4% formaldehyde overnight, embedded in paraffin blocks, and sectioned at 5 µm thickness. We deparaffinized sections and subjected them to hematoxylin and eosin (H&E) staining to assess the degree of morphological changes, as determined using the Nikon Eclipse Ti2 inverted fluorescence microscope. Further examination of proximal tubular necrosis, proximal tubule degradation, and tubular casts served as an indication of morphological damage to the kidney after treatment with cisplatin or cisplatin with 4MP.

Western blotting

Tissue samples were homogenized in ice-cold isolation buffer (pH 7.4) containing 220 mM mannitol, 70 mM sucrose, 2.5 mM HEPES, 10 mM EDTA, 1 mM EGTA, and 0.1% bovine serum albumin. This was followed by total protein measurements with the BCA assay from Pierce Scientific (Waltham, Ma). Western blotting was then carried out with the following

antibodies to cytochrome P450 2E1 (Abcam rabbit polyclonal antibody, Cat. # ab28146). Anti-rabbit or anti-mouse IgG horseradish peroxidase coupled secondary antibodies (Santa Cruz Biotechnology) (1:5000 dilution) and the ECL kit from Amersham (Piscataway, NJ) were then used for the detection of protein bands by chemiluminescence on the LI-COR Odyssey imaging system (LI-COR Biosciences).

Immunostaining of human tissue samples

BCa and bladder normal tissue microarray, containing 35 urothelial carcinomas, 4 squamous cell carcinoma, 1 adenocarcinoma, 10 normal bladder tissue of male and female patients, were obtained from TissueArray (Array number BL1002b Derwood, MD). Normal human kidney tissue sections from male patient donors were obtained from the Jared Grantham KI at KUMC. Then, immunohistochemistry staining of human tissue samples was performed on paraffin-embedded sections cut at 5 µm thickness. Paraffin-embedded tissue sections were then deparaffinized and dehydrated. After sections were washed in phosphate buffer saline (PBS), they were blocked with 5% normal goat serum to reduce nonspecific reactions, followed by an overnight incubation step with the primary anti-CYP2E1 antibody (Cat. # ab28146, AbCam, Boston, MA). After 24 hours of incubation, sections were washed in PBS followed by a 30 min application of SignalStain Boost Detection Reagent (Cell Signaling Technology, Rabbit no. 8114) or 1 h incubation with m-IgG Fc BP-HRP (sc-529765) followed by SignalStain DAB Chromogen detection (Cat. # 8059, Cell Signaling Technology, Danvers, MA) as per manufacturer's instructions. After counterstaining with hematoxylin, slides were imaged on the Nikon Eclipse Ti2 inverted fluorescence microscope applying DAB substrate per manufacture

instruction (vector laboratories) or after nuclei staining with a DAPI-containing mounting medium (Life Technologies).

Cell Culture

Normal kidneys, unsuitable for transplantation, were provided by the Midwest Transplant Network (Kansas City, KS). Normal primary human kidney (NHK) cells from male patient donors were obtained from the Jared Grantham KI at KUMC and cultured, as described in detail by Wallace and Reif [39]. Briefly, cells were seeded on collagen-coated plates in DMEM/F12 + P/S + ITS + 10% fetal bovine serum (FBS) and allowed to attach and proliferate in a humidified 5% CO2 incubator (at 37°C) for 2 days. The adherent NHK cells were washed with sterile PBS, and a starvation media consisting of DMEM/F12 + P/S + ITS + 0.5% FBS was added for 3 hours. NHK cells were treated with either 5, 10, or 20 µM cisplatin with or without 5 mM 4MP for 24 h. All drugs were dissolved in saline.

HTB9 BCa cells were acquired from the American Type Culture Collection (Manassas, VA). The cellular identity of HTB9 cells was confirmed twice yearly via STR analysis (University of Arizona Genetics Core). HTB9 cells were cultured as described in Woolbright et al. [40]. Briefly, cells were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37 degrees Celsius with 5% C02. At 24 h post-seeding, the media was changed until cells were approximately 90% confluent [41]. Cells were washed with sterile PBS and treated for 24 h with either cisplatin, 4MP, or both. All drugs were dissolved in saline.

Biochemical measurements

Plasma samples were used to assess the extent of kidney injury by measuring blood urea nitrogen (BUN) and creatinine with QuantiChrom™ Assay kits from BioAssay Systems (Hayward, CA). Both NHK and HTB9 cells viability was assessed using an MTT (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cell proliferation and cytotoxicity detection assay as described in Flaig et al. [42] and manufacturer instructions with the assay kit from Millipore Sigma (cat. # 11465007001). Cytochrome P450 enzyme activity was assessed in kidney homogenate using a fluorogenic substrate: 7-ethoxy-4-trifluoromethyl coumarin (7-EFC) [43] (Invitrogen, Carlsbad, CA) as described in Akakpo et al. [44].

The Cancer Genome Atlas Program (TCGA) analysis:

Data was acquired from the UALCAN website (http://ualcan.path.uab.edu). UALCAN is a comprehensive, user-friendly, and interactive web resource for analyzing cancer OMICS data[45,46]. Graphs were processed using the UALCAN website.

Statistical analysis

All statistical analyses were performed in SPSS Statistics 25 (IBM Co., Armonk, NY). One-way analysis of variance (ANOVA), followed by Student-Newman-Keul's test, was used to test the significance between multiple groups. Statistical analysis between two groups was performed with the student's two-tailed t-test. If the data were not normally distributed, the Kruskal-Wallis test (non-parametric ANOVA) followed by Dunn's Multiple Comparison Test was used. Differences with values of $p < 0.05$ were considered statistically significant.

RESULTS

Cisplatin-treated C57BL/6J female mice are protected against cisplatin-induced nephrotoxicity.

To assess sex differences in renal damage after cisplatin treatment, both male and female C57BL/6J mice were treated with a supratherapeutic cisplatin dose of 20 mg/kg i.p. or saline (control group) for 3 days. Cisplatin induced a significant elevation of both BUN and creatinine levels in male mice on day 3 of treatment (Figure 1A&B), but these changes were not noted in the female mice. The histological evaluation of renal tissue was consistent with the serum findings consistently demonstrating renal damage as indicated by varying degrees of tubular injury, including tubular dilation (black star) or rupture and tubular necrosis, as well as cast formation (black arrowhead), in male mice with none of these findings in the females, (x200) (Figure 1C). These observations demonstrate that C57BL/6J female mice are resistant to the nephrotoxic effects of cisplatin.

The lack of renal CYP2E1 contributes to the lower susceptibility of female mice to CIN.

