Amelioration of Cisplatin-Induced Nephrotoxicity in Rats by Tetramethylpyrazine, a Major Constituent of the Chinese Herb *Ligusticum wallichi*

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Nephrotoxicity of the anticancer drug, cisplatin (CP) involves enhanced renal generation of reactive oxygen metabolites and lipid peroxidation caused by decreased levels of antioxidants and antioxidant enzymes. Tetramethylpyrazine (TMP) is known to act as a strong antioxidant. Therefore, in the present work, we aimed at testing the possible protective or palliative effect of TMP on CP nephrotoxicity in rats. TMP was given orally at a dose of 80 mg·kg^{-1}·day^{-1} for 7 days. Some of these rats were given a single intraperitoneal injection of CP (or vehicle) at a dose of 6 mg/kg on Day 6 of treatment. Animals were sacrificed 6 days after CP (or vehicle) treatment, and blood, urine, and kidneys were obtained. Nephrotoxicity was assessed biochemically by measuring creatinine and urea in serum, reduced glutathione (GSH) concentration in renal cortex, by urinalysis, and histopathologically by light microscopy. CP significantly increased the concentration of urea and creatinine ($P < 0.05$) by about 128% and 170%, respectively; increased urine volume and *N*-acetyl-β-D-glucosaminidase (NAG) activity; and significantly decreased osmolality and protein concentrations. CP treatment reduced GSH by about 34% ($P < 0.05$) and superoxide dismutase (SOD) and total antioxidant activity (TOX) by about 28% and 21%, respectively ($P < 0.05$). TMP pretreatment significantly mitigated all of these effects. Sections from saline- and TMP-treated rats showed apparently normal proximal tubules. However, kidneys of CP-treated rats had a moderate degree of necrosis. This was markedly reduced when CP was given after pretreatment with TMP. CP cortical concentration was not significantly altered by TMP treatment. The results suggest that TMP ameliorated the histological, physiological, and biochemical indices of nephrotoxicity in rats. Pending further pharmacological and toxicological studies, TMP may potentially be useful as a nephroprotective agent. Exp Biol Med 233:891–896, 2008

Key words: cisplatin; nephrotoxicity; tetramethylpyrazine

Introduction

Tetramethylpyrazine (TMP, also named ligustrazine) is a compound purified from the plant *Ligusticum wallichi* Franchat (family Umbelliferae), a herb that has been used widely in China to treat vascular disorders (1). In rats, it has been found to protect against burn-induced acute myocardial injury (2), ischemia-reperfusion injury (3), nephritis (4), alcohol-induced renal toxicity (5), gastric mucosal injury in a model of acute necrotizing pancreatitis (6), and adriamycin- and gentamicin-induced apoptosis in renal tubular cells (7, 8). It has also been shown to reverse multidrug resistance of the hepatocellular carcinoma, HepG2/ADM *in vitro* (9). Pharmacologically, TMP has been characterized as a phosphodiesterase inhibitor and an antiplatelet agent (10), a true calcium antagonist (11), an antiapoptotic (12), and a strong antioxidant drug (13, 14).

Cisplatin (*cis*-diamminedichloroplatinum (II), CP) is a major antineoplastic drug for the treatment of solid tumors such as metastatic bladder, testicular, and ovarian carcinomas (15). The drug can cause many toxicities (16), but the chief dose-limiting one is nephrotoxicity (17). Several, often interrelated mechanisms of actions have been hypothesized to induce the nephrotoxicity. These include apoptosis (18)

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and inflammatory mechanism (19), production of tumor necrosis factor (TNF)-α by renal parenchymal cells (20), and generation of reactive oxygen species (ROS) that include superoxide dismutase, hydrogen radical, hydrogen peroxide, singlet oxygen, and nitric oxide (21). As TMP is a strong antioxidant agent, and as CP nephrotoxicity involves generation of ROS (22), it was therefore of interest to investigate whether TMP may affect some of the physiological, biochemical, and histological changes induced by CP in rats. Several agents have been used before in both animals and humans in an attempt to either ameliorate or protect against CP nephrotoxicity [reviewed in ref. 23]. However, as far as we are aware, there is no report in the literature on the effect of TMP on the nephrotoxicity of CP. This is the subject of the present work.

