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Protection Against Cisplatin-Induced Nephrotoxicity by a Carbon Monoxide-Releasing Molecule

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ABSTRACT

Nephrotoxicity is one of the main side effects caused by cisplatin (CP), a widely used anti-neoplastic agent. Here, we examined the effect of a novel water-soluble carbon monoxide-releasing molecule (CORM-3) on CP-mediated cytotoxicity in renal epithelial cells and explored the potential therapeutic benefits of carbon monoxide in CP-induced nephrotoxicity *in vivo.* Exposure of LLC-PK1 cells to CP (50 µM) caused significant apoptosis as evidenced by caspase-3 activation and increased number of floating cells. Treatment with CORM-3 (1-50 µM) resulted in a remarkable and concentration-dependent decrease in CP-induced caspase-3 activity and cell detachment. This effect involved activation of the cGMP pathway as ODQ, a guanylate cyclase inhibitor, completely abolished the protection elicited by CORM-3. Using a rat model of CP-induced renal failure, we found that treatment with CP (7.5 mg/kg) caused a significant elevation in plasma urea (6.6-fold) and creatinine (3.1-fold) levels, which was accompanied by severe morphological changes and marked apoptosis in tubules at the cortico-medullary junction. A daily administration of CORM-3 (10 mg/kg i.p.), starting 1 day prior to CP treatment and continuing for 3 days thereafter, resulted in amelioration of renal function as shown by reduction of urea and creatinine levels to basal values, decreased number of apoptotic tubular cells and improved histological profile. A negative control (iCORM-3) that is incapable of liberating CO failed to prevent renal dysfunction mediated by CP indicating that CO is directly involved in renoprotection. Our data demonstrate that CORM-3 can be used as a very effective therapeutic adjuvant in the treatment of CP-induced nephrotoxicity.

Key words: cisplatin, apoptosis, heme oxygenase-1, carbon monoxide, carbon monoxide-releasing molecules (CO-RMs), CORM-3.

Abbreviations: CP; cisplatin, CO; carbon monoxide, CO-RMs; carbon monoxide-releasing molecules.

INTRODUCTION

The chemotherapeutic agent cisplatin (*Cis*-Diammineplatinumdichloride) is an extremely effective anti-neoplastic drug commonly used for the treatment of solid tumours such as head and neck tumours, ovarian cancer and lymphomas (14). Despite its promising pharmacological features, CP-mediated nephrotoxicity is one of the most common side effects encountered in cancer patients leading to severe impairment of renal function after repeated exposure and prolonged treatments (5). CP is retained particularly in proximal tubule epithelial cells at the cortico-medullary junction, which are the main sites where the toxic effect of CP is manifested. Oxidative stress caused by increased generation of free radicals and caspase-mediated apoptosis play a major role in nephrotoxicity and renal dysfunction that progressively develop in response to CP treatment (5, 33). Kidneys, like other organs, are well equipped with diverse inducible antioxidant and anti-apoptotic enzymes which, as part of a ubiquitous and intrinsic natural defence, are engaged to detoxify cells from the stress inflicted by drugs and other xenobiotics introduced into the organism. Among these endogenous defensive systems, heme oxygenase-1 (HO-1) has recently emerged as a pivotal and effective cytoprotective protein because the ubiquitous products of its catalytic activity, carbon monoxide (CO) and biliverdin, exert biologically important functions within the cell. Biliverdin and its reduction product bilirubin are powerful antioxidants (8, 10), whereas CO is a versatile signaling molecule that possesses vasodilatory (19, 25), anti-inflammatory (23) and anti-apoptotic properties (13). In the kidney, the HO-1/CO system has been demonstrated to counteract several pathological conditions including ischemia-reperfusion injury (16), glomerular inflammation (34), renal failure (29) and

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angiotensin-mediated hypertension (1, 4). Notably, the induction of HO-1 has been reported to prevent nephrotoxicity as well as renal tubule apoptosis mediated by CP (3, 30), although the protective contribution of CO and biliverdin remains to be fully investigated. Based on the attractive hypothesis that stimulation of the HO-1/CO pathway could be exploited for therapeutic purposes, we have developed a technology that enables CO to be delivered in a controlled fashion to biological systems. This can be achieved by certain transition metal carbonyls and other carbonylating agents, a novel class of compounds that we initially characterized and termed CO-releasing molecules (CO-RMs) (17, 22). Extensive research has been conducted in our laboratory in order to improve the chemical and pharmacological features of these CO carriers (21). CORM-3 ($Ru(CO)_3Cl(glycinate)$) is a recently synthesized water-soluble compound that, in analogy with CO gas, has been shown to possess potent vasorelaxant properties (11), promote cardio-protection against ischaemia-reperfusion injury (9) and exert anti-inflammatory activities (28). Here we investigated the effects of CORM-3 on CP-mediated apoptosis in renal tubule epithelial cells (LLC-PK1) and utilized a rat model of CP-induced renal failure to explore the potential therapeutic benefits of CO against nephrotoxicity and renal dysfunction.