The protein expression and activity of CYP2E1 have recently been demonstrated to be predominantly localized to the ER of human kidneys [47]. Additionally, work from our group recently corroborated the fact that in the kidney cortex of male mice, renal proximal tubular epithelial cells predominantly express CYP2E1 in the ER [37] . Since it has been demonstrated that *Cyp2e1*$^{-/-}$ mice do not express CYP2E1 [28] and are resistant to CIN like female mice after an acute cisplatin treatment, we examined whether CYP2E1 protein is expressed in the kidneys of female mice. Consistent with previous reports [48], the kidneys of female mice did not show any expression of CYP2E1 when compared to male mice, as revealed by the western blotting in Figure 2A. This data suggests that the lack of renal CYP2E1 expression may be the reason for

female mice's resistance to CIN. To test whether cisplatin could influence CYP2E1 activity, we assessed cytochrome P450 enzyme activities in vitro in kidney homogenate using the fluorogenic substrate 7-ethoxy-4-trifluoromethyl coumarin (7-EFC) (Figure 2B). Cisplatin dose-dependently increased the P450 enzyme activities detected in male tissue with 7-EFC. This suggests that there is a direct effect of cisplatin on CYP2E1 activity in male mice but not female mice, which likely contributes to the sex-specific differences in CIN after a severe cisplatin overdose.

4MP prevented the kidney injury induced by cisplatin.

Since CYP2E1 may be responsible for the development of CIN in male mice (Figures 1&2), the effect of CYP2E1 inhibition with the FDA-approved drug, 4MP, was evaluated. C57BL/6J mice were treated with the acute 20 mg/kg cisplatin dose and either 50 mg/kg 4MP or saline (control) for 3 days. Additional C57BL/6J mice were treated with the chronic 9 mg/kg cisplatin and 50 mg/kg of saline control once a week for 4 weeks. Then, blood and kidney tissue samples were collected to assess the extent of kidney injury. Our data shows that after both the acute and chronic cisplatin treatment, mice developed severe kidney injury as indicated by elevated plasma BUN (Figures 3 and 4A) and creatinine levels (Figures 3 and 4B) when compared to saline-treated control. This is consistent with histological analysis performed on tissue samples collected after both the acute and chronic cisplatin treatment, which shows normal renal corpuscle, glomerulus, and Bowman’s capsule (yellow arrow) surrounded with intact tubules that are lined with high cuboidal cells having rounded nuclei (blue arrow) in control mice tissue (Figure 3 and 4C). The renal tissues from cisplatin-dosed mice also show extensive tubular dilation, tubular necrosis (black star), and cast formation (black arrowhead). Interestingly, the co-treatment of cisplatin with 4MP almost completely prevented cisplatin toxicity after both the

acute and chronic cisplatin treatment, as indicated by lower plasma BUN and creatinine levels, as well as the absence of detectable tubular dilation and necrosis or cast formation (Fig 3 A-C). These data suggest that the inhibition of CYP2E1 with 4MP blocked the development of CIN.

CYP2E1 expression and inhibition in human patients with BCa.

Before the initiation of chemotherapy, BCa patients typically have acceptable creatinine levels, suggesting the absence of kidney injury [3,5]. Thus, we evaluated CYP2E1 expression levels in BCa patients using the extensive TCGA database via UALCAN [45,46] (Figures 6 and 7). CYP2E1 was significantly downregulated in patients with BCa compared to normal control tissue (Figure 6A) at all BCa cancer stages (Figure 6B). Interestingly, the downregulation of CYP2E1 was independent of race (Figure 7A) or sex (Figure 7B). To verify, we subsequently evaluated CYP2E1 expression levels in BCa and bladder normal tissue microarray, containing 35 urothelial carcinomas, 4 squamous cell carcinoma, 1 adenocarcinoma, 10 normal bladder tissue from male and female that we compared to normal kidney from male patients. Immunohistochemistry staining for CYP2E1 revealed appreciable signals in kidney tissue (Figure 5A), consistent with our previous report [37]. However, CYP2E1 expression was undetectable in BCa samples at all stages (Figure 5 B& supplementary figure 1). This indicates that targeting CYP2E1 with 4MP is unlikely to interfere with the antineoplastic effect of cisplatin in BCa patients while protecting kidneys against CIN.

4MP protects against kidney injury without affecting the chemotherapeutic effects of cisplatin on BCa.

To confirm the above assumption derived from analysis of the TCGA data and further evaluate whether targeting CYP2E1-mediated mechanisms of cisplatin-induced renal proximal tubular cell death with 4MP are relevant to humans, we treated NHK cells or HTB9 BCa cells with 5, 10, or 20 μM cisplatin with or without 5 mM 4MP for 24 h. The data shows that cisplatin-induced a dose-dependent cell death in these human kidney cells, which was significantly reduced with 4MP co-treatment. Interestingly, the presence of 4MP did not affect cisplatin-induced HTB9 BCa cell death, as indicated by a similar loss of cell viability in BCa cells exposed to cisplatin alone or cells exposed to the combination of cisplatin + 4MP. This data demonstrates that 4MP-mediated CYP2E1 inhibition appears to be an attractive renoprotective strategy to prevent cisplatin nephrotoxicity without affecting the nchemotherapeutic effects of cisplatin on BCa.

DISCUSSION

The purpose of this study was to investigate whether targeting CYP2E1 reduces CIN without interfering with its antineoplastic effects in BCa. The efficacy of the FDA-approved drug 4MP against CIN was tested in normal human kidney cells and human BCa cells as well as *in vivo* in a murine model.

Bioactivation of cisplatin in the development of CIN

The pathophysiology of CIN acute kidney injury (AKI) involves various molecular and cellular mechanisms, including increased oxidative stress [28,49,50], inflammation [51-54], and apoptosis [26,55,56]. However, the mechanistic events that trigger CIN are not well-established. The renal metabolism and bioactivation of cisplatin to a nephrotoxin have been suggested to play a major role in the

pathophysiology of CIN [18]. After accumulation in the proximal tubule, cisplatin can bind to intrarenal GSH and form the glutathione–cisplatin conjugates [18,19,22,57]. Of note, the conjugate must be transported outside the proximal tubular cells to be consecutively metabolized by the cell surface enzymes, GGT, and DP to a toxic, highly reactive cisplatin-thiol metabolite, which has been suggested to trigger renal proximal tubular cell death [18]. However, CYP2E1 is likely also toxicologically important to the pathology of CIN because its genetic deletion provides novel protection against CIN [28]. Recent results from our group indicate that CYP2E1 is expressed in the ER of renal proximal tubular cells of C57BL/6J male mice [37]. This aligns with previous immunoelectron microscopy experiments with gold particles [28]. Our experimental evidence further shows that cisplatin increases the activity of CYP2E1 (Figure 2B), suggesting a potential direct interaction between the drug and the enzyme. Yet, whether this is replicated *in vivo* is unclear and requires further investigation. Importantly, extracellular transport of cisplatin-GSH is necessary to form cisplatin thiols, while CYP2E1 is expressed intracellularly [28,37]. However, whether the cisplatin influence on CYP2E1 or its renal activation to cisplatin-thiol are the primary events driving CIN is not well established and still remains to be determined.

CYP2E1's influence on the sex-specific toxic renal effect of cisplatin.

