Cilastatin protects against cisplatin-induced nephrotoxicity without compromising its anticancer efficiency in rats

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Cisplatin is an anticancer agent marred by nephrotoxicity; however, limiting this adverse effect may allow the use of higher doses to improve its efficacy. Cilastatin, a small molecule inhibitor of renal dehydropeptidase I, prevents proximal tubular cells from undergoing cisplatin-induced apoptosis *in vitro*. Here, we explored the *in vivo* relevance of these findings and the specificity of protection for kidney cells in cisplatin-treated rats. Cisplatin increased serum blood urea nitrogen and creatinine levels, and the fractional excretion of sodium. Cisplatin decreased the glomerular filtration rate, promoted histological renal injury and the expression of many pro-apoptotic proteins in the renal cortex, increased the Bax/Bcl2 ratio, and oxidative stress in kidney tissue and urine. All these features were decreased by cilastatin, which preserved renal function but did not modify the pharmacokinetics of cisplatin area under the curve. The cisplatin-induced death of cervical, colon, breast, and bladder-derived cancer cell lines was not prevented by cilastatin. Thus, cilastatin has the potential to prevent cisplatin nephrotoxicity without compromising its anticancer efficacy.

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Cisplatin is a potent and reliable chemotherapeutic agent against solid tumors.[1,2] However, cisplatin time- and dose-dependent nephrotoxicity restricts the use of high doses to maximize the therapeutic efficacy. In fact, 25–35% of patients develop evidence of nephrotoxicity following a single dose of cisplatin.[3] Once cisplatin enters the cell, it is bioactivated by hydrolytic reactions. The chloride ligands are replaced by water molecules, transforming platinum (Pt) into mono- and di-aquadiammineplatinum (II) species. Aqua species are more reactive than the neutral molecule and react with DNA, creating intra-strand DNA–cisplatin cross-linking that causes cytotoxic lesions in tumors and other dividing cells.[4] Although nonproliferating cells are relatively well protected, proximal tubule cells are an exception, as they are damaged by cisplatin. Therefore, finding an effective way to prevent cisplatin-induced nephrotoxicity has become a priority.

The mechanisms of tubular cell injury have been studied to develop nephroprotective approaches.[5,6] Several pathways of apoptosis—extrinsic, mediated by death receptors, and intrinsic (or mitochondrial) pathways—have been implicated in cisplatin-induced nephrotoxicity.[6] Cisplatin increases levels of proapoptotic proteins (Bax), decreases anti-apoptotic proteins (Bcl-2), and activates initiator and executioner caspases.[3,6–8] Oxidative stress (OS) also has a critical role in the pathogenesis of cisplatin-induced nephrotoxicity. Reactive oxygen species (ROS)–induced cell death has been reported in renal proximal tubular epithelial cells (RPTECs),[5,6,9] and ROS promote cisplatin-induced renal failure.[10,11] ROS directly damage cell components, including lipids, proteins, and DNA, and activate the mitochondrial pathway of apoptosis, thus highlighting the interactions between both processes.[5,12]

Many studies have assessed the effect of various substances to neutralize cisplatin-induced apoptosis and OS in experimental nephrotoxicity.[11–16] However, in some cases, the effect

on the tumoricidal efficacy of cisplatin is unclear, while in others, it declined in proportion to the grade of nephroprotection showing that OS and apoptosis also have a prominent role in the tumoricidal capacity of cisplatin.[5,6]

We have previously published the ability of cilastatin to inhibit apoptosis induced by a large set of well-known nephrotoxics.[17–19] This property seems to be linked to the interference of the cilastatin–dehydropeptidase I (cilastatin-DHP-I) complex with the signaling of the extrinsic pathway of apoptosis.[17,18] We showed that cilastatin was able to inhibit cilastatin-induced apoptosis but not necrosis in RPTECs, reducing cisplatin–DNA binding, DNA fragmentation, mitochondrial damage, and cell death. However, no changes in the chromatographic profile of Pt-biomolecules were observed.[17]

We explore the potential usefulness of cilastatin as a nephroprotective agent in an animal model of cisplatin-induced nephrotoxicity. Our results may have important relevance for preservation of renal function in cancer patients.

RESULTS

Effect of cilastatin on cisplatin-induced body weight loss

Body weight was not significantly different in rats before the beginning of the treatment. Cisplatin reduced body weight gain compared with the control animals. Cilastatin significantly prevented, but did not normalize, cisplatin-induced weight loss (Table 1). Cilastatin alone did not modify body weight.

Table 1 | Effect of cilastatin on body weight in cisplatin-treated rats

Groups	Initial weight (g)	Final weight (g)	Δ Weight (g)
Control	264 ± 3	279 ± 2	15 ± 1
Control+cil	259 ± 8	273 ± 7	14 ± 2
Cisplatin	272 ± 4	249 ± 5	−23 ± 3[a]
Cisplatin+cil	272 ± 5	259 ± 3	−13 ± 4[a,b]

Abbreviation: cil, cilastatin.
[a]$P<0.0001$ vs. control and control+cil.
[b]$P<0.05$ vs. cisplatin group.
Table shows body weight in the different study groups at the beginning and end of the study. Results are expressed as mean ± s.e.m.; n=7–8 animals per group.

Cilastatin protects from cisplatin-induced renal injury

Cisplatin increased serum blood urea nitrogen and creatinine levels, fractional excretion of sodium and water and decreased the glomerular filtration rate in comparison with the controls at day 5 (Table 2). Cilastatin significantly partially or totally prevented these changes in cisplatin-treated animals. Urinary volume and protein excretion also increased in the cisplatin group but were significantly reduced by cilastatin (Table 2). Cilastatin alone had no effect on these parameters compared with the control group (Table 2).

Cilastatin ameliorates cisplatin-induced morphological renal damage

The toxic effect of cisplatin was also confirmed by the detection of morphologic abnormalities in kidney slices. Histology results for the control rats were normal (Figure 1). The cisplatin group exhibited acute structural damage characterized by tubular necrosis, swelling and tubular dilation, extensive epithelial vacuolization, and hyaline casts in renal tubules (Figure 1). The semiquantitative histological injury score was significantly higher in cisplatin-treated rats than in controls. Cilastatin alone had no effect on renal morphology but reduced the renal injury score in cisplatin-treated rats (Figure 1i).

Cilastatin reduces cisplatin-induced apoptosis

In situ *detection of apoptosis.* Apoptosis in the kidney was assessed using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay. Cisplatin increased the number of apoptotic nuclei compared with the control group (Figure 2). Cilastatin significantly decreased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells.

Expression of caspase-3 and -9. Procaspase-3 levels were high in the renal cortex of rats treated with cisplatin, although they were reduced by cilastatin (Figure 3f and g). Renal sections from the cisplatin-treated group also displayed increased activated cleaved caspase-3 staining in renal tubules compared with untreated kidneys and kidneys treated with cilastatin only (Figure 3a–d). Cilastatin significantly lowered the elevated caspase-3 levels in cisplatin-injected rats, as

Table 2 | Effects of cilastatin on cisplatin-induced nephrotoxicity in rats

Groups	S_{Creat} (mg/dl)	BUN (mg/dl)	GFR (ml/min/100 g)	U_{Vol} (ml/24 h)	U_{Prot} (mg/24 h)	FE_{Na^+} (%)	FE_{H_2O} (%)
Control	0.33 ± 0.02	33.50 ± 3.03	0.83 ± 0.06	15.17 ± 1.38	26.10 ± 3.76	0.47 ± 0.04	0.46 ± 0.03
Control+cil	0.33 ± 0.02	30.20 ± 2.48	0.72 ± 0.06	14.17 ± 2.34	27.72 ± 2.65	0.53 ± 0.04	0.53 ± 0.05
Cisplatin	1.10 ± 0.06[a]	80.00 ± 5.20[a]	0.20 ± 0.02[a]	29.14 ± 2.04[a]	44.90 ± 5.62[a]	1.65 ± 0.17[a]	4.64 ± 0.37[a]
Cisplatin+cil	0.67 ± 0.08[a,b]	50.00 ± 4.12[a,b]	0.38 ± 0.04[a,c]	17.50 ± 3.70[b]	29.00 ± 1.57[b]	0.76 ± 0.08[b,d]	1.61 ± 0.41[b,e]

Abbreviations: BUN, blood urea nitrogen; cil, cilastatin; FE, fractional excretion; GFR, glomerular filtration rate; S_{Creat}, serum creatinine; U_{Prot}, urinary protein; U_{Vol}, urinary volume.
[a]$P<0.005$ vs. control and control + cil.
[b]$P<0.005$ vs. cisplatin.
[c]$P<0.05$ vs. cisplatin.
[d]$P<0.05$ vs. control group.
[e]$P<0.05$ vs. control and control+cil.
Results are expressed as mean ± s.e.m.; n = 7–8 animals per group.