Materials and Methods

Animals and Treatment. Twenty-four male Wistar rats, aged 8 weeks and initially weighing 193 ± 10 g, were given a standard laboratory diet and water *ad libitum*. They were randomly divided into four equal groups and individually housed in metabolic cages, to facilitate urine collection, at a temperature of 23 ± 2°C, relative humidity of 50%–60% and a 12:12-hr light:dark cycle. An acclimatization period of 6 days was allowed for the rats before experimentation. The rats were weighed at the beginning of the experiment and just before killing. Rats were cared for under a protocol approved by the Animal Research Ethics Committee of our college and according to the NIH Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985.

The rats in the four groups were treated as follows:

Group 1: Normal saline (control, 0.5 ml per rat) given orally for 7 consecutive days, and on Day 7 also given a single intraperitoneal injection of saline (0.5 ml per rat);

Group 2: Normal saline (control, 0.5 ml per rat) given orally for 7 consecutive days, and on Day 7 also given a single intraperitoneal (ip) injection of CP (6 mg/kg), dissolved in normal saline, at a concentration of 1 mg/ml;

Group 3: TMP (50 $mg \cdot kg^{-1} \cdot day^{-1}$) given orally once daily for seven consecutive days, and on Day 7 also given a single ip injection of saline (0.5 ml per rat);

Group 4: TMP (50 $mg \cdot kg^{-1} \cdot day^{-1}$) given ip once daily for 7 consecutive days, and on Day 7 also given a single ip injection of CP (6 mg/kg), dissolved in normal saline, at a concentration of 1 mg/ml.

One day before the rats were sacrificed, urine of each rat was collected over a 24-hr period, and the volume was measured. Six days after CP or vehicle administration, rats were anesthetized with a combination of ketamine (60 mg/kg) and xylazine (5 mg/kg) given ip. Blood was collected from the inferior vena cava in plain plastic tubes, left to stand at 4°C for 1 hr, and centrifuged at 900 *g* at 5°C for 15 mins to separate serum. The serum obtained was stored frozen at –80°C to await biochemical analyses, and the rats were sacrificed by an overdose of anesthesia. The kidneys were removed from the rats, washed with ice-cold saline, blotted with a piece of filter paper and weighed. A small piece from the left kidney was fixed in 10% buffered formalin. The cortex of the right kidney was excised from the medulla. Part was stored immediately deep frozen (–80°C) for measuring platinum concentration, and part was rapidly homogenized in ice-cold saline to produce 10% (w/v) tissue homogenate.

Biochemical Analysis. The concentrations of urea and creatinine in serum were spectrophotometrically measured using commercial kits (Humana, Spain). Total antioxidant activity was measured in serum by using a commercial kit (Oxford Biomedical Research, Oxford, MI). Urine osmolality was measured by the freezing point depression method (–70°C) using an Advanced Digimatic osmometer (Advanced Instruments Inc, Norwood, MA), and *N*-acetyl-β-glucosaminidase (NAG) activity by kits from Diazyme (General Atomics, San Diego, CA). Urine protein concentration was measured spectrophotometrically using a kit from Humana. In renal cortex homogenates, glutathione (GSH) concentration (24) and superoxide dismutase (SOD) activity were measured spectrophotometrically (Randox, Antrim, UK). The concentration of CP (as platinum) in cortical tissue was measured by a flameless atomic absorption spectrophotometer (Perkin-Elmer, 3300 DV ICP-OES equipped with a cross-flow nebulizer, in addition to an ultrasonic nebulizer; Waltham, MA). The procedure involves mineralization of the kidney cortex tissue with a mixture of concentrated HNO_3 and H_2O_2, followed by determination of platinum in the extract, using inductively coupled serum optical emission spectrometry, at an emission wavelength of 265.945 nm.

Histopathology. Fixed sections (5 μm thick) were cut and stained with haematoxylin and eosin and Periodic Acid Schiff (PAS). Coded slides were examined using light microscopy by a pathologist unaware of the treatments and assigned a score as described by Mohan *et al*. (18), which represents the approximate extent of necrotic area in the cortical proximal tubules on a scale of 0–4 (0 = no necrosis; 1 = a few focal necrotic spots; 2 = necrotic area about one-half; 3 = necrotic spots about two-thirds; 4 = nearly all of the area necrotic).

