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## Novel Strategies in Drug-Induced Acute Kidney Injury

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### 1. Introduction

#### 1.1 Renal toxicity

Renal toxicity associated with commonly prescribed drugs lengthens hospital stay, worsens prognosis, and limits the potential benefits obtained from therapy (Peracella, 2011; Servais et al., 2008).

Proximal tubule preservation is a clue in strategies aimed to prevent nephrotoxicity. The proximal tubule is a target for filtered drugs that are reabsorbed by solvent drag or pinocytosis, but also for drugs that are secreted into the luminal side.

Proximal tubules recover more than 60% of total filtered load, i.e., a single molecule of toxin that is filtered and reabsorbed will pass through the proximal tubule cell more than 50 times per day. Such a high degree of exposure implies a risk of cell damage causing a variety of clinical syndromes, from proximal acidosis and acquired Fanconi syndrome to tubular cell necrosis (Oh, 2010). This spectrum of diseases is known as acute kidney injury (AKI), which also includes cell death by apoptosis, anoikis, necrosis, or cell dysfunction (Lorz et al., 2006).

Nephrotoxicity can often be expected with certain drugs, such as vancomycin, gentamicin, foscarnet, cisplatin, cyclosporine A (CsA), and tacrolimus. Less often, the toxic effect is unexpected and not predictable, as is the case with iodinated contrast agents and paracetamol.

#### 1.2 Cell death mediation

Intrinsic pathway-mediated apoptosis and extrinsic pathway-mediated apoptosis are both involved in toxic proximal tubule cell death (Pabla & Dong, 2008; Servais et al., 2008; Xiao et al., 2011). With most of toxins, cell death is followed by detachment and anoikis. Paracetamol is a notable exception to this behavior. Caspases activation, mitochondrial depolarization, release of cytochrome C from mitochondria, cell membrane modification, and nucleosome formation are all hallmarks of apoptosis that are regularly observed in toxin-damaged proximal tubules (Camano et al., 2010). Nitric oxide, soluble oxygen radicals, and proinflammatory cytokines are released by damaged proximal tubules, thus amplifying the lesion.

### 1.3 Nephrotoxicity prevention strategies

Overhydration is the most common maneuver to prevent toxic concentrations in urine and, consequently, inside the cell. However, nephrotoxicity usually requires dose adjustment or drug withdrawal, thus limiting effectiveness.

Other strategies aimed at inhibiting cell drug transport or interfering with mediation of apoptosis also tend to interfere with the therapeutic targets and, consequently, limit the effectiveness of therapy (Pabla & Dong, 2008; Servais et al., 2008).

During the last 5 years, our work on nephrotoxicity has enabled us to better understand the role of proximal tubule behavior in the adaptation of the kidney to toxic aggressions (Camano et al., 2010; Camaño-Paez et al., 2008; Neria et al., 2009; Perez et al., 2004; Tejedor et al., 2007).

Therefore, not surprisingly, the search for alternative protective strategies against toxic damage to the proximal tubule is an important area of investigation today.

### 1.4 Ability of cilastatin to prevent drug toxicity targeting the proximal tubule

Cilastatin is an inhibitor of brush border dehydropeptidase I (DHP-I), which is present in renal proximal tubular epithelial cells (RPTECs). It was initially designed to inhibit hydrolysis and uptake of the carbapenem antibiotic imipenem, thus enabling it to be more easily recovered from urine (Birnbaum et al., 1985; Norbby et al., 1983). However, cilastatin is also able to inhibit uptake of CsA and cisplatin by RPTECs by decreasing in a dose-dependent way the toxic effect of CsA and cisplatin on RPTECs (Camano et al., 2010; Perez et al., 2004). Clinical studies also support this protective role of cilastatin against CsA-induced nephrotoxicity (Carmellini et al., 1997, 1998; Gruss et al., 1996; Markewitz et al., 1994; Mraz et al., 1987, 1992; Tejedor et al., 2007). Experimental evidence suggests that cilastatin binding to brush border DHP-I could interact with apical cholesterol lipid rafts (Camano et al., 2010; Perez et al., 2004; Tejedor et al., 2007).

The aim of this brief report is to determine whether cilastatin is able to interfere with the direct toxic effect of several known nephrotoxic drugs on cultured RPTECs. We investigated the effect of cilastatin on the toxicity of gentamicin, vancomycin, iodinated contrast agent, amphotericin B, foscarnet, cisplatin, mannitol, chloroform, paracetamol, CsA and tacrolimus.