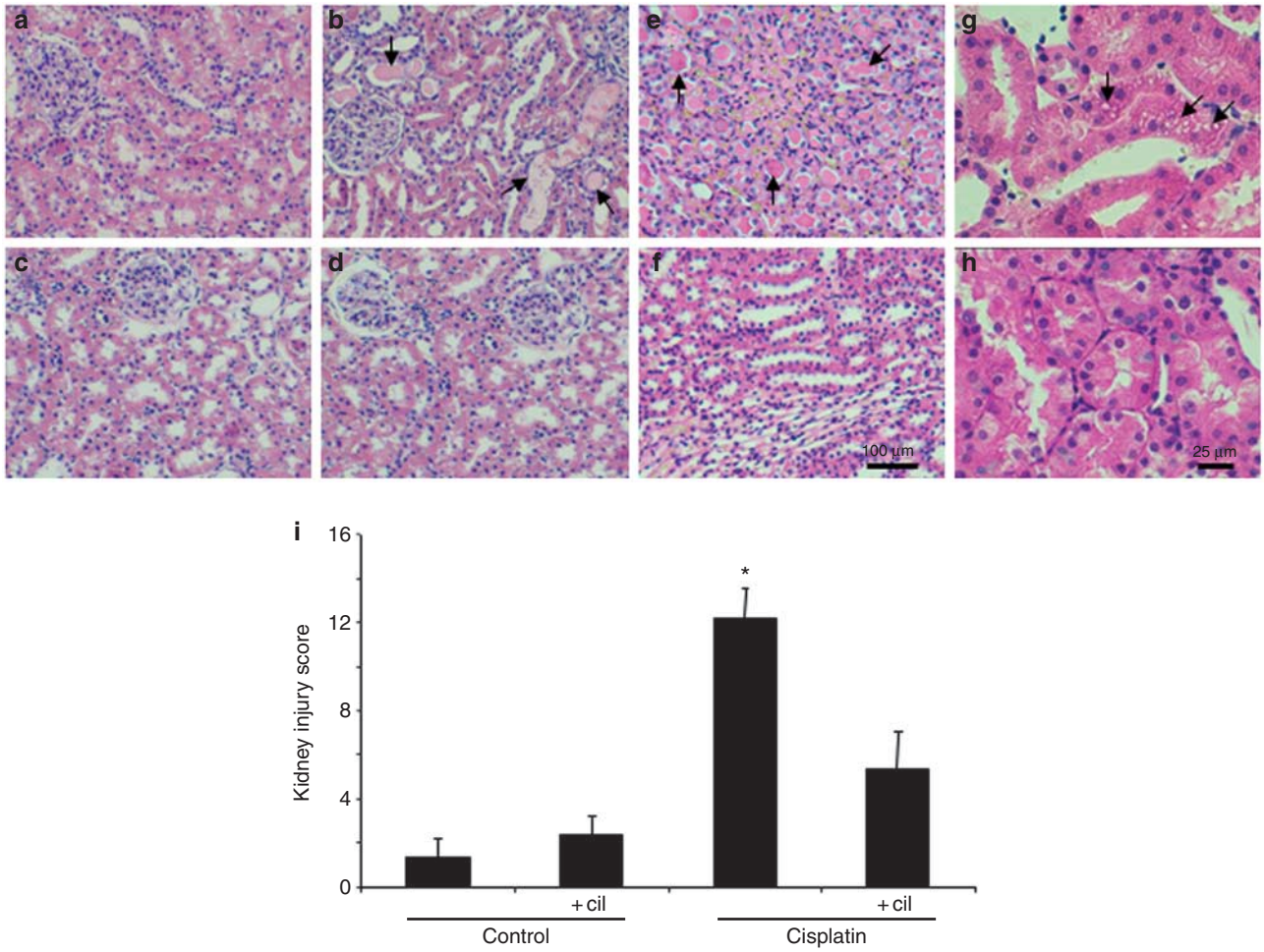

Figure 1 | Cilastatin (cil) improves renal histology in cisplatin-injected rats. Representative images of the renal pathology (hematoxylin–eosin staining, magnification × 20) on day 5 after administration of cisplatin. (**a**) Control rats, (**b**, **e**) cisplatin (renal cortex and medulla, respectively), (**c**) control + cilastatin, and (**d**, **f**) cisplatin + cilastatin (renal cortex and medulla, respectively). Control groups show normal renal structure; cisplatin-injected kidneys show marked injury with sloughing of tubular epithelial cells, loss of brush border, dilation of tubules, vacuolization, and intratubular cast formation (arrows). These changes were significantly reduced by treatment with cilastatin, with the kidneys showing almost normal morphology; bar = 100 μm. (**g**, **h**) Details of renal tubules of cisplatin and cisplatin + cilastatin groups, respectively (magnification × 60), showing the presence of vacuolization (arrows). Cilastatin completely reduced the damage; bar = 25 μm. (**i**) Semiquantitative renal injury score (see Materials and Methods section). Results are expressed as mean ± s.e.m.; $n = 7$–8 animals per group.*$P < 0.01$ compared with other groups.

assessed both by immunohistochemistry and by western blot of the renal cortex (Figure 3f and h). Renal cortex procaspase-9 and cleaved caspase-9 were also markedly increased after administration of cisplatin; both were also attenuated by cilastatin (Figure 4).

Expression of Bax/Bcl-2 proteins. Apoptotic Bax and anti-apoptotic Bcl-2 expressions were measured in the renal cortex. Bax increased significantly in the cisplatin-treated group compared with the control group, and this increase was significantly diminished by cilastatin (Figure 5a and b). No changes were observed with respect to the controls in Bcl-2 protein levels in cilastatin-treated cisplatin-injected rats or cisplatin-treated rats (Figure 5a and c). The ratio of Bax to Bcl-2 was calculated as an index of the susceptibility of the cells to apoptosis: an increased ratio indicates predisposition to apoptosis. This was significantly higher in the kidneys of cisplatin-treated rats than in control rats. Cilastatin reversed the increase in the Bax/Bcl-2 ratio in cisplatin-injected rats (Figure 5d).

The extrinsic pathway of apoptosis. We investigated whether cilastatin modulated the Fas/Fas ligand (FasL) system and activator caspase-8. Cisplatin increased renal cortex Fas, FasL, and caspase-8 levels compared with untreated animals (Figure 6). Cilastatin significantly decreased protein expression of Fas, FasL, and caspase-8 (Figure 6). Cilastatin alone had no effect on any of the above-mentioned apoptotic variables.

Effect of cilastatin on cisplatin-induced OS

Evidence of OS was found both in urine and in the kidneys of cisplatin-treated rats.