Statistical Analysis. Values reported are mean ± SEM (from six rats). Differences between means of the four groups were estimated by a one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. A value of $P < 0.05$ was considered statistically significant.

Drug and Chemicals. Cisplatin (Ebewe Pharma, Untrech, Austria) was obtained from SQU Hospital Pharmacy. Tetramethylpyrazine and platinum standard solution were obtained from Sigma-Aldrich (St Louis, MO), total antioxidant kits were from Oxford Biomedical Research, and SOD kits were from Randox. Protein, creatinine, and urea spectrophotometric kits were bought

Table 1. Effect of Treatment With Cisplatin (CP) Either With or Without Tetramethylpyrazine (TMP) on Body and Kidney Weights of Rats[a]

Group	Initial weight (g)	Final weight (g)	Change (%)	Kidney weight (g)	Kidney weight (%)
Saline (control)	193.9 ± 12.3[a]	228.6 ± 18.7[a]	17.9 ± 2.6[a]	1.49 ± 0.1[a]	0.70 ± 0.3[a]
CP + saline	194.5 ± 9.9[a]	178.8 ± 11.7[a]	−8.1 ± 1.6[b]	1.60 ± 0.1[a]	0.89 ± 0.6[b]
TMP + saline	195.8 ± 14.3[a]	223.2 ± 13.7[a]	14.0 ± 3.2[a]	1.43 ± 0.2	0.64 ± 0.5[a]
TMP + CP	192.8 ± 10.4[a]	188.2 ± 17.7[a]	−2.3 ± 0.9[c]	1.49 ± 0.2	0.79 ± 0.6[c]

[a]Values are mean ± SD ($n = 6$). Values (among the four groups) followed by different letters are significantly different ($P < 0.05$). Either saline or TMP (80mg/kg/day) was given intraperitoneally for 7 days. On day 7, some of these rats were given a single intraperitoneal injection of either CP (6 mg/kg) or saline. Rats were weighed (to the nearest gram), then killed 6 days after CP treatment.

Table 2. Effect of Treatment With Cisplatin (CP) Either With or Without Tetramethylpyrazine (TMP) on Serum Urea and Creatinine Concentrations, Total Antioxidant Activity (TOX), and Reduced Glutathione (GSH) and Superoxide Dismutase (SOD) in Renal Cortex[a]

Group	Urea (mmol/L)	Creatinine (μmol/L)	TOX* (μg/l)	SOD (U/g)	GSH (μg/g)
Saline (control)	5.8 ± 0.5[a]	35.3 ± 2.3[a]	0.585 ± 0.32[a]	1.43 ± 0.1[a]	7.34 ± 0.46[a]
CP + saline	13.5 ± 1.4[b]	94.5 ± 8.7[b]	0.461 ± 0.45[b]	1.02 ± 0.1[b]	5.17 ± 0.44[b]
TMP + saline	5.9 ± 0.6[a]	37.5 ± 3.0[a]	0.621 ± 0.52[a]	1.57 ± 0.2[a]	7.83 ± 0.57[a]
TMP + CP	8.3 ± 0.9[c]	51.1 ± 5.4[c]	0.503 ± 0.58[c]	1.39 ± 0.2[a]	6.93 ± 0.53[c]

[a]Values are mean ± SD ($n = 6$). Values (among the four groups) followed by different letters are significantly different ($P < 0.05$). Either saline or TMP (80 mg/kg/day) was given intraperitoneally for 7 days. On day 7, some of these rats were given a single intraperitoneal injection of either CP (6 mg/kg) or saline. Rats were weighed (to the nearest gram), then killed 6 days after CP treatment.
* m*M* uric acid equivalents.