MATERIALS AND METHODS

Reagents and solutions. *Cis* diamminplatinumdichloride (cisplatin, CP), biliverdin and all other reagents were purchased from Sigma (Sigma-Aldrich, Poole, UK) unless otherwise specified. Stock solutions of CP (5 mM) were freshly prepared in dimethylsulphoxide (DMSO) on the day of the experiment. CORM-3 was synthesized and prepared freshly in distilled water (or sterile water for injection if used in animals) as previously reported by our group (9). An inactive compound (iCORM-3) was prepared by adding CORM-3 (2.5 mM) to a phosphate buffer solution (pH=7.4), left at room temperature for 2 days and finally bubbled with nitrogen to remove the residual solubilized CO (9, 11). The inhibitor of heme oxygenase activity, tin protoporphyrin IX (SnPPIX), was purchased from Frontier Scientific (Carnforth, Lancashire, UK) and prepared freshly as previously described (19). The guanylate cyclase inhibitor (1H-Oxadiazole [4, 3-a] quinoxaline-1-ore) (ODQ) was obtained from Alexis Corporation (UK).

Caspase-3 activity assay. Caspase-3 specific activity was measured by using a colorimetric assay (Promega-UK) and performed according to manufacturer's instructions. Briefly, cells were collected, resuspended in cell lysis buffer and subjected to 3 cycles of freezing and thawing before centrifugation. The collected supernatant (20 μg proteins) was used to measure caspase-3 activity. The colorimetric substrate (Ac-DEVD-pNA) was labeled with the chromophore *p*-nitroaniline (pNA) which is released from the substrate upon cleavage by DEVDase (caspase-3) and produces a yellow color

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that is monitored spectrophotometrically at 405 nm. Caspase-3 activity was expressed as pmol pNA liberated/h/µg protein.

TUNEL assay. DNA fragmentation as an index of apoptosis was detected by TdT mediated dUTP nick end labeling (TUNEL) using a DeadEnd Colorimetric TUNEL Assay kit (Promega, UK) according to manufacturer's instructions. Briefly, cells were initially grown in 4-well glass slide chambers (Fisher, UK); after each treatment, they were fixed with formalin and permeabilized with 0.2% Triton X100 in PBS. Cells were then reacted with deoxynucleotidyl transferase (TdT) and biotin nucleotides, and the reaction terminated with 2 X SSC solutions. Endogenous peroxidases were blocked with 0.3% H_2O_2 solution prior to incubation with streptavidin horse radish peroxidase (HRP) and stained with diamniobenzidine (DAB) substrate. Apoptotic cells were stained dark brown and were observed by light microscopy.

Cell culture and experimental protocol. Porcine renal tubule epithelial cells (LL-CPK1) were obtained from the European Collection of Animal Cell Culture (Salisbury, UK) and cultured as previously described (7). Confluent cells were exposed for 16 h to CP (50 µM) alone or in combination with increasing concentrations of either CORM-3 or iCORM-3 (1, 5, 10 or 50 µM). At the end of the incubation period, caspase-3 activity was measured as a marker of apoptosis. To investigate the possible involvement of cGMP, the inhibitor of guanylate cyclase ODQ (10 µM) was incubated for 30 min prior to addition of CP and CORM-3. Since biliverdin is generated alongside CO by heme

oxygenase, additional experiments were performed to examine the effect of biliverdin (0.5-10 µM) on CP-mediated caspase-3 activation.