Cilastatin blunts cisplatin-induced increased ROS formation and enhances antioxidant defenses. OS results from the imbalance between ROS generation and destruction. To

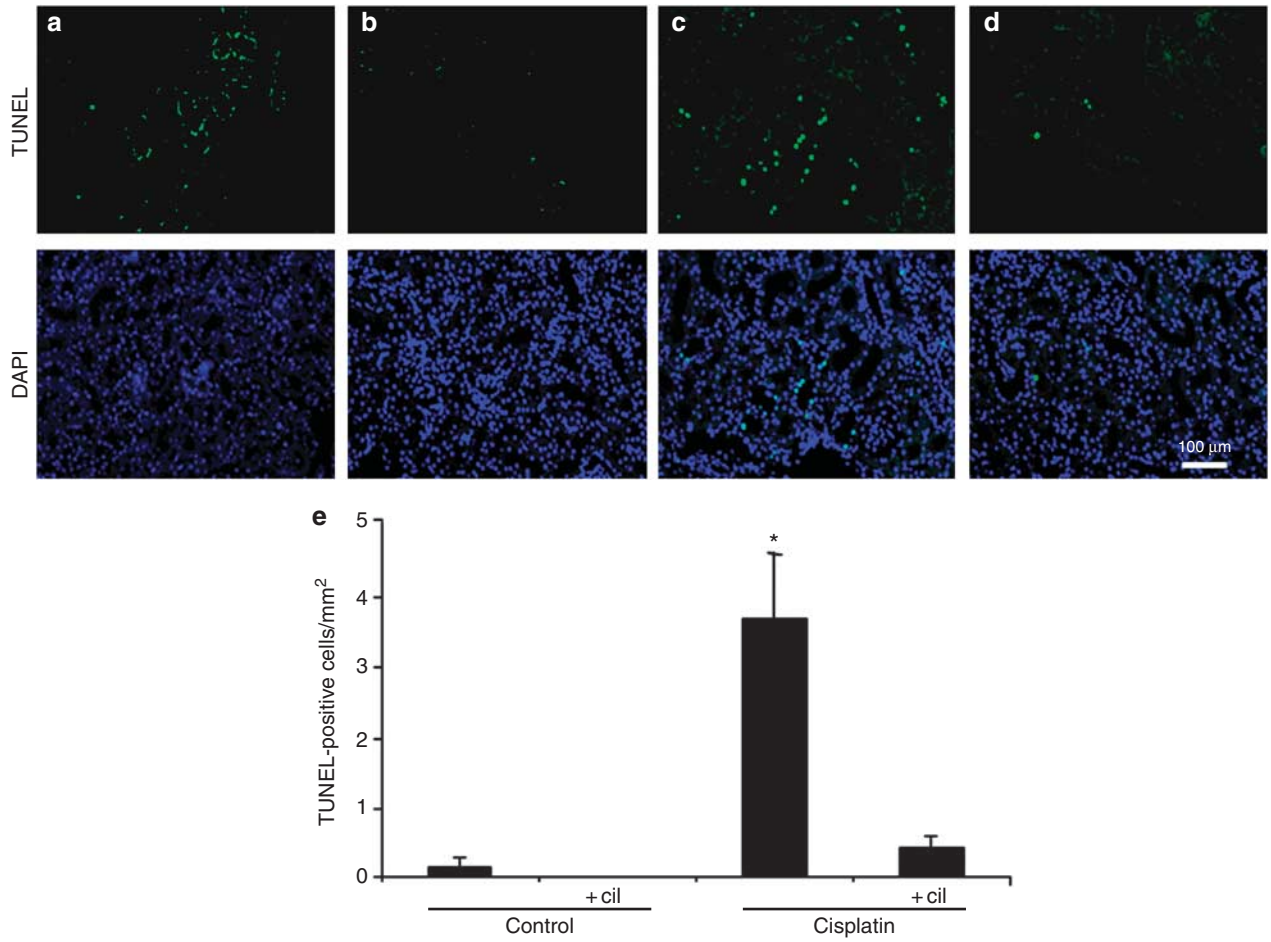

Figure 2 | Cilastatin (cil) decreases tubular cell apoptosis in cisplatin-injected rats. Photomicrographs of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining in the kidneys. (**a**) Control rats, (**b**) control + cilastatin, (**c**) cisplatin, and (**d**) cisplatin + cilastatin. Green fluorescent staining indicates TUNEL-positive nuclei, and blue staining (4,6-diamidino-2-phenylindole (DAPI)) represents all nuclei in the sample (magnification × 20). (**e**) Quantitative analysis of TUNEL-positive cells. Results are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. *$P \leqslant 0.001$ vs. all other groups.

evaluate the role of cilastatin in countering cisplatin-induced OS, we measured ROS and antioxidant capacity in urine. Urine from cisplatin-treated rats contained a larger amount of hydrogen peroxide than the control group, and this was decreased by cilastatin (Figure 7a). Furthermore, cisplatin significantly decreased antioxidant capacity in urine, which was partially improved by cilastatin (Figure 7b).

Catalase (an antioxidant enzyme) expression was decreased in renal cortex of cisplatin-treated rats. Cilastatin restored catalase expression (Figure 7c and d).

Heat shock protein 27 (HSP 27) protects cells from apoptosis and enhances antioxidant defenses. Expression of HSP 27 was markedly greater in cisplatin-treated rats than in controls. Cilastatin significantly decreased kidney HSP 27 levels (Figure 7e and f).

Cilastatin reduces cisplatin-induced lipid peroxidation. 4-Hydroxy-2-nonenal and malondialdehyde (MDA) are products of fatty acid oxidation. Cisplatin enhanced 4-hydroxy-2-nonenal staining (Figure 8a–e) and increased MDA levels (Figure 8f–h) in the kidneys when compared with the controls. This was completely prevented by cilastatin.

Cilastatin alone did not modify these variables compared with the control group.

Pharmacokinetics

Cisplatin pharmacokinetics was studied following a single intraperitoneal (i.p.) injection in the presence or absence of cilastatin (Table 3 and Figure 9c). The area under the curve (AUC) of serum cisplatin was not significantly different between animals treated with or without cilastatin. Cilastatin decreased the cisplatin elimination rate constant and increased Pt half-life ($t_{1/2}$) (Table 3). Although maximum Pt concentration (C_{max}) was attained at similar time points in both the cases, C_{max} was slightly lower in the cilastatin-treated group ($P < 0.05$).

Cilastatin reduces incorporation of cisplatin in the renal cortex

As previously described in RPTECs exposed to cisplatin,[17] *in vivo* cilastatin also decreased renal cortical tissue Pt in rats