In this study, we show that male mice develop severe kidney injury after cisplatin treatment when compared to females. This is consistent with a previous study demonstrating that C57 BL/6J female mice are less susceptible to CIN [22]. In fact, most of the experimental studies have reported that female rodents are more resistant than males to both acute and chronic cisplatin treatments [58-60]. Thus, in animal models, sex difference has a significant influence on the severity of nephrotoxicity induced by cisplatin. This is clinically relevant because prior studies have

highlighted that there is also a sex-specific difference in the susceptibility to CIN in humans and noted an increased susceptibility in male patients [29]. In fact, age-stratified patient data revealed that peri- and post-menopausal women have a greater risk of developing CIN that is greater than men of the same age [32]. Moreover, clinical epidemiology shows that BCa mainly affects post-menopausal women with a median age of diagnosis of 73 [3,61]. However, the mechanism underlying the sex-dependent susceptibility to CIN in patients is still poorly understood. Understanding this mechanism is crucial for optimizing treatment strategies and minimizing adverse effects. In this study, we provide evidence for a significant role of CYP2E1 in mediating the sex-specific differences in the susceptibility to CIN since female C57BL/6J mice lack renal CYP2E1 expression and are resistant to CIN (figure 1&2). The data suggest that renal CYP2E1 expression in male mice may lead to enhanced bioactivation of cisplatin, resulting in a greater cisplatin nephrotoxic effect, which is not observed in female mice. Yet, our data do not rule out the possibility that additional confounding factors may contribute to the resistance of female mice to CIN. For instance, there are sex-related differences in the organic cation transporter (OCT2) [62], which is responsible for cisplatin uptake into renal tubular cells and significantly higher in male kidneys, thereby increasing the accumulation and exposure time of cisplatin in proximal tubules. Sex-specific differences have also been noted in the expression of GGT [63,64]. The enzyme is higher in male than female mice and may facilitate the formation of cisplatin-thiol for a potential potentiation of CIN. Females have lower oxidative stress [17,65] than males, and this variation may additionally play a role in the female mice's resistance to CIN. This further supports the implication of CYP2E1 in CIN since the enzyme plays a significant role in oxidative stress, which is a well-documented contributor to CIN [49,66]. While the enzyme is constitutively expressed intracellularly in proximal tubules, it can also be upregulated in certain

pathophysiologic conditions such as diabetes [67], starvation [68], and obesity [69]. This sex-specific difference in susceptibility in humans could be due to GSH synthesis [22], but the estrous cycle regulation of CYP2E1 may also play a role [70]. Further investigation is needed to evaluate the influence of CYP2E1 in the resistance of female mice to CIN and determine if targeting the enzyme would be beneficial in managing CIN clinically.

4MP and the prevention of CIN clinically

The preceding discussion builds a case for CYP2E1 expression and activity being responsible for the higher susceptibility of kidneys to CIN. Moreover, our report that 4MP does not impact the antitumor effect of a severe acetaminophen overdose on commonly used 4T1 breast tumor and Lewis lung carcinoma tumor models [34], together with the lack of detectable CYP2E1 expression in BCa tissue of human samples (Fig. 5), indicate that the mechanism of cell death in BCa cells is independent of CYP2E1, unlike the kidney**.** This implies that therapeutic strategies targeting CYP2E1 may be beneficial in reducing CIN, the major use-limiting factor, in cancer patients. In the clinic, BCa patients generally have acceptable kidney function prior to the initiation of cisplatin therapy [5,71]. Additionally, the data presented in this study also shows that BCa patients do not express CYP2E1 in tumors. Thus, targeting CYP2E1 appears to be a logical nephroprotective strategy to reduce the occurrence of CIN while not interfering with the antineoplastic effect of cisplatin. We provide evidence that inhibition of CYP2E1 activity by 4MP co-treatment with an acute or chronic cisplatin dose protects male mice against CIN (figure 3). To evaluate if 4MP reduction in cisplatin nephrotoxicity was specific to the kidney, we looked for potential reductions in the chemotherapeutic efficacy of cisplatin on human HTB9 BCa cells. Our translational experiments further revealed that while 4MP dose-dependently

prevents CIN in NHK cells, it does not interfere with cisplatin toxicity in human BCa cells. Since 4MP is already an FDA-approved drug, our data suggests that 4MP is a promising drug candidate that could be rapidly repurposed to prevent CIN without interfering with the cisplatin antineoplastic effect. However, further translational investigations in additional human BCa cell lines and clinically relevant BCa cancer mouse models are required to provide strong evidence for the potential benefit of 4MP in the clinic.

In conclusion, cisplatin is the first line of platinum-based chemotherapy for the treatment of various cancers [70,72], including BCa. However, nephrotoxicity, characterized by extensive tubular injury and rapid loss of renal function, occurs in around 30% of patients who are receiving cisplatin chemotherapy. Unfortunately, there is currently no FDA-approved treatment to reduce or prevent CIN and no defined second-line chemotherapy agent to treat BCa. To the best of our knowledge, this is the first study to demonstrate the potential protective effects of 4MP against the possible primary mechanism of CIN, where the influence of cisplatin on CYP2E1 activity drives CIN. It is suggested that *in situ* activation of cisplatin within the kidney is a primary event that precedes cisplatin-thiol activation in the pathomechanism of CIN. Early treatment with 4MP prior to initiation of cisplatin could be a viable prophylactic therapeutic option to prevent CIN clinically because it could completely prevent kidney injury after acute or chronic cisplatin treatments by inhibiting cytochrome P450 2E1 without interfering with cisplatin's toxic effects on the cancer cells. Future research will focus on clarifying the precise molecular mechanisms by which CYP2E1 contributes to sex-specific differences in cisplatin nephrotoxicity. Future work will also determine whether 4MP can reverse injury progression after acute or chronic treatments of cisplatin.

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FOOTNOTES

The authors declare no conflict of interest.

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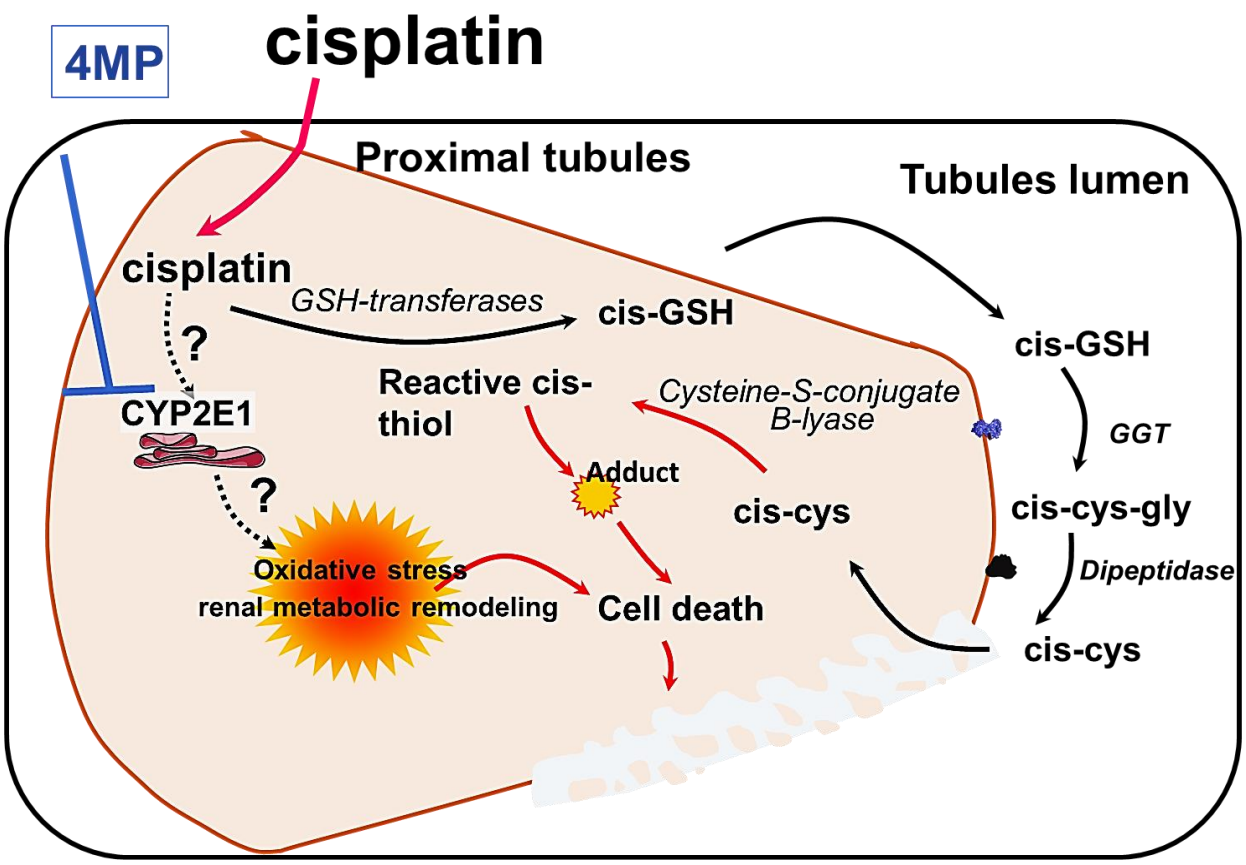