Animal studies and experimental protocol. Male Wistar rats weighing 300-400 gm were used in all experiments. Rats were divided into 6 groups (*n*=5 each*)*: a) the control group received sterile distilled water (i.p.) daily for 4 days; b) the second group received a single dose of CP (7.5 mg/kg i.p); c) the third group received CORM-3 (10 mg/kg i.p.) daily for 4 days; d) the fourth and fifth groups, respectively, received CORM-3 or iCORM-3 daily starting 24 h prior to CP treatment and continuing for 3 days thereafter; To evaluate a possible involvement of heme oxygenase-1 induction following CORM-3 treatment (32), an additional set of experiments was investigated using tin protoporphyrin IX (SnPPIX), an inhibitor of heme oxygenase activity. Specifically, a sixth group received CORM-3 in combination with SnPPIX (10 mg/kg i.p.) daily starting 24 h prior to CP treatment and continuing for 3 days thereafter. After each treatment, blood samples were collected for measurements of plasma urea and creatinine levels. The right kidney was harvested and fixed with formalin for histological examination (H&E) and TUNEL assay.

Histopathological scoring

Histopathological scoring was assessed by a pathologist in a blinded manner and scores are reported as mean of 5 independent experiments. The following parameters were evaluated: glomerular necrosis, tubular necrosis, tubular dilatation, epithelial sloughing vascular congestion and extravasation. All these parameters were assessed

according to the degree of changes involved using a 0 to 4 grading system. A score of 0 corresponded to the absence of change, a score of 1 indicated marginal changes, a score of 2 corresponded with minimal changes, and 3 and 4 indicated moderate and severe changes, respectively.

Statistical analysis. Differences in the data among different groups were analyzed by one way analysis of variance (ANOVA) combined with Bonferroni test. Values were expressed as mean ± SEM and the differences were considered to be significant at $P<$ 0.05.

RESULTS

Confluent renal tubule epithelial cells (LLC-PK1) exposed to CP for 16 h displayed a significant increase in caspase-3 activity (Figure 1A). This effect was accompanied by severe damage as evidenced by an increased number of cells floating in the culture media (>70%) compared with control (no detachment). Addition of CORM-3 (1-50 µM), which rapidly releases CO in the cell media (9, 21), prevented the increase in caspase-3 activity in a concentration-dependent manner. CORM-3 was very effective in protecting cells from CP-mediated damage since both the increase in caspase-3 activity and cell detachment were completely abolished at concentrations as low as 10 µM (Figure 1A). Interestingly, an inactive form of CORM-3 (iCORM-3), which is incapable of liberating CO, did not have any effect on cell disruption and caspase-3 activation caused by CP. The protective effect of CO appears to be mediated by cGMP since CORM-3 added to cells in the presence of an inhibitor of the guanylate cyclase pathway (ODQ, 10 µM) failed to suppress caspase-3 activity induced by CP (Figure 1B). Of note is that biliverdin, which is generated alongside CO during the degradation of heme by heme oxygenase, did not have any effect on CP-mediated caspase-3 activation (Figure 2) indicating that CO is specifically required to prevent nephrotoxicity inflicted by the anti-neoplastic drug. We further tested whether similar results could be reproduced *in vivo* and found that administration of CORM-3 completely prevented acute renal failure and kidney tissue damage induced by CP. Specifically, CP caused a significant ($p < 0.05$) increase in plasma urea (from 7.0 ± 0.5 to 46.0 ± 6.5 mmol/L) and creatinine (from 57 ± 5 to 179 ± 49 µmol/L) levels; this effect was totally prevented by CORM-3 as plasma urea (5.3 ± 0.5 mmol/L) and creatinine (62 ± 3 µmol/L) concentrations were reduced to levels

similar to the control group (Figures 3A and 3B). The protective action of CORM-3 is mediated by CO as the negative control iCORM-3 failed to ameliorate renal dysfunction caused by CP. This negative control by itself did not have any effect on the basal levels of urea and creatinine (data not shown). In addition, administration of a heme oxygenase inhibitor (SnPPIX) in combination with CORM-3 only partially restored the increase in urea and creatinine levels following CP treatment suggesting that CO liberated from CORM-3 primarily contributes to the observed renal protection (Figure 3A and 3B). Histological analysis of the kidneys showed that treatment with CP resulted in severe tissue damage that affected mainly the tubules, while the glomeruli maintained their normal structure (Figure 4B). There was marked tubular necrosis, cellular desquamation, vacuolisation and blockade of tubular lumen by proteinacious materials. Interestingly, CORM-3 greatly improved the histological profile of cortex and medulla (Figure 4C), which appearance was similar to the control kidneys (Figure 4A); iCORM-3 once again failed to protect against the damage to the kidney inflicted by CP (Figure 4D) strongly indicating CO as potent effector of the pharmacological activity elicited by CORM-3. As shown in Table 1, histological changes assessed by a scoring system used by a pathologist in a blinded manner revealed that CORM-3 significantly improved the detrimental effects of CP on tubular necrosis and to a lesser extent on extravasation. A similar profile was observed when renal tissue was analyzed for apoptosis using a TUNEL assay. Treatment with CP caused extensive apoptotic cell death which mainly affected tubule epithelial cells of the cortico-medullary junction (see brown staining in Figure 5B). Administration of CORM-3, but not iCORM-3, completely prevented CP-mediated apoptosis (see Figure 5C and 5D, respectively). It has to be noted that the