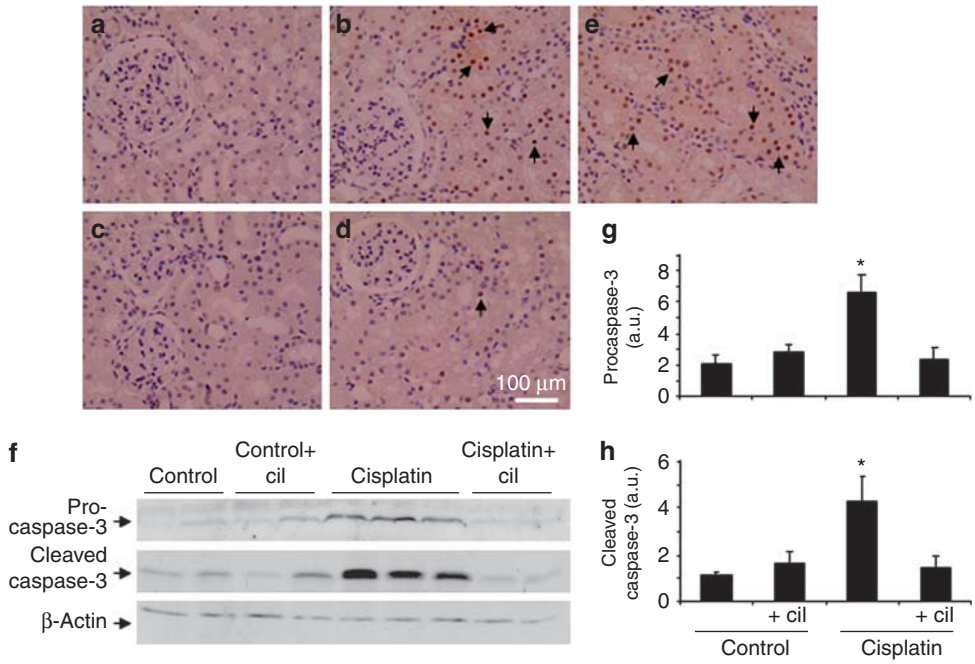

Figure 3 | Cilastatin (cil) diminishes cisplatin-induced caspase-3 expression. Localization of active cleaved caspase-3 in kidney sections. (**a**) Control rats; (**b**, **e**) cisplatin; (**c**) control + cilastatin; and (**d**) cisplatin + cilastatin. Note that renal tubules are the main site of caspase-3 activation in cisplatin-treated animals (arrows, magnification ×20). Cilastatin significantly reduced expression of active caspase-3 induced by cisplatin. (**f**) Representative western blots of procaspase-3 and active cleaved caspase-3 in renal cortex. (**g**, **h**) Densitometric analysis of western blots of procaspase-3 and active cleaved caspase-3, respectively. Data are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. *$P \leqslant 0.01$ vs. all other groups. a.u., arbitrary units.

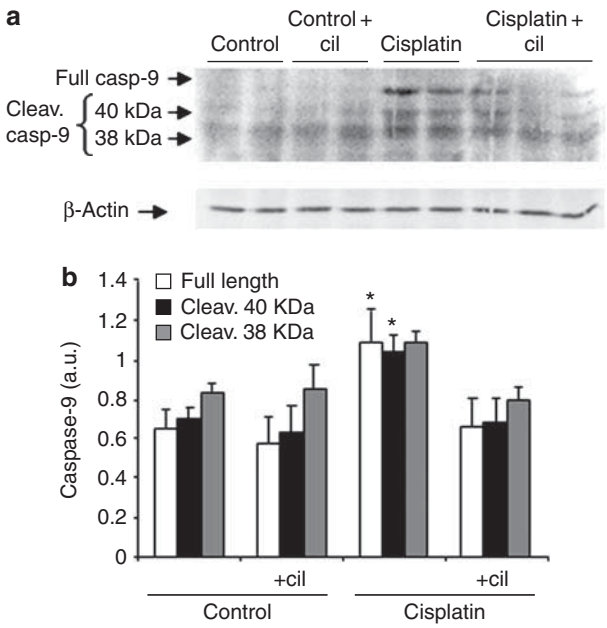

Figure 4 | Cilastatin (cil) prevents caspase-9 (casp) upregulation in cisplatin-injected rats. (**a**) Representative photomicrographs of western blots of procaspase-9 and active cleaved (Cleav) caspase-9 in renal cortex. (**b**) Densitometric analysis of procaspase-9 and active caspase-9 levels on western blots. Data are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. *$P \leqslant 0.05$ vs. all other groups. a.u., arbitrary units.

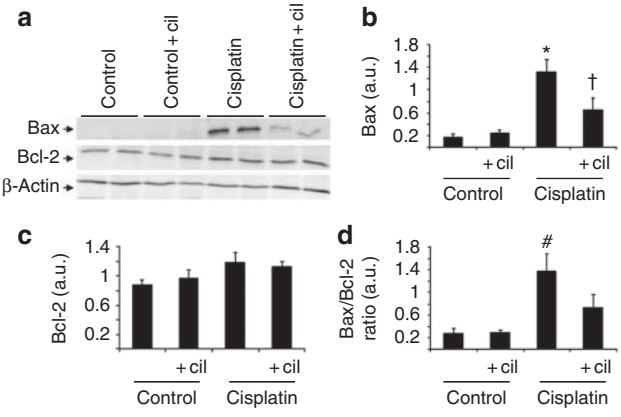

Figure 5 | Cilastatin (cil) diminishes Bax upregulation induced by cisplatin. (**a**) Representative photomicrographs of western blot of Bax and Bcl-2 in renal cortex. (**b**–**d**) Densitometric analysis of Bax, Bcl-2, and Bax/Bcl-2 ratio levels, respectively, on western blots. Data are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. *$P \leqslant 0.01$ vs. all other groups; †$P = 0.046$ vs. control; #$P < 0.05$ vs. all other groups. a.u., arbitrary units.

following administration of cisplatin (Figure 9a). Decreased cortical Pt probably reflects the specific lower accumulation of Pt in RPTECs, because no differences were observed in medullary Pt accumulation. Indeed, medullary Pt was lower than cortical Pt content in both groups of rats.

There was no qualitative change in the chromatographic profile of Pt-containing proteins, irrespective of whether rats

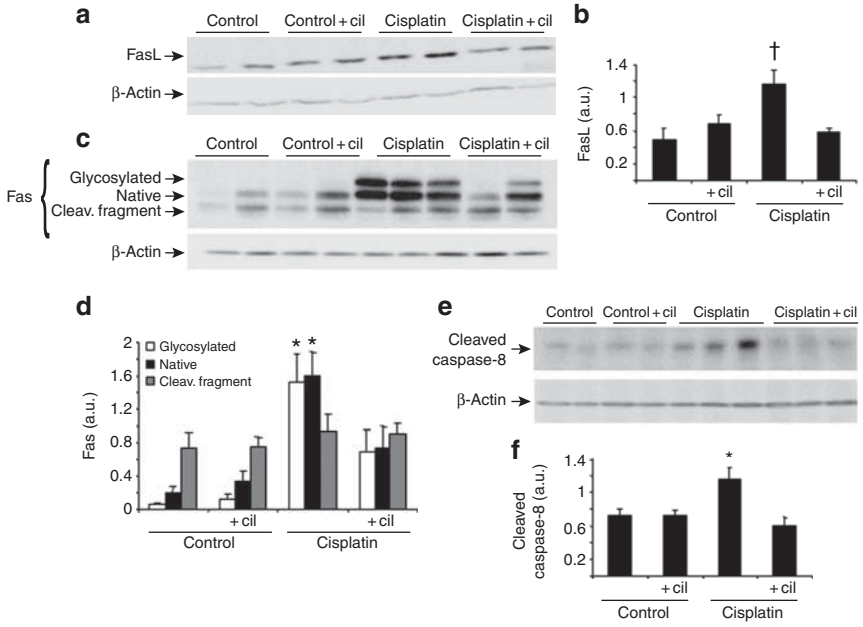

Figure 6 | Cilastatin (cil) diminishes cisplatin-induced Fas/Fas ligand (FasL) apoptotic pathway upregulation. (**a**, **c**, **e**) Representative photomicrographs of western blot of FasL, Fas, and cleaved (Cleav) caspase-8, respectively, in renal cortex. (**b**, **d**, **f**) Densitometric analysis of FasL, Fas, and caspase-8 levels on western blots. Data are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. $^{\dagger}P < 0.001$ vs. all other groups; $^{*}P < 0.05$ vs. all other groups. a.u., arbitrary units.

were treated with cilastatin (Figure 9b). Pt-bound proteins were observed within the whole separation range (3–70 kDa), and the most intense peaks corresponded to species around 20 and 65 kDa. Moreover, no significant peaks were observed for intact cisplatin or the intracellular monoaquo complex, indicating that all Pt is bound to biomolecules.

Effects of cilastatin on cancer cells

To evaluate if cilastatin reduction in cisplatin nephrotoxicity was specific for the kidney, we looked for potential reductions in chemotherapeutic efficacy of cisplatin on different tumor cell lines. In Figure 10, the effect of cisplatin on the apoptosis of HeLa cells is depicted. Increasing doses of cisplatin caused a dose-dependent loss of cells, a rise in the percentage of cells with apoptosis-like nuclei (Figure 10a and b) and caspase-3 activation (Figure 10c). Cilastatin was unable to revert any of these effects. DHP-I activity was measured in HeLa cells and compared with cultured RPTECs (Figure 10d). The specific DHP-I activity was very low in HeLa cells and was not inhibited by cilastatin. However, in the presence of cilastatin we observed a 24% reduction in the intracellular content of cisplatin in HeLa cells (Figure 10e). This reduction apparently was neither related to the protective properties of cilastatin nor its ability to inhibit DHP-I.