Summary Figure: Effect of 4MP on the metabolism of cisplatin in the kidney. Cisplatin enters cells and binds to DNA, which is toxic to dividing BCa cells (A) but not to quiescent proximal tubular epithelial cells (PTECs) in the kidney (B). In PTECs, cisplatin forms cisplatin-glutathione conjugates (Cis-GSH), which is then cleaved to a cisplatin-cysteinyl-glycine conjugate (cis-cys-gly) by GGT expressed on the cell surface. Cis-cys-gly is further cleaved to a cisplatin-cysteine conjugate (cis-gly) by aminodipeptidase, also expressed on the cell surface. The platinum-cysteine conjugate is taken up into the cell, where it is converted to a highly reactive thiol by cysteine S-conjugate β-lyase (cis-thiol). The binding of the reactive thiol to essential proteins within the cell is toxic. Cisplatin also influences tubular cell death via unknown mechanisms that involve CYP2E1 expressed in the ER of the cell. 4MP protects PTECS against cisplatin-induced nephrotoxicity.

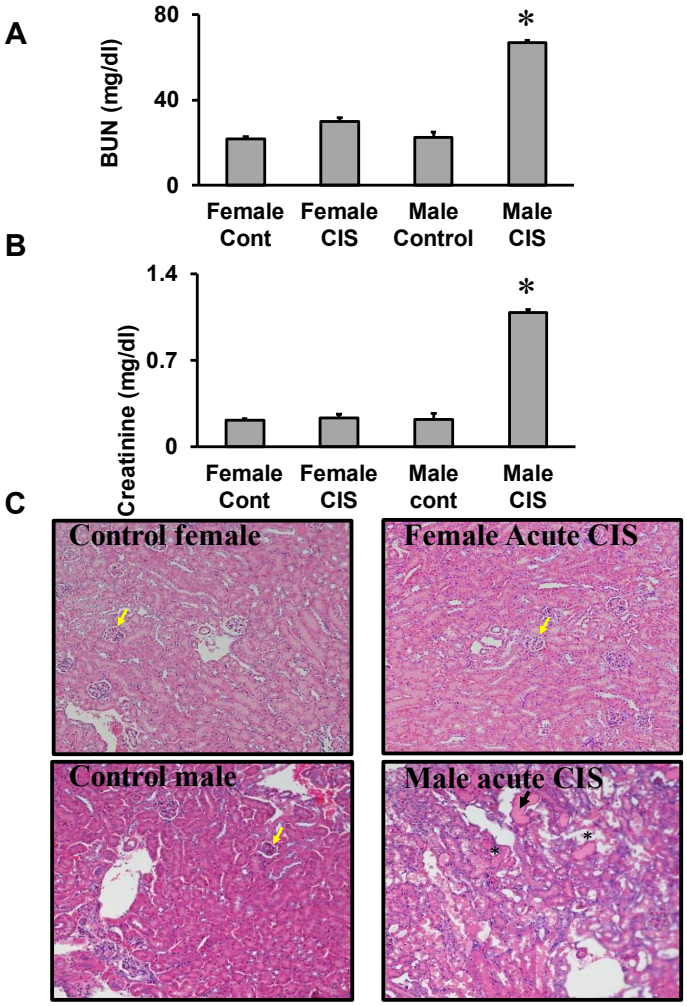

Figure 1: Sex-specific susceptibility to cisplatin-induced nephrotoxicity in C57BL/6J mice. C57BL/6J mice were treated with 20 mg/kg cisplatin or saline (control) for 3 days to collect blood and kidney tissue samples. Graphs are means ± SEM for plasma Blood Urea Nitrogen (BUN) (A) and creatinine levels (B). n= 6 animals per group. *$p < 0.05$ (compared to female control). Tissue sections were stained for H&E to visualize kidney morphology. Representative images of H&E staining of kidney sections (C) in control show normal renal corpuscle, glomerulus, and bowman's capsule (yellow arrow). In contrast, tissue from cisplatin-dosed mice shows dilated tubules (black star) and cast formation (black arrowhead) (x200).