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difference in weight before and after the various treatments directly correlated with the extent of renal dysfunction. Specifically, treatment with CP resulted in a 7.6±1.2% weight loss which was significantly ($p < 0.05$) attenuated by CORM-3 (1.7±1.9%), but not iCORM-3 (7.1±2.0%).

DISCUSSION

The multiplicity of beneficial effects elicited by carbon monoxide (CO), a signaling mediator generated by constitutive (HO-2) and inducible (HO-1) heme oxygenase enzymes (12), encompasses vasodilatory, anti-inflammatory and anti-apoptotic activities (13, 19, 23). Its key involvement in the regulation of important cellular processes in physiology and disease has been underlined by recent studies reporting that CO gas could be used as a therapeutic agent to counteract vascular dysfunction and other conditions driven by inflammatory reactions (12, 23). In this context, we have identified and characterized a class of CO-releasing molecules (CO-RMs) that liberate small quantities of CO in biological systems and provided strong scientific evidence for their pharmacological activities in a number of experimental models (9, 11, 17, 21, 28). Of particular interest is that CORM-3, a water-soluble ruthenium carbonyl complex, has been demonstrated to protect against myocardial ischemia-reperfusion injury (9), heart graft rejection (9), vasoconstriction (11) and inflammatory reactions (28). In the present study we report for the first time that CORM-3 can protect both renal tubule epithelial cells and kidneys against the structural and functional impairment induced by CP, a widely used anti-neoplastic drug that is also known for its nephrotoxic side effects (5, 14). Specifically, our main findings show that: 1) CORM-3 suppressed CP-mediated increase in caspase-3 activation and cell detachment in cultured tubule epithelial cells, an effect that was completely abolished by inhibition of the guanylate cyclase pathway; 2) administration of CORM-3 *in vivo* prevented acute renal failure caused by CP as evidenced by a significant reduction in rat plasma urea and creatinine levels; 3) the protective effects of CORM-3 against renal dysfunction inflicted by CP *in vivo* was

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associated with preservation of kidney morphology, decrease in apoptotic tubule epithelial cells of the cortico-medullary junction and a significant prevention of weight loss. Specifically, we observed a marked protective effect by CORM-3 against CP-induced tubular necrosis and to a lesser extent against extravasation. The fact that iCORM-3, an inactive negative control that does not release CO, failed to prevent both caspase-3 activation *in vitro* and nephrotoxicity *in vivo* strongly implicates CO as mediator of the observed pharmacological effect. We cannot exclude a possible contribution of endogenously generated CO following CORM-3 treatment since previous studies have shown that this transition metal carbonyl complex induces HO-1 protein expression and increases heme oxygenase activity *in vitro* and *in vivo* (28, 32). In fact, our results show that administration of a heme oxygenase inhibitor (SnPPIX) in combination with CORM-3 only partially restored the increase in urea (56%) and to a much lesser extent in creatinine (26%) levels following CP treatment. These data indicate that CORM-3-mediated activation of HO-1, and by inference increased endogenous CO, participates in the improved outcome of kidney function following CP-induced acute renal failure. However, our results demonstrate that CO liberated from CORM-3 has a marked additive effect to the protection elicited by HO-1 activation and that CO-RMs could be used therapeutically as an alternative to HO-1 inducers for the protection against drug-induced nephrotoxicity. The bioactivity of CO could be complementary or additive to the protective effect elicited by bilirubin, the other end product of heme oxygenase which has been shown to prevent oxidative stress-mediated injury in various organs including the kidney (1, 2, 8). Our data are in line with previous reports demonstrating that induction of HO-1 protects human renal tubule cells from CP-