Moreover, cisplatin reduced cell survival in cervical cancer (Figure 11a), colon cancer (Figure 11b and f), breast cancer (Figure 11c and e), and bladder cancer (Figure 11d) derived cell lines, and this effect was time dependent. Cilastatin did not protect any of these cells from cisplatin-induced cell death.

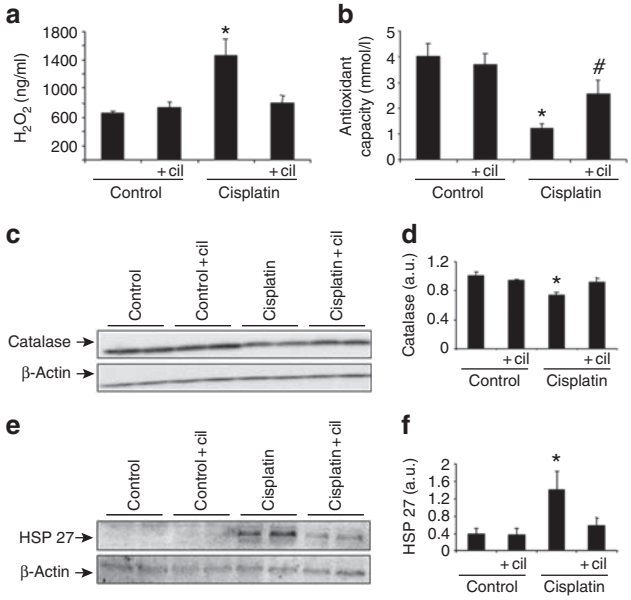

Figure 7 | Cilastatin (cil) decreases cisplatin-induced oxidative stress. (**a**) Urine hydrogen peroxide (H_2O_2) production and (**b**) antioxidant capacity in urine were measured in rats using specific kits. H_2O_2 concentration was higher in cisplatin-injected rats than in cisplatin-injected rats treated with cilastatin and control rats. In contrast, antioxidant capacity had the opposite effect. (**c**, **d**) Representative photomicrographs of western blot of catalase in renal cortex and densitometric analysis, respectively. (**e**, **f**) Representative photomicrographs of western blot of HSP 27 and densitometric analysis in renal cortex, respectively. All results are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. $^{*}P \leqslant 0.05$ vs. all other groups; $^{\#}P < 0.05$ vs. control. a.u., arbitrary units; HSP, heat shock protein.

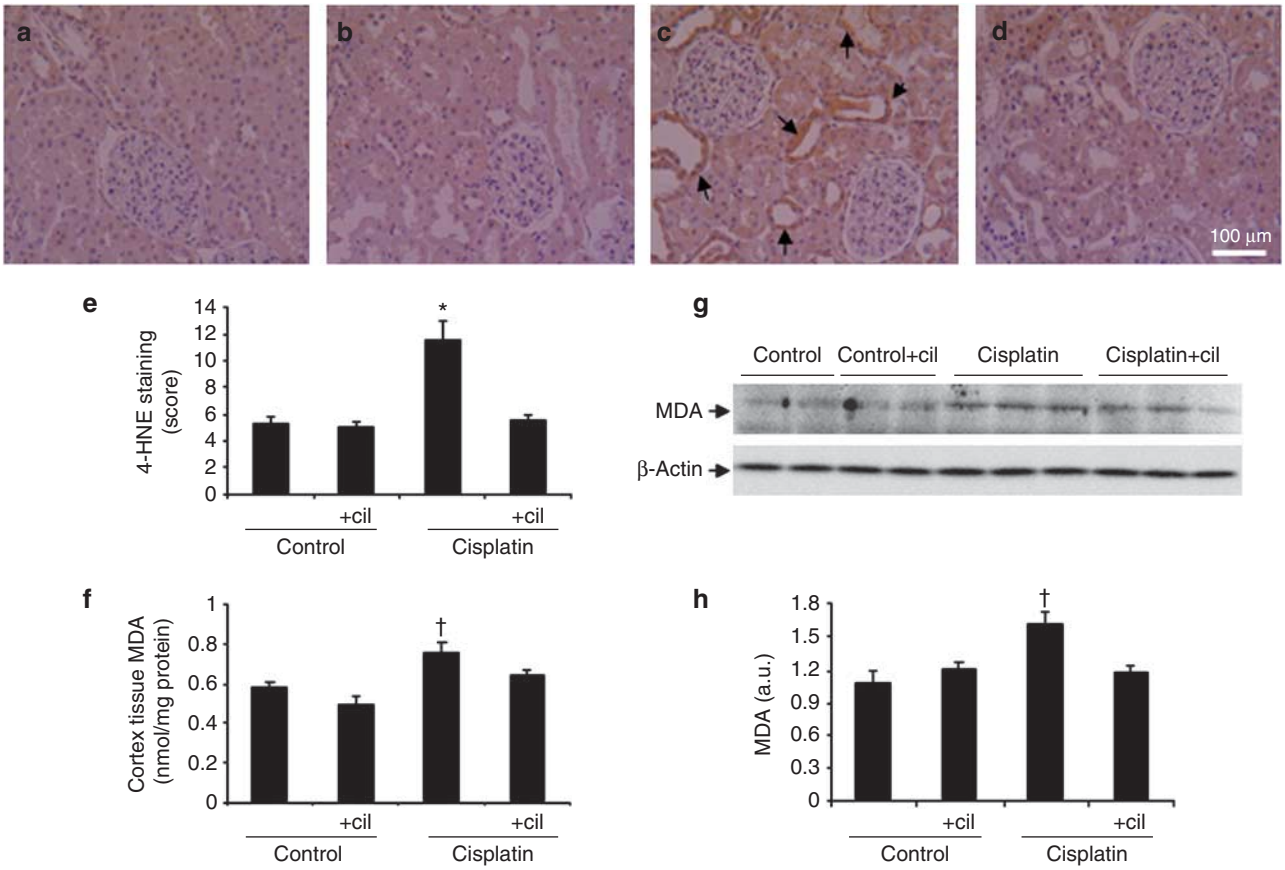

Figure 8 | Cilastatin (cil) decreases cisplatin-induced lipid peroxidation. Localization of 4-hydroxy-2-nonenal (4-HNE) in kidney sections of (**a**) control rats, (**b**) control + cilastatin, (**c**) cisplatin, and (**d**) cisplatin + cilastatin. Note increased tubular staining in cisplatin-injected rats (arrows) compared with cisplatin + cilastatin and control rats (magnification ×20); bar = 100 μm. (**e**) Semiquantification of 4-HNE immunostaining in renal cells. (**f**) Renal cortex malondialdehyde (MDA) content in the groups of rats studied. (**g**, **h**) Representative photomicrographs of western blot of MDA and densitometric analysis in renal cortex, respectively. All results are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. *$P < 0.0001$ vs. all other groups; †$P < 0.05$ vs. all other groups. a.u. arbitrary units.

Table 3 | Pharmacokinetic parameters estimate for cisplatin and cisplatin plus cilastatin in rats

Parameters	Unit	Cisplatin	Cisplatin+cilastatin	*P*-value
C_{max}	μg/l	3335 ± 181	2300 ± 56	<0.05
t_{max}	Min	15 ± 0	15 ± 0	NS
$t_{1/2}$	Min	135 ± 15	279 ± 27	<0.05
Elimination ratio constant	μg/ml/min	5.23 ± 0.57	2.50 ± 0.24	<0.05
AUC	(μg min)/ml	361.0 ± 11.1	402.7 ± 6.2	NS

Abbreviations: AUC, area under the curve; C_{max}, maximum platinum concentration; NS, nonsignificant; t_{max}, time to obtain C_{max}; $t_{1/2}$, half-life of platinum. The pharmacokinetic parameters were calculated using Microsoft Excel fitted with pharmacokinetic functions written for Excel (see Materials and Methods for details).
Results are expressed as mean ± s.e.m.; n=4 animals per group.