We describe for the first time the effects of a drug that specifically targets the renal proximal tubule brush border and seems to be able to reduce accumulation and toxicity of the main nephrotoxic drugs by inhibiting internalization of brush border-bound lipid rafts.

## 2. Methods

### 2.1 Drugs

We used commercially available parenteral formulations of gentamicin (powder, Guinama, Alboraya, Spain), vancomycin (powder, Combino Pharm, Barcelona, Spain), iodinate contrast agent (iopamidol, Laboratorios Farmacéuticos Rovi, Madrid, Spain), amphotericin B (Bristol Myers Squibb, Madrid, Spain), foscarnet (Foscavir, AstraZeneca, Madrid, Spain), cisplatin (Pharmacia, Barcelona, Spain), mannitol 20%, (Osmofundin®, Braun Medical S.A., Barcelona, Spain), chloroform (Scharlau, Barcelona, Spain), CsA (Sandimmun Neoral®,

Novartis Farmaceutica S.A., Spain), tacrolimus (Prograf®, Fujisawa S.A., Spain), and paracetamol (Perfalgan, Bristol Myers Squibb). The concentrations used were similar to the pharmacologically active recommended plasma level.

Crystalline cilastatin was provided by Merck Sharp & Dohme S.A. (Madrid, Spain). A dose of 200 µg/ml was chosen, because it is cytoprotective and falls within the reference range for clinical use (Camano et al., 2010; Perez et al., 2004).

All drug dilutions were performed with sterile culture medium and cilastatin, and the tested drugs were added simultaneously.

## 2.2 Primary cultures of renal proximal tubule epithelial cells

Porcine RPTECs were obtained as previously described (Camano et al., 2010; Perez et al., 2004). Briefly, the cortex was sliced and incubated for 30 minutes at 37°C with 0.6 mg/ml of collagenase A (Boehringer Mannheim, Germany) in Ham's F-12 medium. Digested tissue was then filtered through a metal mesh (250 µm), washed 3 times with Ham's F-12 medium, and centrifuged using an isotonic Percoll gradient (45% [v/v]) at 20,000g for 30 minutes. Proximal tubules were recovered from the deepest fraction, washed, and resuspended in supplemented DMEM/Ham's F-12 at a 1:1 ratio (with 25 mM HEPES, 3.7 mg/ml sodium bicarbonate, 2.5 mM glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin,  $5 \times 10^{-8}$  M hydrocortisone, 5 mg/ml insulin-transferrin-sodium selenite media supplement, and 2% fetal bovine serum). Proximal tubules were seeded at a density of 0.66 mg/ml and incubated at 37°C in a 95% air/5% CO<sub>2</sub> atmosphere. Culture medium was renewed every 2 days. RPTECs were used after they had reached confluence (80%).

## 2.3 Cell death studies

### 2.3.1 Nuclear morphology

Cell nuclei were visualized following DNA staining with the fluorescent dye DAPI (Sigma-Aldrich, Missouri, USA). Briefly, cells were seeded on cover slips in a 24-well plate, fixed in 4% formaldehyde for 10 minutes, and permeabilized with 0.5% Triton X-100. They were then rinsed with PBS and incubated with DAPI (12.5 µg/ml) for 15 minutes. Excess dye was removed. Cells imaging was performed with the 40X PL-APO 1.25 NA oil objective of a Leica-SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany). DAPI was excited with a 405 nm laser-diode. Emission between 420 nm and 490 nm was collected following the manufacturer's recommendations. Six fields with ~200 cells per field were examined in each condition to estimate the percentage of nuclei with an apoptosis-like appearance.

### 2.3.2 Nucleosomal quantification

To evaluate DNA fragmentation in the context of apoptosis, RPTECs were incubated for 48 hours under specific conditions with the nephrotoxic compounds selected. At the end of this period RPTECs were lysed and centrifuged at 200g for 10 minutes to remove cell debris. DNA and histones present in the soluble fraction were quantified using an enzyme-linked immunosorbent assay (*Cell Death Detection ELISA<sup>PLUS</sup>* kit, Boehringer Mannheim, Germany), as previously described (Camano et al., 2010; Perez et al., 2004).

### 2.3.3 Cell viability assay

The cell survival assay relies on the capacity of cells to reduce 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Calbiochem, California, USA) to colored formazan in metabolically active cells. RPTECs were seeded onto 96-well plates and incubated with toxins alone or in combination with cilastatin. Twenty-four hours later, 0.5 mg/ml of MTT was added, plates were incubated for 3 hours in the dark at 37°C, and 100  $\mu$ L of 50% dimethylformamide in 20% SDS (pH 4.7) was added. Plates were incubated at 37°C overnight, and absorbance was measured at 595 nm. All assays were performed in triplicate.