mediated apoptosis and preserves renal hemodynamic functions in rats treated with this chemotherapeutic agent and other clinically used drugs (3, 24, 30). In the present study we also showed that CP-mediated caspase-3 activation is not affected by biliverdin and that the anti-apoptotic effect of CORM-3 is completely abolished by inhibition of the guanylate cyclase pathway, highlighting the specificity of heme oxygenase-derived CO to counteract CP-mediated renal toxicity. This is consistent with findings showing that CO gas protects vascular smooth muscle cells against caspase-3 activation and apoptosis via a cGMP-dependent mechanism (15). The fact that guanylate cyclase is a preferential target for the vascular activity of CO liberated from CORM-3 also supports this concept (11). Since apoptosis caused by CP is associated with an increased production of free radicals (33), we cannot exclude a priori that the anti-apoptotic effects of CORM-3 may also involve the suppression of crucial pathways that trigger oxidative stress (31). Our data on the renoprotective effects of CORM-3 against CP-mediated increase in creatinine and urea levels emphasise the potential of using CO carriers for therapeutic purposes. The concentration of plasma urea and creatinine, two classical markers of renal function *in vivo*, were dramatically reduced to basal levels by CORM-3 in CP-treated rats, an effect that was accompanied by a complete suppression of tubular cell apoptosis and preservation of the renal tubule morphology. Our results are consistent with previous studies revealing a pharmacological effect on kidney by all CO-RMs that were originally identified by our group (9, 17, 21). In fact, both water-soluble (CORM-3) and lipid-soluble (CORM-1 and CORM-2) CO-releasing agents markedly improved renal hemodynamics in rats (6) and limited renal damage in a mouse model of ischemia-induced acute renal failure (32). Moreover, more recently we have produced

data showing that addition of CO-RMs to cold storage solutions during the preservation of kidneys improves mitochondrial respiration at reperfusion (27). A direct effect of CO-RMs on the activity of cytochromes, oxygen uptake and phosphorylation in isolated mitochondria from kidneys has also been reported (26). Thus, CO-RMs appear to be excellent candidates in the development of pharmaceuticals for the treatment of nephrotoxicity and renal dysfunction (18, 20). In conclusion, our results demonstrate for the first time that the injurious effects mediated by CP on renal tissue can be mitigated by delivering small amounts of CO into the organism suggesting that CO-RMs could be utilized as effective adjuvants to prevent nephrotoxicity in cancer patients undergoing treatment with platinum-derived chemotherapeutic agents.

ACKNOWLEDGMENTS

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Table 1. Histopathological scoring of renal tissue.

	Glomerular necrosis	Tubular necrosis	Tubular dilatation	Epithelial sloughing	Vascular congestion	Extravasation
Control	0	0	0	0	0	0
Cisplatin (CP)	0	4	0	0	3	3
CORM-3+CP	0	0	0	0	0	1
iCORM-3+CP	3	4	3	3	0	2

Cisplatin (CP, 7.5 mg/kg i.p.), CORM-3 (10 m/kg i.p.) or the inactive control (iCORM-3, 10 mg/kg i.p.) were administered to Wistar rats. Kidneys harvested at the end of each treatment and H&E stained sections were assessed for histological changes as described in Materials and Methods. Values are expressed as the mean of 5 independent experiments.

FIGURE LEGENDS

Fig 1. Inhibition by CORM-3 of cisplatin-induced caspase-3 activation in renal tubule cells involves cGMP. (A) Confluent LLC-PK1 cells were exposed to CP (50 µM) alone or in the presence of increasing concentrations of CORM-3 (1-50 µM). The inactive compound iCORM-3 was used as negative control. After 16 h incubation caspase-3 activity was measured as described in Materials and Methods. Each bar represents the mean ± SEM of 5 different experiments. **(B)** Cells were treated with CP (50 µM) plus CORM-3 (1-50 µM) in the presence of 10 µM ODQ, an inhibitor of guanylate cyclase, and caspase-3 activity was measured after 16 h incubation. * $p < 0.05$ vs. control (no treatment); † $p < 0.05$ vs. CP-treated cells.

Fig. 2. Effect of biliverdin on cisplatin-induced caspase-3 activation in LLC-PK1 cells. Confluent LLC-PK1 cells were exposed to CP (50 µM) alone or in the presence of increasing concentrations of biliverdin (0.5-10 µM). After 16 h incubation caspase-3 activity was measured as described in Materials and Methods. Each bar represents the mean ± SEM of 5 different experiments. * $p < 0.05$ vs. control (no treatment).