DISCUSSION

Nephrotoxicity is the main adverse effect of cisplatin, primarily induced by accumulation of Pt in kidney cells,[20] followed by apoptosis, and, in a minor proportion, necrosis.[6,17,21] Renal tubules are the main targets in cisplatin-induced nephrotoxicity,[6] and both intrinsic and extrinsic pathways have been involved in proximal tubular cell apoptosis.[5,6,8,17,22,23] OS and inflammation have also been involved in critical roles.[5,6,21,24]

In several *in vitro* models of toxicity on RPTECs, cilastatin incubation resulted in marked protection.[17–19] As only RPTECs have brush border-bound renal DHP-I, our results suggested that protection would be specific for RPTECs, but we had no evidences on the clinical relevance of these findings in an animal model. Here, we show that cilastatin significantly protects against cisplatin-induced nephrotoxicity and attenuates cisplatin-induced apoptosis and OS *in vivo*, without affecting its antitumor activity.

The rat model of acute cisplatin renal dysfunction induced by a single dose of drug was chosen because of its reproducibility and similarity to clinical situations.[3,16,25–27] A single injection of cisplatin in rats causes tubular cell

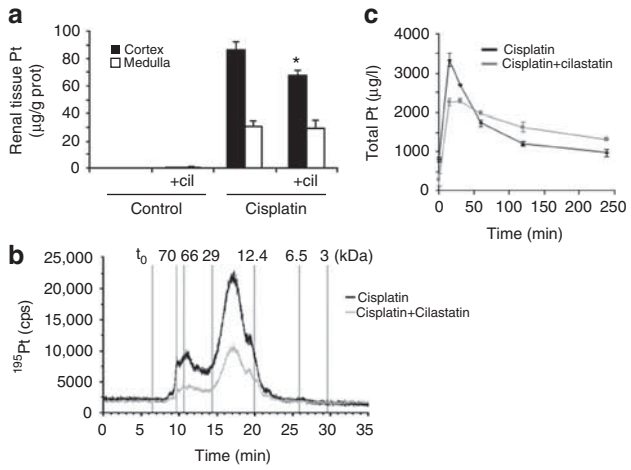

Figure 9 | Effect of cilastatin (cil) on platinum (Pt), speciation analysis, and pharmacokinetics in cisplatin-injected rats. (**a**) Pt concentration in the renal cortex and medulla of the animals 5 days after cisplatin administration. Cilastatin reduces renal cortex Pt accumulation by 22%. Data are expressed as mean ± s.e.m.; $n = 7$–8 animals per group. *$P < 0.005$ vs. cisplatin group. (**b**) Size exclusion/inductively coupled plasma mass spectrometry (^{195}Pt signal) chromatograms from renal cortex of cisplatin-injected rats and cilastatin-treated cisplatin-injected rats. Cilastatin reduces the height of detected peaks without changing their retention times, distribution, or relative proportion. (**c**) Serum cisplatin pharmacokinetic profile followed for 4 h after intraperitoneal injection of cisplatin with or without coadministration of cilastatin ($n = 4$). Effect of cilastatin kinetic parameters are displayed in Table 3 and discussed in the text. Prot, protein.

injury, decreased glomerular filtration rate, and increased serum creatinine, blood urea nitrogen, and fractional excretion of sodium. The reduction in body weight following cisplatin administration was caused by polyuria resulting from tubular injury, which in turn leads to dehydration, although gastrointestinal toxicity may also contribute.[28,29] Cilastatin significantly improved—in part or completely—all these manifestations of cisplatin-induced nephrotoxicity. Histopathology score of damage were also much improved.

Our results show that the protection afforded *in vivo* by cilastatin is associated with reversal of cisplatin-induced apoptosis and changes in apoptotic regulatory protein levels or state of activation. Specifically, cisplatin increased levels of renal native and glycosylated Fas, FasL, Bax, and cleaved caspase-3, -8, and -9, all of which were reversed by cilastatin. Cisplatin did not modify Bcl-2, finding that is consistent with previous observations.[3,12,30]

Cisplatin generates ROS and inhibits the activity of antioxidant enzymes in renal tissue.[11,13,14,16,22] In our study, we also showed increased hydrogen peroxide levels and marked deterioration of the endogenous antioxidant profile in the kidneys and urine of cisplatin-treated rats. Administration of cilastatin improved both findings. Lipid peroxidation via ROS generation is usually associated with cisplatin-induced nephrotoxicity.[16,25,30,31] In this regard, cilastatin also decreased the amount of kidney MDA and 4-hydroxy-2-nonenal observed in cisplatin-treated rats. Similar results were obtained when antioxidants such as curcumin, resveratrol, lycopene, and others were used in experimental cisplatin-induced nephrotoxicity.[13,16,25,29] Unfortunately, many of the studies cited above did not analyze whether these approaches would limit the tumoricidal efficacy of cisplatin, limiting its potential clinical use. Very recently, it has been reported that quercitin prevents the nephrotoxic effect of cisplatin without affecting its antitumor activity in rats.[32] Pabla *et al.*[33] have also shown that the inhibition of protein kinase Cδ enhances the chemotherapeutic effect of cisplatin in several tumor models while protecting kidneys from nephrotoxicity.

HSPs may also have an important role in protecting cells from injury, and their expression correlates strongly with the degree of OS in rat proximal tubules.[30,34] In fact, HSP 70 protects against cisplatin-induced cytotoxicity in tubular cells.[35] The increased levels of HSP 60 and 90 observed after treatment with cisplatin are decreased by antioxidants that ameliorate cisplatin-induced acute renal failure.[30,36] The most plausible explanation is that cells with decreased levels of injury fail to upregulate compensatory cell protection mechanisms, because these are no longer needed. Consistent with these observations, cisplatin increased HSP 27, an anti-apoptotic molecule that inhibits Fas-induced apoptosis and modulates ROS and glutathione levels.[37] Cilastatin restored normal levels of HSP 27, and we hypothesize that this is a consequence of its cytoprotective effect.

Pharmacokinetics showed similar AUC for cisplatin with or without cilastatin. However, the cisplatin elimination rate constant was slightly slower in the presence of cilastatin. As long as creatinine clearance was better preserved with cilastatin, changes in excretion suggested a higher reabsorption or a reduced secretion. From our previous experiences, we knew cilastatin interfered with cisplatin accumulation into RPTECs.[17] We did not know if this effect was relevant to nephroprotection, specific for proximal tubule or related to the inhibition of DHP-I. In our *in vivo* model, 5 days after cisplatin injection we were able to recover Pt from kidney homogenates. Simultaneous treatment with cilastatin did not make any difference with cisplatin content in medulla, but reduced the cisplatin recovered from renal cortex by 22%. We have confirmed these findings by laser ablation inductively coupled plasma mass spectrometry on renal slices obtained from our model: kidneys obtained from animals treated with cilastatin and cisplatin displayed lower content of Pt[38] and copper in the cortex, but not in medulla, where levels of both metals were similar to those observed in animals treated with cisplatin alone (data not shown). Cilastatin is able to inhibit certain basolateral organic anion transporters in proximal tubule cells.[39–41] But reduction in intracellular content of Pt and copper in renal cortex, even if modest in quantity, suggest that it may also inhibit other basolateral transporters like Ctr1, involved in Pt and copper basolateral transport in proximal tubules.[42]

Interestingly, our studies on cancer-derived HeLa cell lines displayed very low levels of specific DHP-I activity, and