Table 3. Effect of Treatment With Cisplatin (CP) Either With or Without Tetramethylpyrazine (TMP) on Urinalysis[a]

Group	Volume (ml)	NAG (U/L)	Protein (g/100 ml)	Osmolality (osmol)
Saline (control)	12.8 ± 1.2[a]	3.0 ± 0.2[a]	5.8 ± 0.3[a]	2032.4 ± 101.7[a]
CP + saline	18.3 ± 1.9[b]	10.2 ± 1.5[b]	8.9 ± 0.9[b]	1275.3 ± 126.3[b]
TMP + saline	11.5 ± 1.4[a]	2.8 ± 0.3[a]	5.4 ± 0.4[a]	2134.4 ± 161.1[a]
TMP + CP	13.6 ± 2.3[c]	6.0 ± 0.4[c]	7.4 ± 0.8[c]	1439.2 ± 133.7[c]

[a]Values are mean ± SD ($n = 6$). Values (among the four groups) followed by different letters are significantly different ($P < 0.05$). Either saline or TMP (80 mg/kg/day) was given intraperitoneally for 7 days. On day 7, some of these rats were given a single intraperitoneal injection of either CP (6 mg/kg) or saline. Rats were weighed (to the nearest gram), then killed 6 days after CP treatment.

from Humana, and NAG kits were from General Atomics. The rest of the chemicals were analytical grade.

Results

Rats given CP lost about 8% of their initial body weight, while their respective controls and TMP-treated rats gained about 15–18% of their initial body weight. However, rats given both TMP and CP lost about 2% of their initial body weight (Table 1).

Table 2 shows the effects of treatment with saline, CP, TMP + saline and TMP + CP on the concentrations of serum urea and creatinine and SOD and TOX activities, and GSH concentration in renal cortical homogenates. Values obtained from rats treated with TMP alone were not significantly different from those treated with saline ($P > 0.1$), although GSH concentration and SOD activity in TMP-treated rats tended to be higher than in saline-treated rats. On the other hand, CP significantly increased the concentrations of urea and creatinine ($P < 0.05$) by about 167% and 133%, respectively. CP treatment reduced cortical GSH concentration by about 30% ($P < 0.05$) and the activities of SOD and TOX by about 28% and 21%, respectively ($P < 0.05$). TMP significantly mitigated these effects.

Table 3 depicts the effects of treatment of rats with CP either with or without TMP on urinalysis. Compared with saline-treated animals, CP and CP + TMP treatments increased the 24-hr urine volume by about 36% ($P < 0.05$) and 8% ($P > 0.1$), respectively. Urinary NAG activity was raised by about 225%, compared with saline-treated controls ($P < 0.001$) and by 79% in rats treated with CP + TMP ($P < 0.05\%$). Urine osmolality in CP-treated rats was decreased by about 37% compared with saline-treated controls. In rats treated with TMP + CP, the decrease was 18% ($P < 0.05$). TMP alone had no significant effect on urine osmolality ($P > 0.1$).

Figure 1 shows representative histological micrographs of renal cortex from the four groups used. Sections from the saline-treated rats (control) and TMP-treated rats showed