Fig. 3. Effect of CORM-3 on plasma urea and creatinine levels *in vivo* following cisplatin-induced acute renal failure. Acute renal failure in rats was induced by a single intraperitoneal injection of cisplatin (CP, 7.5 mg/kg b.w.). CORM-3 alone (10 mg/kg i.p.) or in combination with tin protoporphyrin IX (SnPPIX, 10 mg/kg i.p.) was administered daily to animals starting 1 day prior CP treatment and continuing for 3 days thereafter. Plasma urea **(A)** and creatinine **(B)** levels were measured at the end of the

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treatment. Each bar represents the mean ± SEM of at least 5 experiments. *$p < 0.05$ vs. control group (CON); †$p < 0.05$ vs. CP or iCORM-3 groups.
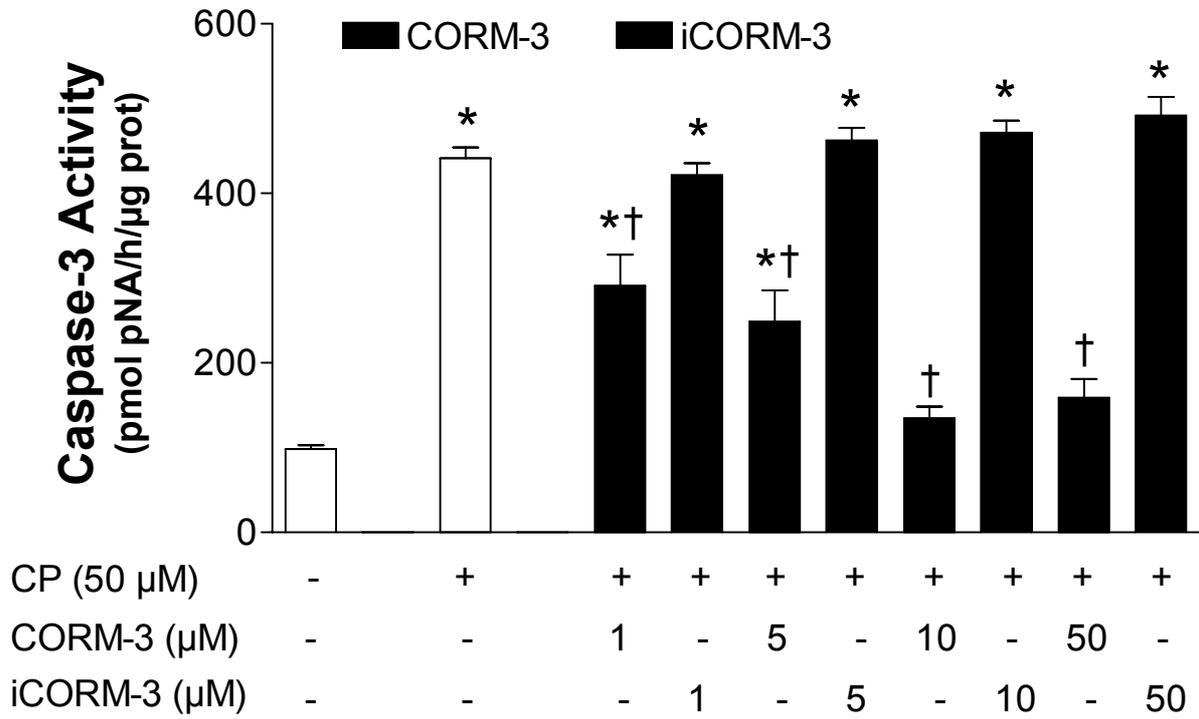
Fig. 4. Effect of CORM-3 on kidney morphology *in vivo* following cisplatin (CP) treatment. Rats were treated with a single intraperitoneal injection of CP (7.5 mg/kg) and CORM-3 (or iCORM-3) was administered as described in Figure 1. The control group received water for injection. At the end of each treatment, renal tissue was collected for histological examination (see also Materials and Methods). As shown, while the control group displays normal kidney histology **(A)**, extensive tubule epithelial cellular necrosis, desquamation, vacuolization and swelling were observed in CP-treated kidneys **(B)**. Administration of CORM-3 resulted in remarkable improvement in the histological appearance and reduction in tubule cell damage **(C)**, whereas the negative control iCORM-3 was totally ineffective **(D)**.