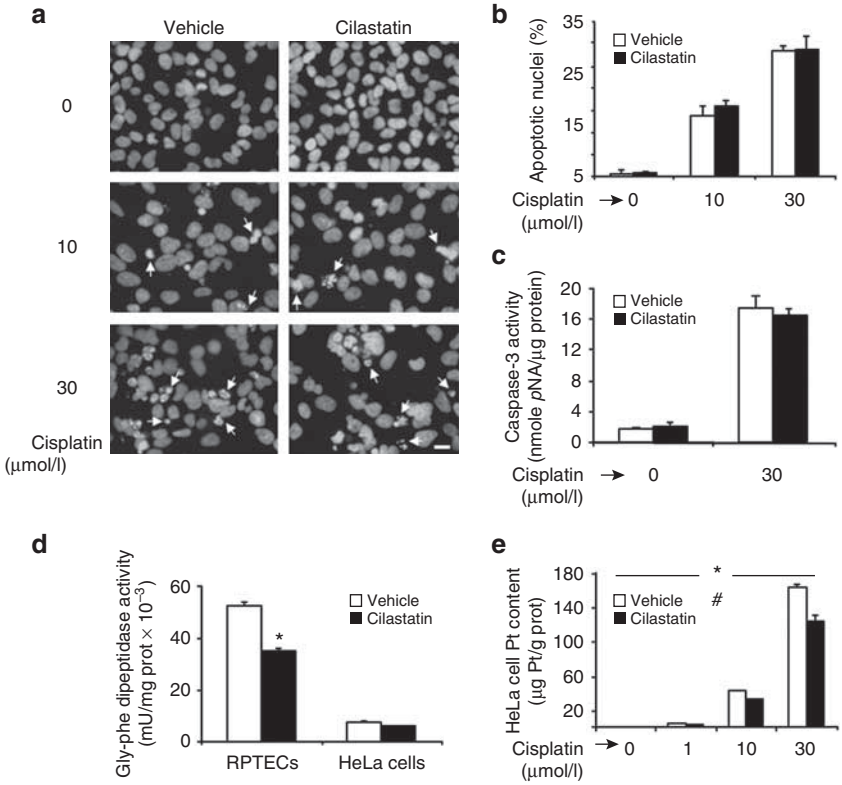

Figure 10 | Effect of cilastatin on cisplatin-induced cell death in HeLa cancer cells. HeLa cells were treated with cisplatin alone (1, 10, and 30 μmol/l) or in combination with 200 μg/ml cilastatin for 24 h. (**a**) Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI). Cells were stained with DAPI to study apoptotic-like nuclear morphology. Arrows point to fragmented apoptotic nuclei. Bar = 20 μm. (**b**) Quantitative approach to the images presented in **a**. (**c**) Activation of caspase-3. (**d**) Dehydropeptidase I (DHP-I)–specific activity in cultured renal proximal tubular epithelial cells (RPTECs) and HeLa cells. Activity was determined by the hydrolysis of 1 mmol/l gly-phe-*p*-nitroanilide in the presence or absence of 200 μg/ml cilastatin. Data are expressed as mean ± s.e.m. of at least three separate experiments and show that cilastatin does not interfere with the cytotoxic effect of cisplatin on tumor cells; *$P < 0.0005$ vs. same data without cilastatin. (**e**) Platinum (Pt) was measured in HeLa-soluble fractions using quadrupole inductively coupled plasma mass spectrometry. Values were expressed as the mean ± s.e.m. of Pt concentration, expressed in micrograms of Pt per gram of protein (prot; $n = 4$ different experiments). Analysis of variance model, $P < 0.0001$; *cilastatin effect, $P \leqslant 0.05$; #dose effect, $P \leqslant 0.05$.

cilastatin did not modify either DHP-I activity or cisplatin-induced cell death. Similarly, several cancer cell lines were not protected by cilastatin against the cytotoxic effects of cisplatin. However, we observed an inhibition of 24% in the intracellular content of cisplatin on HeLa cells treated with cilastatin. These findings complete our previous observations about the effect of cilastatin on tumor-derived cells.[17] Table 4 compares the inhibitory effects of cilastatin on different parameters of RPTECs or HeLa cells treated with cisplatin.

Cilastatin causes a modest inhibition of Pt cell transport that is not specific to kidney cells. This reduction in Pt intracellular content is not related with DHP-I presence or activity, and does not seem to have a major role on the intensity of induced apoptosis. On the other hand, cilastatin protection and DHP-I inhibition by cilastatin seem to be specifically restricted to proximal tubule cells.

Fas-mediated cell death requires the clustering of the Fas receptor in lipid rafts.[43,44] Exposure of human colon carcinoma cells to cisplatin induces formation of Fas aggregates and their redistribution into plasma membrane lipid rafts, together with the intracellular signaling molecules, Fas-associated protein with death domain and procaspase-8.[45] Furthermore, disruption of rafts abolishes the initiation of Fas death signaling in thymocytes and Jurkat cells.[46,47] We have recently observed that binding of cilastatin to proximal tubule renal DHP-I inhibits internalization of brush border lipid rafts.[17,19] In the presence of cisplatin, cilastatin inhibits internalization of Fas–FasL bound to cell membrane lipid rafts.[17] Here, we have observed that levels of Fas and FasL are decreased when cilastatin is present, and it could be related to reductions in caspase-8, -3, and apoptosis.

In summary, this study provides evidence that cilastatin reduces *in vivo* cisplatin nephrotoxicity by interference with extrinsic apoptotic mediators. Cilastatin does not interfere with the cytotoxic effects of cisplatin on tumor cell targets (as long as they do not have renal DHP-I) and does not modify the cisplatin AUC. Therefore, cilastatin has the potential to preserve renal function in cisplatin-treated cancer patients without compromising the efficacy of anticancer therapy.

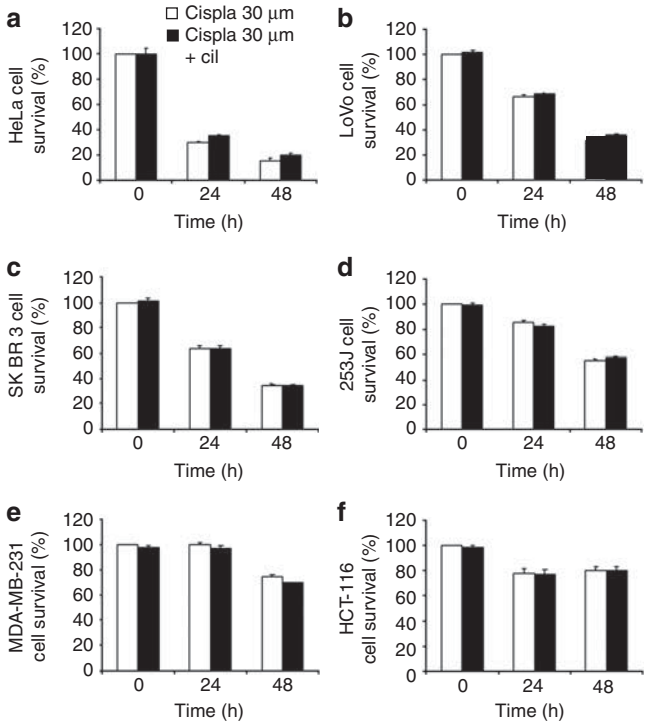

Figure 11 | Effect of cilastatin on the antitumor efficacy of cisplatin. (**a**) HeLa, (**b**) LoVo, (**c**) SK BR3, (**d**) 253J, (**e**) MDA-MB-231, and (**f**) HCT-116 cancer cells were treated with cisplatin (cispla) alone (30 μmol/l) or in combination with 200 μg/ml cilastatin (cil) at 24 and 48 h and cell viability was determined by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells incubated in the absence of cisplatin and cilastatin were used as 100%. Data are expressed as mean ± s.e.m. of four separate experiments and show that cilastatin does not interfere with the cytotoxic effect of cisplatin on tumor cells. Analysis of variance model, $P<0.0005$. Factors: cisplatin effect, $P<0.0005$; cilastatin effect, nonsignificant (NS); time effect, $P<0.0005$; cell type effect, $P<0.0005$. Interactions: cell type × cisplatin effect, $P<0.0005$; cisplatin × cilastatin effect, NS; cilastatin × time, NS.