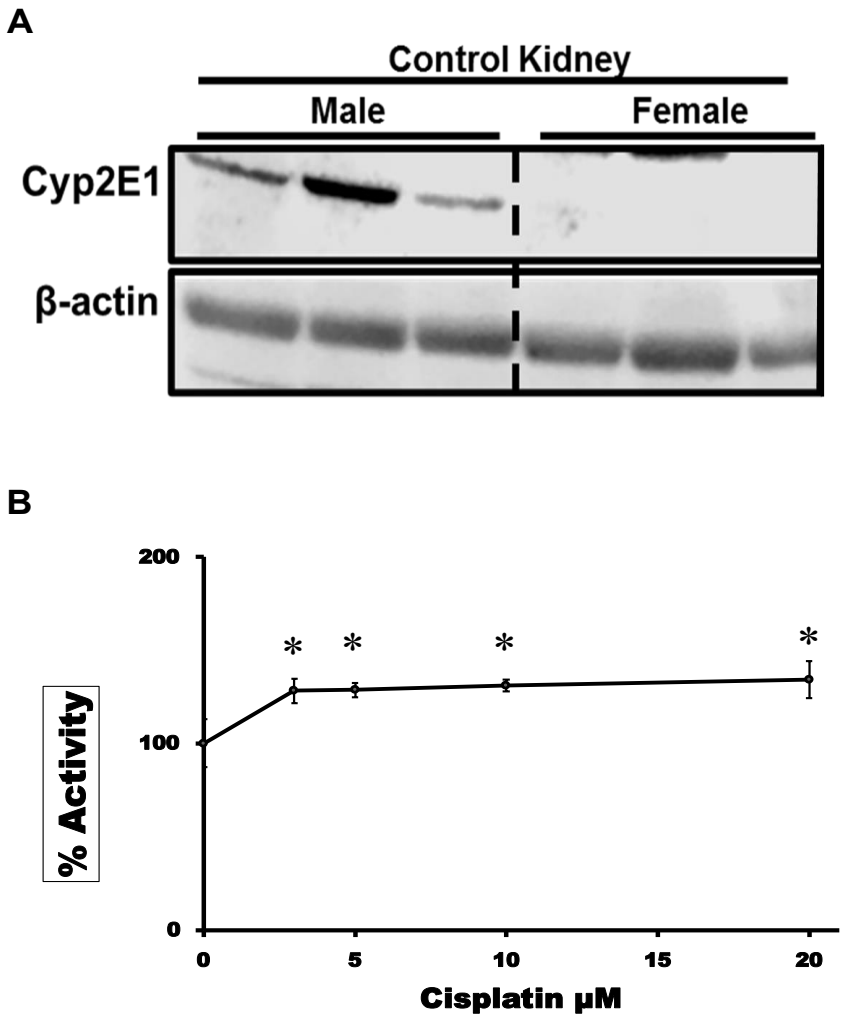

Figure 2: CYP2E1 contributes to the lower susceptibility of female mice to CIN. Kidney homogenates from untreated C57BL/6J male and female mice were subjected to western blot analysis to assess baseline expression of CYP2E1 (A). Cytochrome P450 enzyme activities were assayed in kidney homogenate using the fluorogenic substrate, 7-ethoxy-4-trifluoromethyl coumarin (7-EFC), which detects CYP2E1 and CYP1A2 activities, in the presence or absence of 3, 5, 10 and 20 μM 4MP. The enzyme activities of homogenates with cisplatin were expressed in RFU/min/mg protein and normalized to 100% of the control activity of homogenates without cisplatin. Data represent means ± SEM of 3 separate measurements. *$p < 0.05$ (compared to respective control).

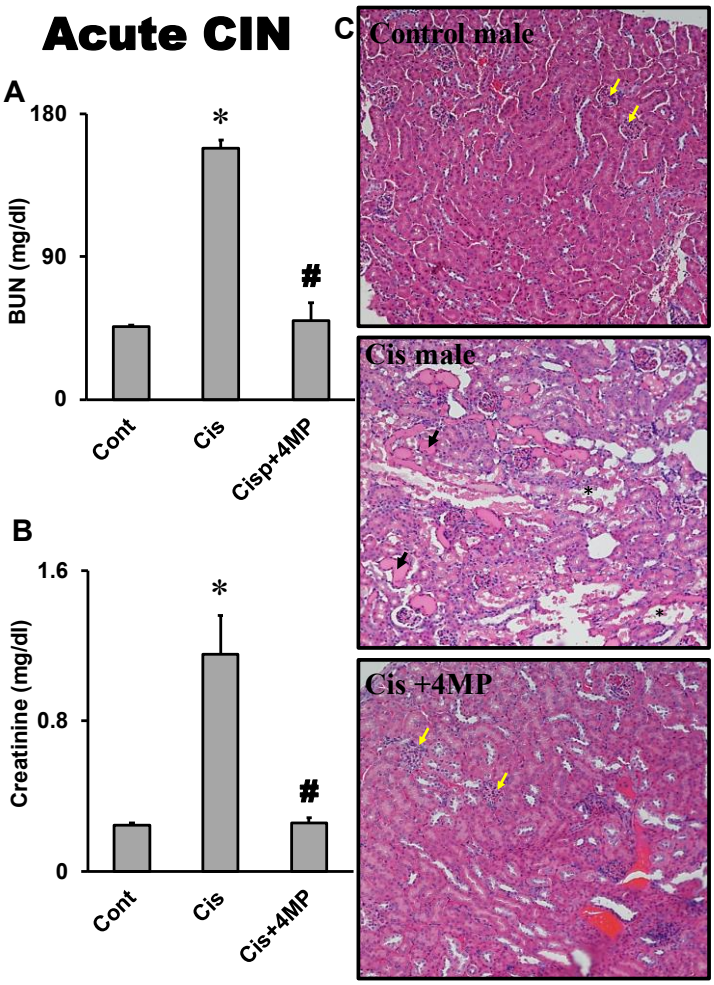

Figure 3: 4MP protects against cisplatin-induced acute kidney injury in C57BL/6J mice. C57BL/6J mice were treated with 20 mg/kg cisplatin and either 50 mg/kg 4MP or saline (control) for 3 days to collect blood and kidney tissue samples. Graphs are means ± SEM for plasma blood urea nitrogen (BUN) (A) and creatinine levels (B). n= 6 animals per group. *$p< 0.05$ (compared to female control). #$p<0.05$ (compared to cisplatin). Tissue sections were stained for H&E to visualize kidney morphology. Representative images of H&E staining of kidney sections (C) in control show normal renal corpuscle, glomerulus, and Bowman's capsule (yellow arrow). In contrast, tissue from cisplatin-dosed mice shows dilated tubules, tubular necrosis (black star), and cast formation (black arrowhead) (x200).