Fig. 5. Effect of CORM-3 on renal tubule apoptosis *in vivo* following cisplatin (CP) treatment. Rats were treated with a single intraperitoneal injection of CP (7.5 mg/kg) and CORM-3 (or iCORM-3) was administered as described in Figure 1. The control group received water for injection. At the end of each treatment, renal tissue was collected for the assessment of apoptosis using a TUNEL assay (see also Materials and Methods). As shown, kidneys from the control group displayed very little apoptosis **(A)**. Treatment with CP significantly increased the number of tubule apoptotic cells mainly in the corticomedullary junction **(B)** and CORM-3 administration significantly reduced this

effect **(C)**. The negative control iCORM-3 failed to reduce tubule apoptosis in CP-treated
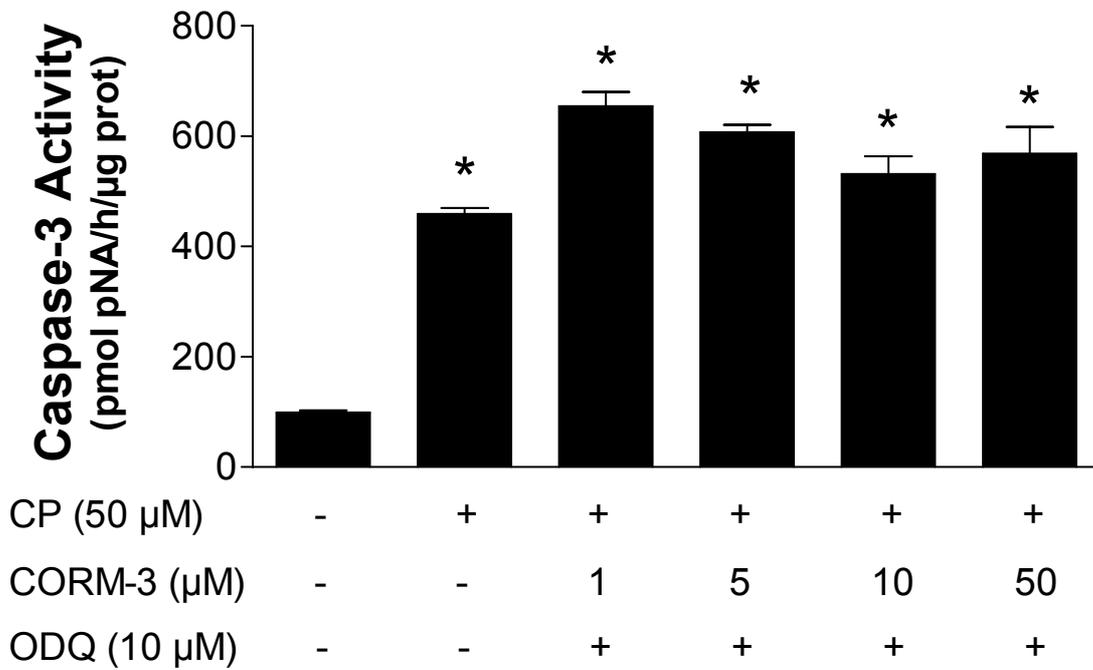
rats **(D)**.