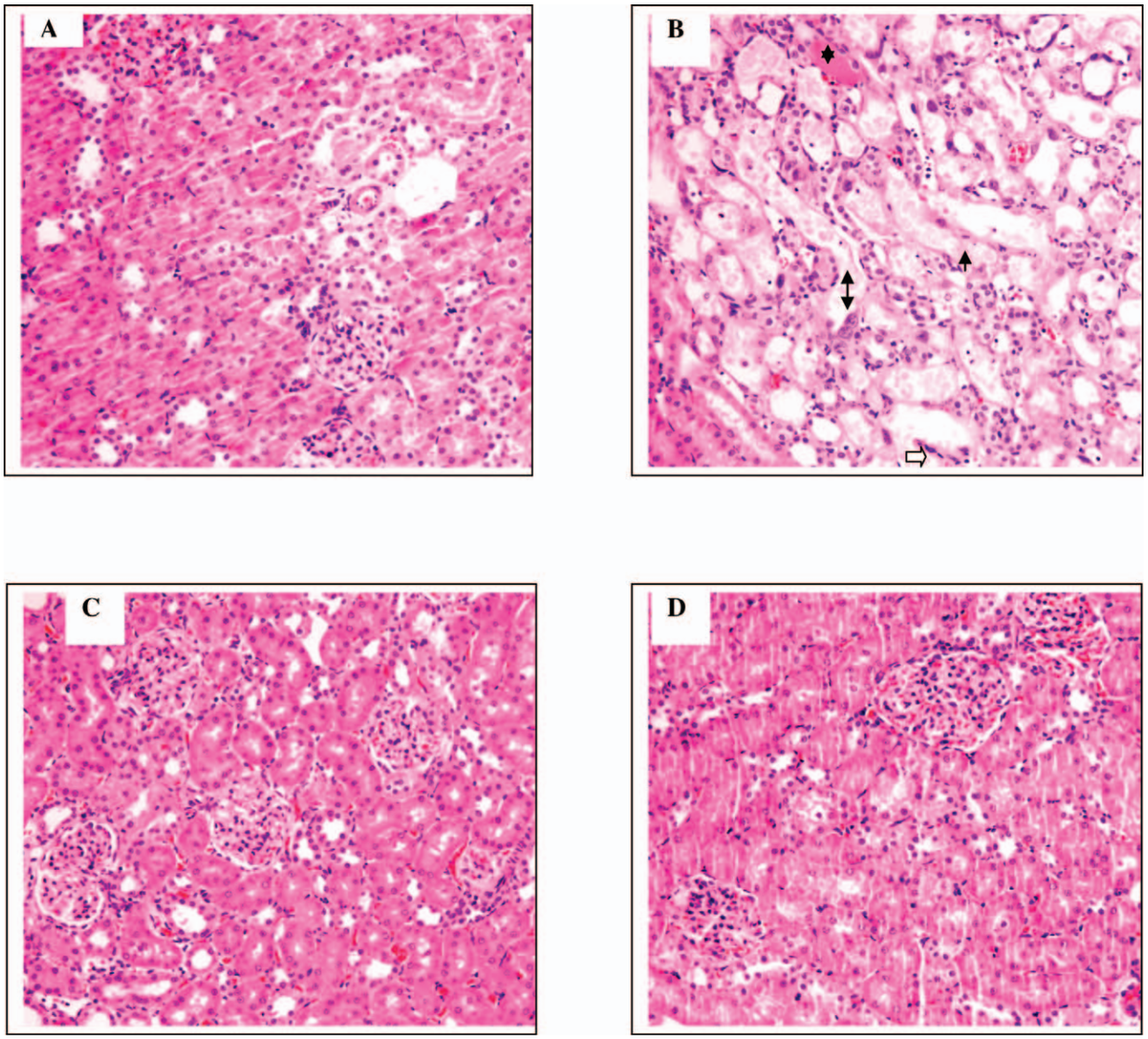

Figure 1. Light microscopy photomicrographs depicting sections from kidneys of rats receiving (A) saline control; (B) cisplatin given at a single oral dose (6 mg/kg); (C) tetramethylpyrazine (80 mg·kg^{-1}·day^{-1} intraperitoneally for 7 days); and (D) cisplatin + tetramethylpyrazine, at the above doses. Treatment with either saline or tetramethylpyrazine alone did not cause renal damage. Kidneys of cisplatin-treated rats caused marked renal injury with marked necrosis (filled arrows), loss of epithelial cells (double arrow), vacuolization (open arrow), and cast formation (star). Tetramethylpyrazine together with cisplatin markedly reduced the severity of the renal damage. A color version of the figure is available in the online journal.

apparently normal histology (Fig. 1A and D). CP-treated rats had extensive epithelial cell vacuolization, swelling, desquamation, and necrosis, occurring primarily in the proximal convoluted tubules (Fig. 1B). These changes were markedly decreased when TMP was given prior to CP (Fig. 1C). Table 4 shows a semiquantitative analysis of the histology of the kidney of rats treated with CP with or without TMP.

The concentration of platinum in the renal cortex of rats given TMP + CP was not significantly different from that in rats treated with saline + CP ($P > 0.1$) (data not shown).