MATERIALS AND METHODS

Drugs

Cisplatin was obtained from Pharmacia (Barcelona, Spain). Crystalline cilastatin was provided by Merck Sharp and Dohme SA (Madrid, Spain). Both cisplatin and cilastatin were dissolved in normal 0.9% saline (vehicle).

Proximal tubular primary cell culture and cancer cell line cultures

Porcine RPTECs were obtained as described previously[17,19] (further detail in Supplementary Information).

Breast (MDA-MB-231 and SKBR3), colon (LoVo and HCT-116), and bladder (253J) cancer cells lines were generously provided by Francisco del Real (CNIO, Madrid, Spain). Cells were cultured equally that HeLa cervical tumor cells[17] plus 1% sodium pyruvate.

Cancer cells viability was measured using MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described.[17]

Cellular cisplatin uptake and cell death studies in HeLa cells

HeLa cells incubated for 24 h with increasing concentrations of cisplatin in the presence or absence of cilastatin (200 μg/ml) were used to measure cell death (apoptotic nuclei and caspase-3 activity) and uptake of cisplatin as previously described.[17] Further details are found in Supplementary Information.

Table 4 | Comparison of cytotoxicity inhibition by cilastatin between renal proximal tubular epithelial cells and HeLa cancer cells

Parameter	Cultured renal proximal tubular epithelial cells (%)	Cultured HeLa cells (%)
Intracellular platinum	23.8[a17]	24.4[a]
DNA-bound platinum (detached cells)	43.7[a17]	0.0[b17]
Apoptosis (nuclear damage)[c]	63.8[a17]	0.0[b17]
Apoptosis (mitochondrial damage)[d]	20.6[a17]	4.4[b17]
Caspase-3 activity	32.7[a17]	5.4[b]

[a]Inhibition percentage different from 0, $P \leqslant 0.05$.
[b]Inhibition statistically nonsignificant.
[c]Nuclear damage was estimated from 4,6-diamidino-2-phenylindole staining data.
[d]Mitochondrial damage was estimated from MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay data.
Table shows the effect of cilastatin in reducing parameters previously elevated by cisplatin treatment in both cell types. Results are expressed as % of reduction.

DHP-I activity assay

DHP-I activity assay was realized in RPTECs and HeLa cells as previously described.[17]

Animals and experimental protocols

Studies were performed on 7-week-old male Wistar rats weighing 250–270 g (Criffa, Barcelona, Spain). The animals were housed under controlled light (12-h light–dark cycle), temperature, and humidity with free access to food and water. Rats were weighed at the beginning of the experiment and just before killing. The study was approved by the Institutional Board for Animal Experiments. Animals were handled at all times according to the applicable legal regulations in RD 1201/2005, of 10 October, on the protection of animals used for experimentation and other scientific purposes.

The study population comprised 30 animals, which were randomized into four groups: untreated control rats ($n=7$); cilastatin-treated rats ($n=7$); cisplatin-injected rats ($n=8$); and cilastatin-treated cisplatin-injected rats ($n=8$). Cisplatin (5 mg/kg) or its vehicle was administered by a single i.p. injection to rats in the same manner and volume (1 ml/100 g). Cilastatin was injected i.p. at 75 mg/kg twice daily from the day of cisplatin administration and until the day of killing. The first dose of cilastatin was administered just before administration of cisplatin. Cilastatin was substituted by vehicle (0.25 ml/100 g) in the other groups. The dose and administration period of cisplatin were selected based on the proven effectiveness of the drug in inducing nephrotoxicity.[3,12,16,25,26] The dose of cilastatin was selected from pilot studies based on previous experience,[48,49] which showed that imipenem/cilastatin reduced cyclosporin-induced nephrotoxicity. One day before the rats were killed, the urine of each animal was collected over a 24-h period and the volume measured. On the fifth day after the cisplatin or saline injection, all animals were anesthetized with ketamine (10 mg/kg) and diazepam (4 mg/kg) and killed. Blood and kidney samples were collected and stored for the different studies (further detail in Supplementary Information).

Renal function monitoring
Serum creatinine, blood urea nitrogen levels, and other parameters were measured using the Dimension RxL autoanalyzer (Dade-Behring, Siemens, Eschborn, Germany). The glomerular filtration rate was calculated using the creatinine clearance rate. Total protein in urine was measured using sulfosalicylic acid method.[50]

Renal histopathological studies
For light microscopy, paraffin-embedded renal sections (4-μm thick) were stained with hematoxylin–eosin (Sigma-Aldrich, St Louis, MO). The kidney injury score was calculated using a previously described semiquantitative index.[51]

Immunohistochemistry
Immunohistochemistry was carried out as described previously.[51] The primary antibodies used are found in Supplementary Information.

Western blotting analysis
Western blotting was performed as described previously.[17,50] The primary antibodies used are found in Supplementary Information.

In situ detection of apoptosis
DNA fragmentation as an index of apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling in paraffin-embedded kidney tissue sections using a Fluorescein FragEL DNA Fragmentation Detection Kit (Calbiochem, San Diego, CA) following the manufacturer's protocol (Supplementary Information).

Assessment of lipid peroxidation
MDA in renal homogenate was measured by the method of Recknagel *et al.*[52] Details are found in Supplementary Information.

Urine levels of antioxidant and hydrogen peroxide
Urine levels of antioxidant and hydrogen peroxide were measured using the commercially available Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, MI) and Colorimetric Hydrogen Peroxide Kit (Assay Designs, Stressgen, Ann Arbor, MI), respectively, according to the manufacturer's protocol.

Renal Pt determination and speciation analysis
Renal tissue was pulverized and digested with lysis buffer (further detail in Supplementary Information). The supernatant was analyzed for total protein content and cisplatin (as Pt) using inductively coupled plasma mass spectrometry as previously described.[17]

Pt-biomolecule speciation was analyzed using high-performance liquid chromatography/inductively coupled plasma mass spectrometry as described elsewhere.[17]

Pharmacokinetics
A catheter was inserted into the tail vein under anesthesia (ketamine 50 mg/ml and xylacine 2%). Rats were given a single i.p. injection of cisplatin (5 mg/kg, $n=4$), or cisplatin plus cilastatin (75 mg/kg, i.p., $n=4$). Cilastatin was substituted by vehicle in the cisplatin group. Blood samples were collected at 0, 15, 30, 60, 120, and 240 min after injections via the catheter. Total Pt was measured in serum aliquots by inductively coupled plasma mass spectrometry. AUC, C_{Max}, time to maximal Pt concentration (t_{Max}), elimination rate constant, and $t_{1/2}$ were estimated for each animal. The program used was Microsoft Excel fitted with pharmacokinetics functions written for Excel by Joel I Usansky, Atul Desai, and Diane Tang Liu (Allergan, Irvine, CA); (http://www.boomer.org/).

Statistical analysis
Quantitative variables were summarized as the mean ± s.e.m. Equality of variances was tested with Levene's text. Normally distributed continuous variables with equal variances were analyzed with analysis of variance. If variances were not equal, the Kruskal–Wallis test was performed. Differences were considered statistically significant for bilateral α-values <0.05. Tests were performed using SPSS 11.5 software package (SPSS, Chicago, IL).

DISCLOSURE
AT, AL, SC and JAL: the use of cilastatin as a renal protector against toxic injuries is under patent (PCT International Application No. ES2008/070137).

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SUPPLEMENTARY MATERIAL
Supplementary Information.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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