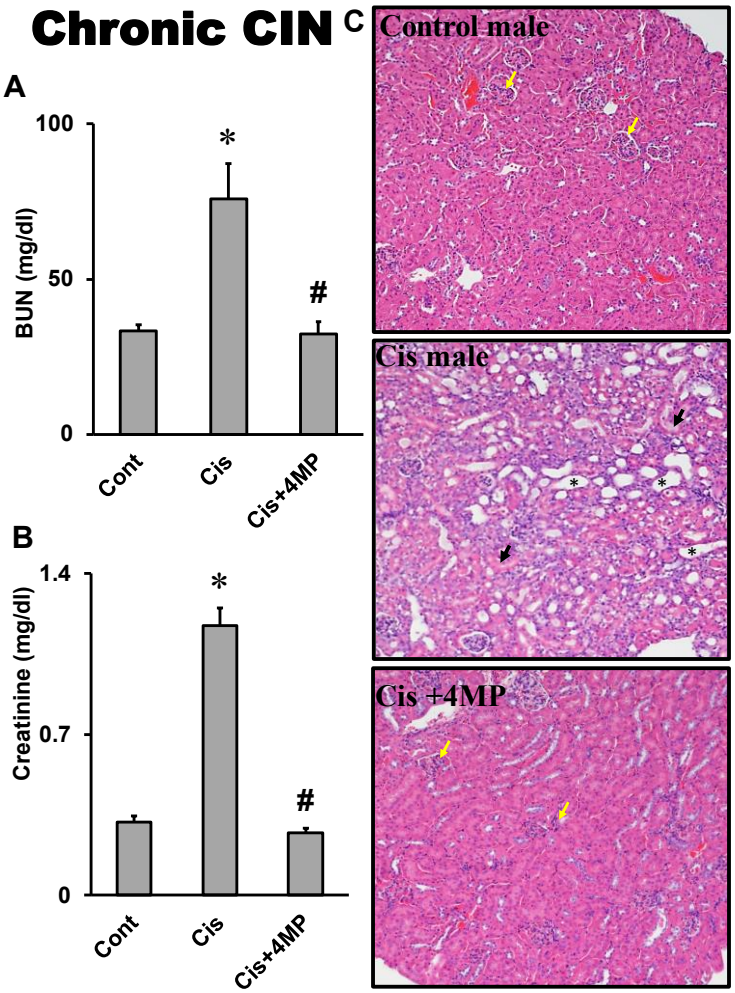

Figure 4: 4MP protects against cisplatin-induced chronic kidney injury in C57BL/6J mice. C57BL/6J mice were treated with 9 mg/kg cisplatin and 50 mg/kg of saline control once a week for 4 weeks to collect blood and kidney tissue samples. Graphs are means ± SEM for plasma blood urea nitrogen (BUN) (A) and creatinine levels (B). n= 6 animals per group. *$p< 0.05$ (compared to female control). #$p<0.05$ (compared to cisplatin). Tissue sections were stained for H&E to visualize kidney morphology. Representative images of H&E staining of kidney sections (C) in control show normal renal corpuscle, glomerulus, and bowman's capsule (yellow arrow). In contrast, tissue from cisplatin-dosed mice shows dilated tubules, tubular necrosis (black star), and cast formation (black arrowhead) (x200).

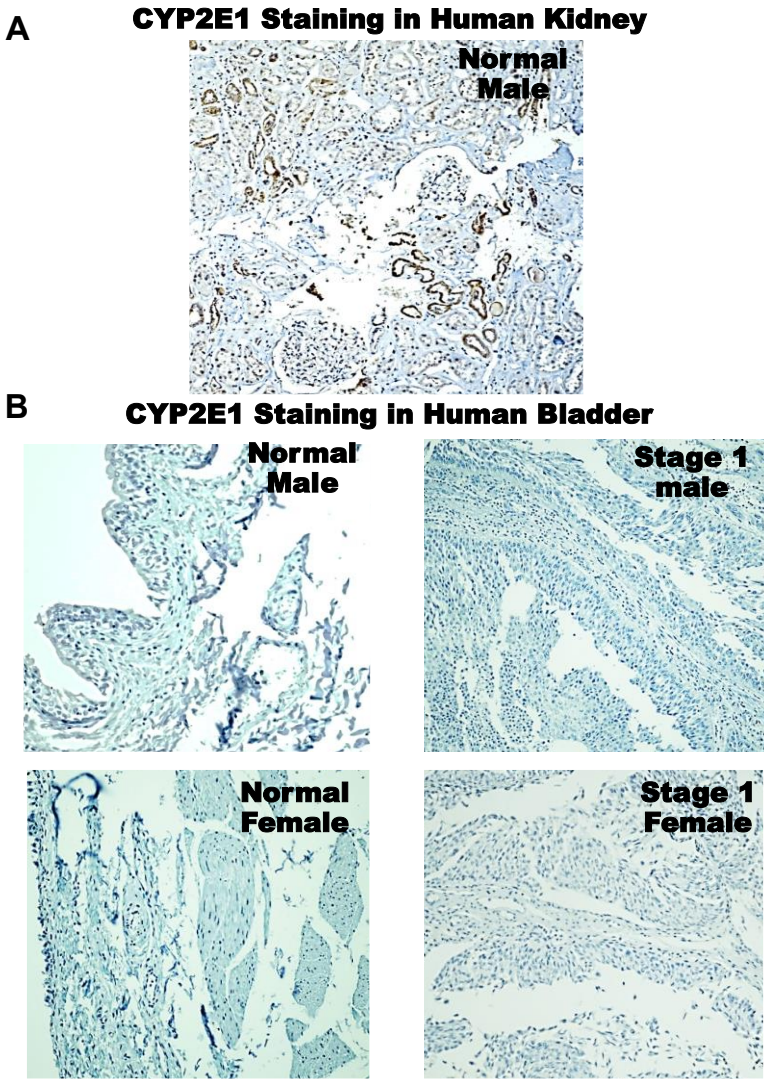

Figure 5: CYP2E1 expression in the kidney and bladder of human tissue sections.

Immunohistochemistry staining of CYP2E1 male kidney tissue (magnification: x200). (A). Immunohistochemistry staining of CYP2E1 in normal and stage 1 BCa tissue sections (B) (magnification: x200). Kidney image is representative of 3 male donors per group. Bladder images are representative of male and bladder carcinoma or normal tissue derived from a tissue microarray, containing 35 urothelial carcinoma, 4 squamous cell carcinoma, 1 adenocarcinoma, 10 normal bladder human tissue. Unstained microarray slide was obtained from TissueArray (BL1002b) and stained for CYP2E1.