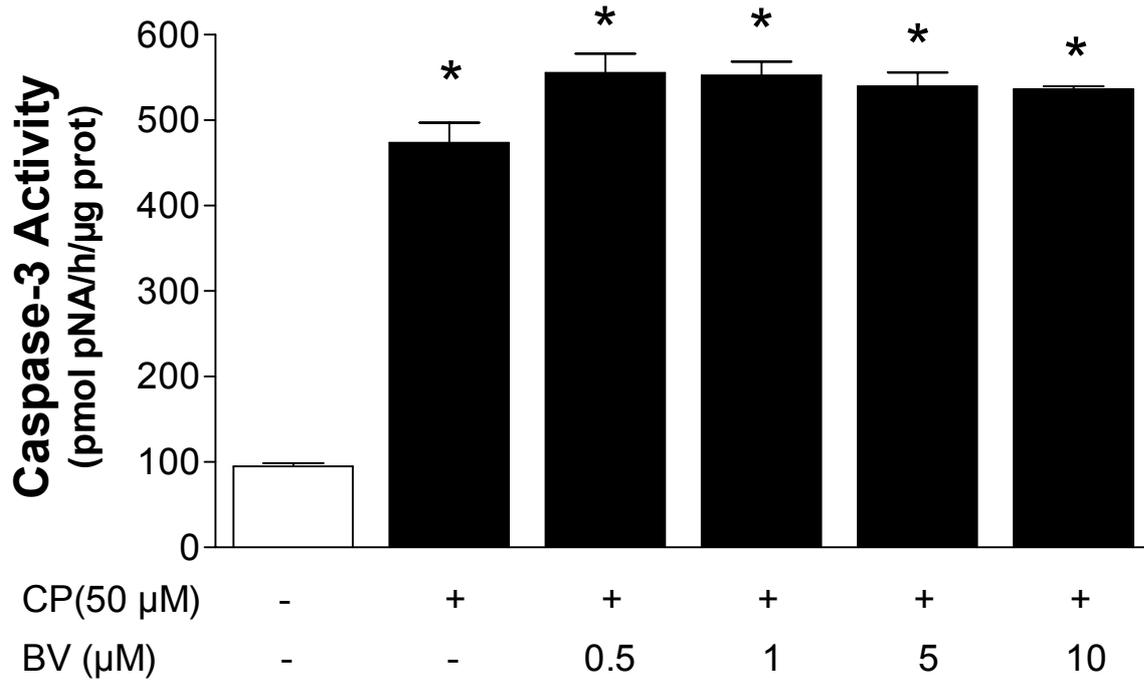FIGURE 1

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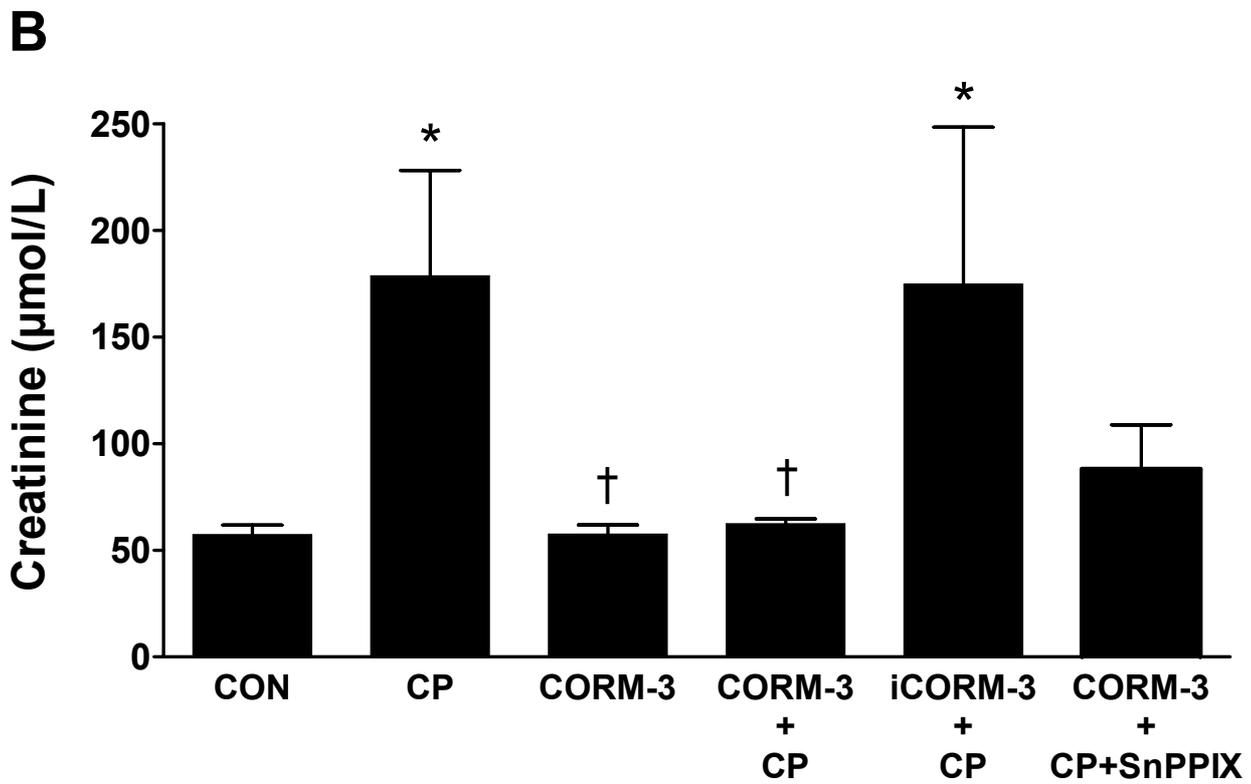**A**

B

FIGURE 2

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FIGURE 3

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A

B

FIGURE 4

FIGURE 5