Alternatively, MTT assays were performed in real time, following MTT reduction on single cells, with an Olympus IX70 inverted microscope fitted to a spectrofluorometer SLM AMINCO 2000. MTT was measured by reading cell absorbance at 570 nm.

### 2.4 Cell viability: Quantification of colony-forming units

RPTECs were treated for 24 hours with CsA, tacrolimus or paracetamol in the presence or absence of cilastatin (200  $\mu$ g/ml). Adherent cells were washed in saline serum, harvested with trypsin-EDTA, seeded in Petri dishes (100 mm), and cultured for 7 days in drug-free complete medium. Surviving adherent cells were fixed for 5 minutes with 5% paraformaldehyde/PBS and stained with 0.5% crystal violet/20% methanol for 2 minutes. Excess dye was rinsed with PBS. Finally, the intracellular dye was eluted with 50% ethanol/50% sodium citrate 0.1 M (pH 4.2) and quantified by spectrometry at 595 nm.

### 2.5 Cellular drug transport and accumulation

RPTECs incubated for 24 hours with increasing concentrations of CsA, tacrolimus or paracetamol in the presence or absence of cilastatin (200  $\mu$ g/ml), were scraped and lysed in 400  $\mu$ L of lysis buffer at 70°C (2.22% [w/v] SDS; 19.33 % [v/v] glycerol [87% v/v]; 790 mM Tris HCl pH 6.8 in dH<sub>2</sub>O, phenylmethylsulfonyl fluoride, and protease inhibitors). Cell lysates were heated at 100°C for 5 minutes, homogenized in ice, and centrifuged at 12,000g for 5 minutes at 4°C. The supernatant was analyzed for total protein content and the presence of nephrotoxins. The concentrations of CsA, tacrolimus and paracetamol were measured using fluorescence polarization immunoassay technology on a TDX Chemistry Analyzer (Abbot Laboratories, USA) in accordance with the instructions provided by the manufacturer. The calibrators and controls supplied with each kit were applied, and the results were expressed as ng drug/ $\mu$ g protein.

### 2.6 Localization of lipids rafts by immunofluorescence

To study the interaction of cilastatin with cholesterol lipid rafts, we used FITC-conjugated cholera toxin B (Molecular Probes, Oregon, USA), as its internalization is mediated by lipid rafts.

RPTECs cultured on glass coverslips were preincubated with culture medium alone or cilastatin 200  $\mu$ g/ml for 15 minutes. The cells were then incubated with 10  $\mu$ g/ml FITC-labelled cholera toxin B for 1 and 2.5 hours. Cells were washed with PBS and fixed in 4%

formaldehyde in PBS for 10 minutes before being rinsed with PBS. The nuclei were counterstained with DAPI. After washing, cells were mounted in fluorescent mounting medium (Dako North America, Inc., Carpinteria, California). Images of the distribution of cholera toxin immunolocalization across membranes were obtained with the 20X PL-APO 0.7-numerical aperture objective of a Leica-SP2 confocal microscope (Leica Microsystems).

## 2.7 Dehydropeptidase I and IV activity assays

RPTECs were incubated overnight with Gly-Phe-*p*-nitroanilide (DHP-I substrate; Sigma-Aldrich) 1mM in PBS for DHP-I activity determination or with Gly-Pro-*p*-nitroanilide (DHP-IV substrate; Sigma-Aldrich) 1 mM for DHP-IV activity determination. Both activities were measured in the presence or absence of cilastatin (200 µg/ml). *P*-Nitroanilide was quantified in aliquots from supernatants by measuring at 410 nm absorbance.

## 2.8 Statistical analysis

Quantitative variables were expressed as the mean  $\pm$  standard error of the mean (SEM). Differences were considered statistically significant for bilateral alpha values less than 0.05. Factorial ANOVA was used when more than 1 factor was considered. When a single factor presented more than 2 levels and the model showed significant differences between factors, a post-hoc analysis (least significant difference) was performed. When results are shown, they represent a minimum of at least 3 repeats. When possible, a quantification technique (e.g. dye recovery) was used to illustrate reproducibility. When figures illustrated an effect, paracetamol was chosen as the example.