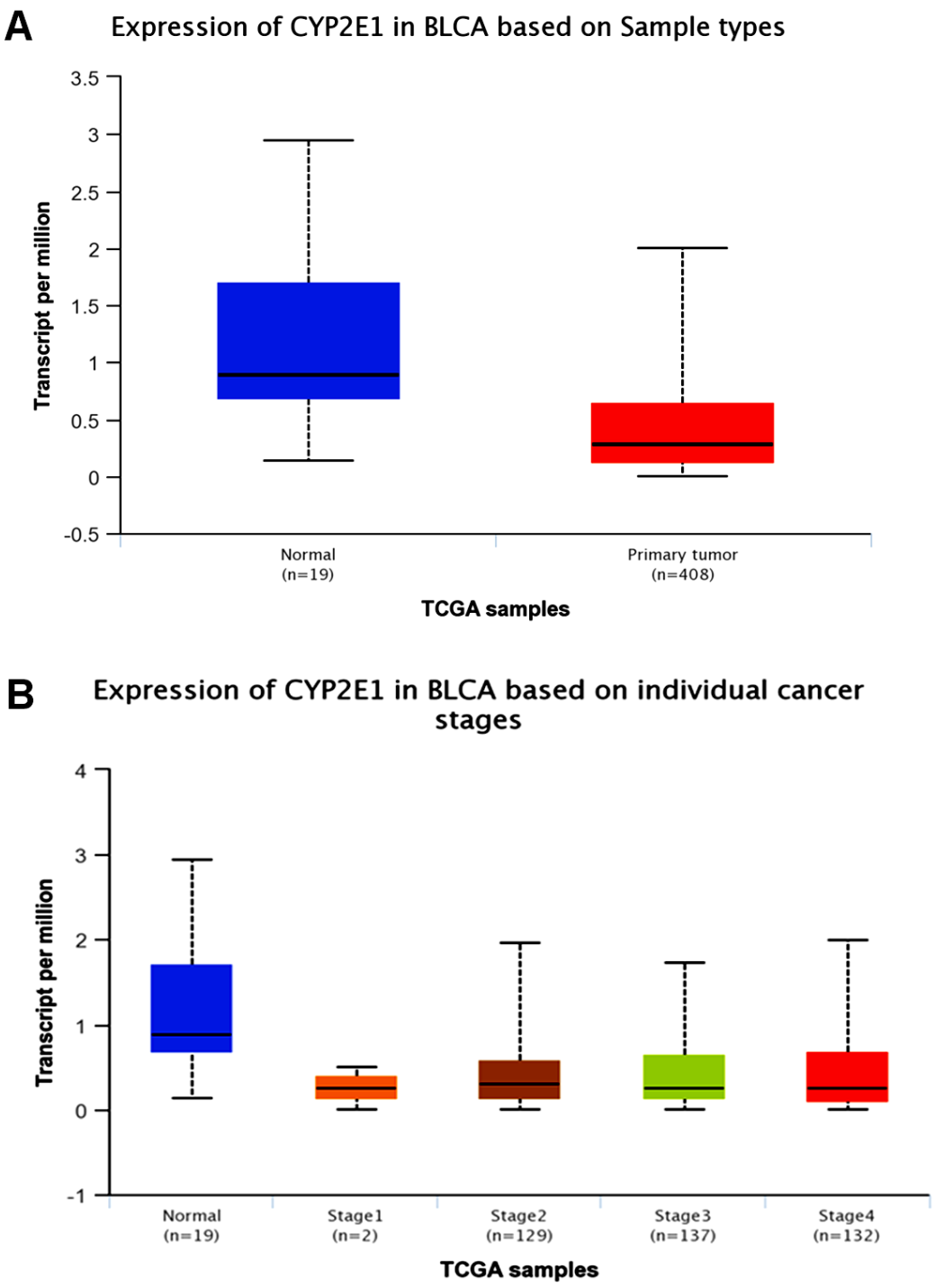

Figure 6: CYP2E1 expression in patients with primary BCa tumor. UALCAN was used to analyze CYP2E1 expression in TCGA patients with BCa based on sample type (A) or individual cancer stages (B).

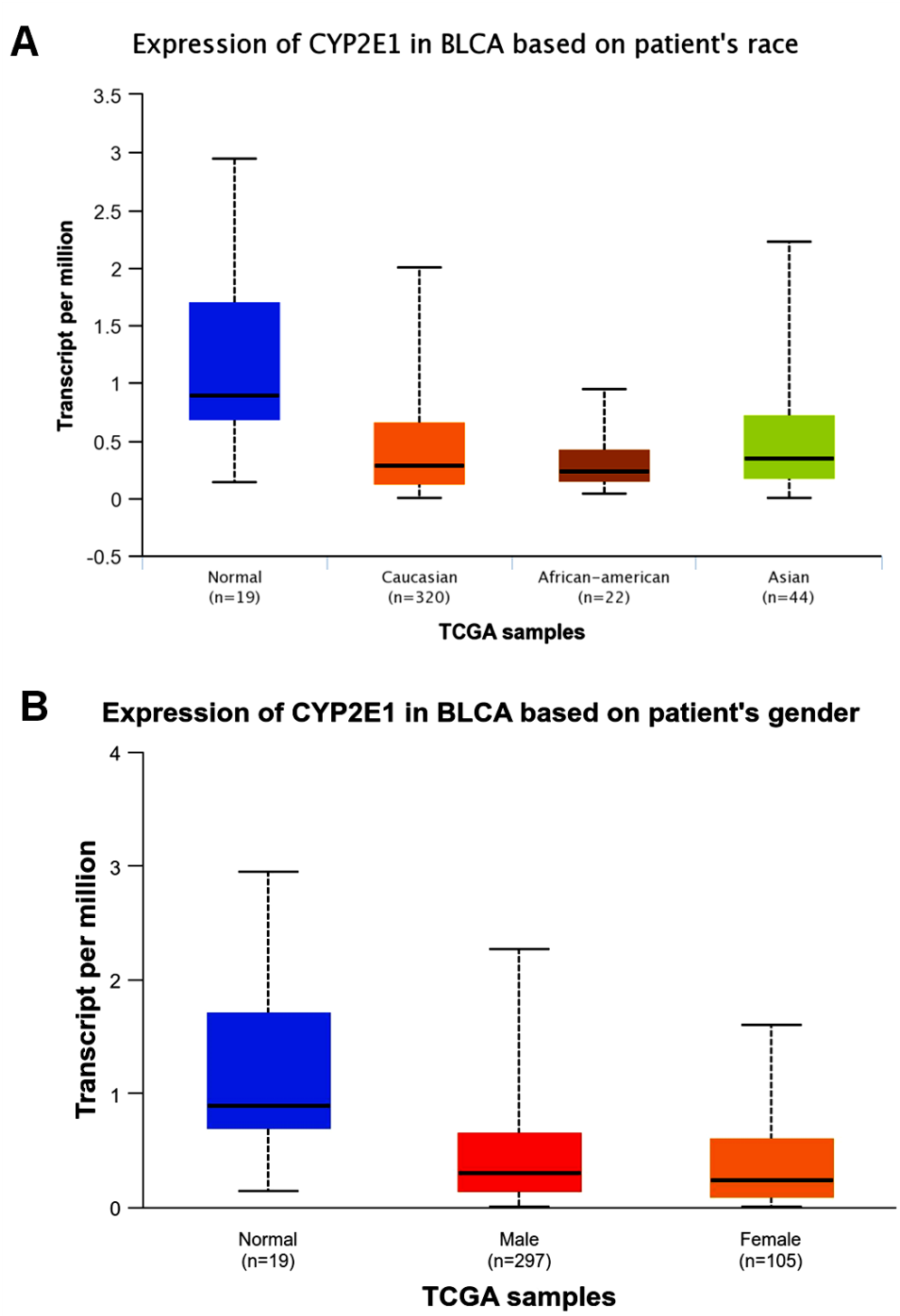

Figure 7: Race and sex and differences in CYP2E1 expression of BCa human patients. UALCAN was used to analyze CYP2E1 expression in TCGA patients with BCa based on patients' race (A) or patient's sex (B).