Table 4. Semiquantitative Analysis of Histology of Kidney of Rats Treated With Cisplatin (CP) Together With or Without Tetramethylpyrazine (TMP)

Group	Treatments	Score
1	Saline (control)	+0
2	CP + saline	+3
3	TMP + saline	+0
4	TMP + CP	+1

Discussion

Cisplatin is an important anticancer agent for treating various solid tumors. The key limitation of this drug is nephrotoxicity (17, 19, 23). In this work, all rats survived the experimental period until they were sacrificed. CP administration at a dose of 6 mg/kg resulted in acute renal failure, similar to many previous studies (e.g. Refs. 17, 25). The reduction in body weight following CP treatment may possibly be due to the injured renal tubules and the subsequent loss of the tubular cells to reabsorb water, leading to dehydration and loss of body weight. The above, and the increased urine volume voided might be some of the reasons behind the loss of body weight. Treatment with TMP antagonized this reduction significantly, but not completely. This alleviation of CP-induced body weight reduction is a reflection of the general palliative effect of TMP on the nephrotoxicity.

TMP has widely been used in China to treat a number of vascular disorders (1). Besides vasodilatory actions and antiplatelet activity, TMP has strong effects to remove reactive oxygen species (ROS), and the ROS decrement may be associated with increased activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (13, 14), and CP nephrotoxicity involves generation of ROS (22). This is the first report, as far as we are aware, on the effect of TMP in cisplatin-induced nephrotoxicity.

The results of this work indicate that TMP, at the dose used, was effective in mitigating the biochemical, physiological, and histological changes induced by CP.

Further support for the salutatory action on the renal function was obtained from the alleviation of the increase in serum concentrations of creatinine and urea and the decrease in renal GSH concentration and SOD and TOX activities. We have confirmed that plasma concentrations of some oxidative stress markers are elevated at an early stage in chronic kidney disease and regularly increase with its progression (26). The urine analysis in this work confirmed that CP caused a polyuric acute renal failure, as judged by significant increases in the volume of urine voided, and in the activity of NAG and protein concentration, concomitant with a significant decrease in osmolality.

These effects induced by CP were significantly reversed, albeit not completely, by treatment with TMP, adding further evidence that this antioxidant has the potential to be used to ameliorate CP nephrotoxicity. The moderate necrosis of the renal tissues seen in the slides of kidneys from rats treated with CP was mitigated by pretreatment with TMP. This is in line with the biochemical changes observed in serum and urine. The mechanism by which TMP exerted its partial nephroprotective action is not certain. However, it is well established that TMP has a strong antioxidant effect in several organs (22). From both *in vivo* studies and cell culture, the antioxidant GSH has been shown to protect against CP nephrotoxicity, and CP has been recently confirmed to be metabolized to a nephrotoxicant through a GSH-conjugate intermediate (18). Oxidative stress in kidney plays an important role in CP-induced renal damage (22, 23), and several antioxidants and thiol compounds have been shown to protect against CP nephrotoxicity (23). The present work confirms that CP depletes renal GSH concentration and SOD and TOX activities, leaving the renal tissues vulnerable to damage by oxygen radicals that are responsible for the induction of tubular epithelial cell death.

The present data show that pretreatment of rats with the antioxidant agent TMP increased GSH concentration and SOD and TOX activities. These actions may have led to the amelioration of the histological, serum, and urine biochemical indices of renal damage studied here.

The kidneys accumulate CP in the renal tissue more than in any other organ, resulting in necrosis of the terminal portion of the proximal renal tubules and apoptosis in the distal nephron (17). TMP treatment did not significantly alter the concentration of CP in the renal cortex in rats treated with CP. This suggests that the nephroprotective effect of TMP is not mediated through a reduction in the accumulation of the drug in the kidney cortex, and furthermore implies that the antitumor activity of CP may not be adversely affected by the coadministration of TMP.

In conclusion, we have shown that some signs of CP nephrotoxicity in rats are mitigated by pretreatment with TMP. Pending further pharmacological and toxicological studies to confirm TMP safety and its efficacy to protect against CP nephrotoxicity, it can be concluded that TMP is a potentially promising nephroprotectant in animals and humans. Further studies will be conducted to verify the nephroprotectant action of TMP in experimental tumor animal models and that the coadministration of TMP with CP does not diminish its oncological effect.

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