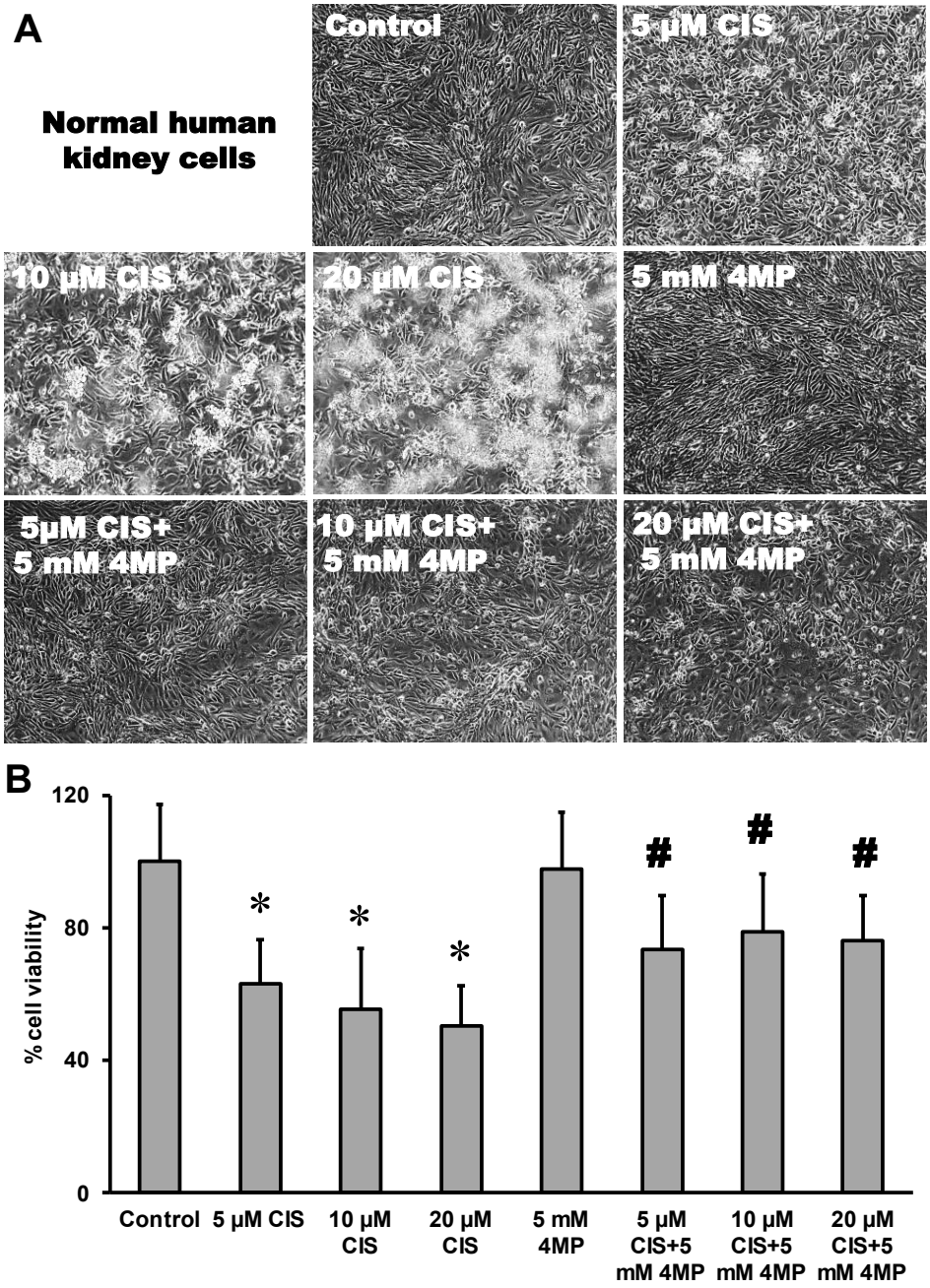

Figure 8: Effect of 4MP in cisplatin-treated NHK cells. Cells were treated with 5, 10, or 20 µM cisplatin alone or in combination with 5 mM 4MP. Phase contrast images of control and treated cells (A) (x200). % MTT cytotoxicity assay was performed to determine % cell viability (B). Bars represent means ± SEM for three separate experiments. *$p < 0.05$ versus control; #$p < 0.05$ versus cisplatin alone treatments. (CIS=cisplatin).

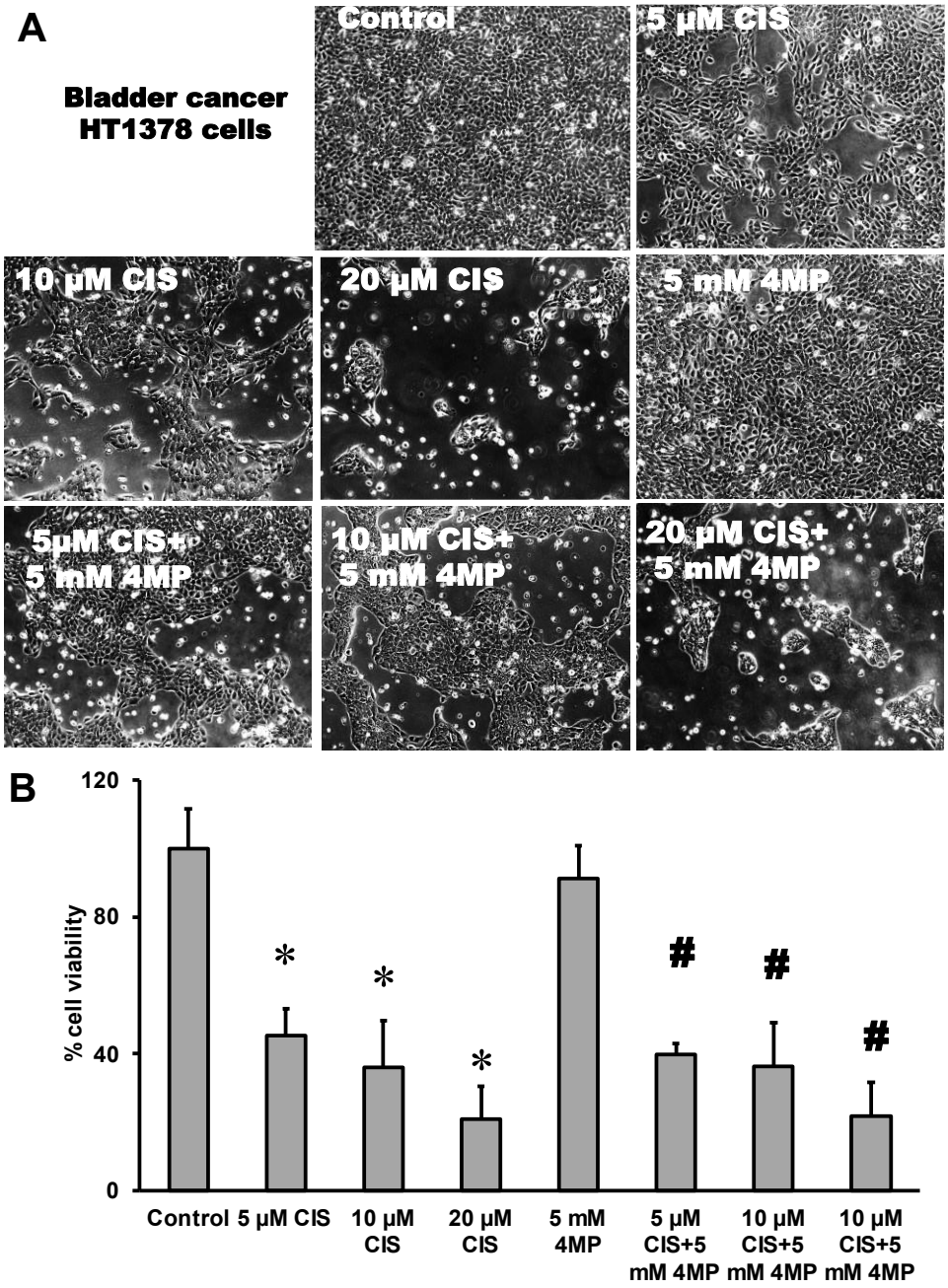

Figure 9: Effect of 4MP in cisplatin-treated Bladder cancer HTB9 cells. Cells were treated with 5, 10, or 20 µM cisplatin alone or combined with 5 mM 4MP. Phase contrast images of control and treated cells (A) (x200). MTT cytotoxicity assay was performed to determine % cell viability (B). Bars represent means ± SEM for three separate experiments. *$p < 0.05$ versus control; #$p < 0.05$ versus 4MP alone treatment. (CIS=cisplatin).