## 3. Results

### 3.1 Cilastatin as a broad nephroprotective drug: reduction of toxin-induced proximal tubular cell death

After 48 hours of exposure to the drugs tested, apoptosis of RPTECs measured as nucleosomal DNA fragmentation and migration from nuclei to cytosol was quantified and compared with apoptosis under the same conditions, although in the presence of cilastatin (Fig. 1). RPTECs exposed to toxins present different increases in the number of nucleosomes recovered from cytosol. Cilastatin significantly partially or totally prevented these changes in most of the selected drugs (Fig. 1).

When the magnitude of cilastatin protection was plotted against the magnitude of basal cell death under every treatment tested, a clear linear trend was observed ( $r=0.839$ ,  $p<0.0005$ ). None of the drugs tested differed significantly from this trend (Fig. 2).

We made a detailed study of the effect of 3 of these drugs: CsA, tacrolimus and paracetamol. A more selective qualitative estimation of apoptotic cell death was also obtained in adherent cells treated with CsA, tacrolimus, and paracetamol and stained with DAPI (Fig. 3). Incubation with toxins led to cell shrinkage with significant nuclear condensation, fragmentation, and formation of apoptosis-like bodies (see arrows). Cilastatin was able to reduce nuclear damage in all cases. Apoptosis-like nuclei are quantified in Fig. 3B, C, and D.

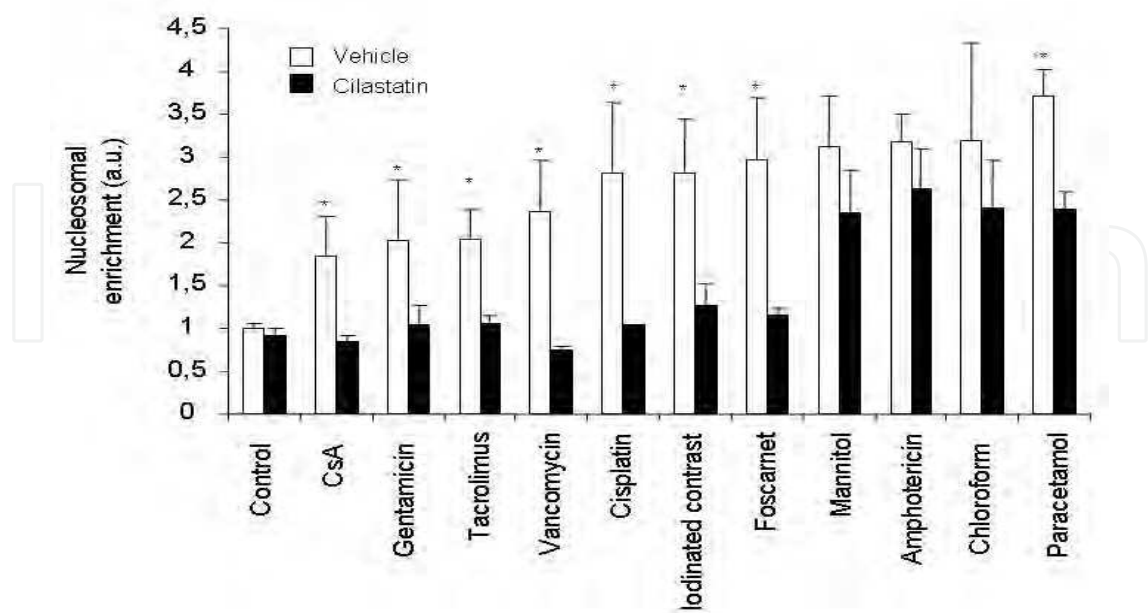


Fig. 1. Effect of cilastatin on nephrotoxin-induced apoptosis. Renal proximal tubular epithelial cells were exposed to CsA (1  $\mu\text{g/ml}$ ), gentamicin (20  $\text{mg/ml}$ ), tacrolimus (50  $\text{ng/ml}$ ), vancomycin (600  $\mu\text{g/ml}$ ), cisplatin (10  $\mu\text{M}$ ), iodinated contrast (1  $\text{mg/ml}$ ), foscarnet (1  $\text{mM}$ ), mannitol (100  $\text{mosm/l}$ ), amphotericin B (10  $\mu\text{g/ml}$ ), chloroform (100  $\mu\text{g/ml}$ ), and paracetamol (300  $\mu\text{g/ml}$ ) with and without cilastatin (200  $\mu\text{g/ml}$ ) for 48 hours. Oligonucleosomal DNA fragmentation was detected by ELISA. Data are represented as the mean  $\pm$  SEM of at least 3 separate experiments. ANOVA model:  $p < 0.0001$ . \* $p < 0.05$  vs. same data with cilastatin.

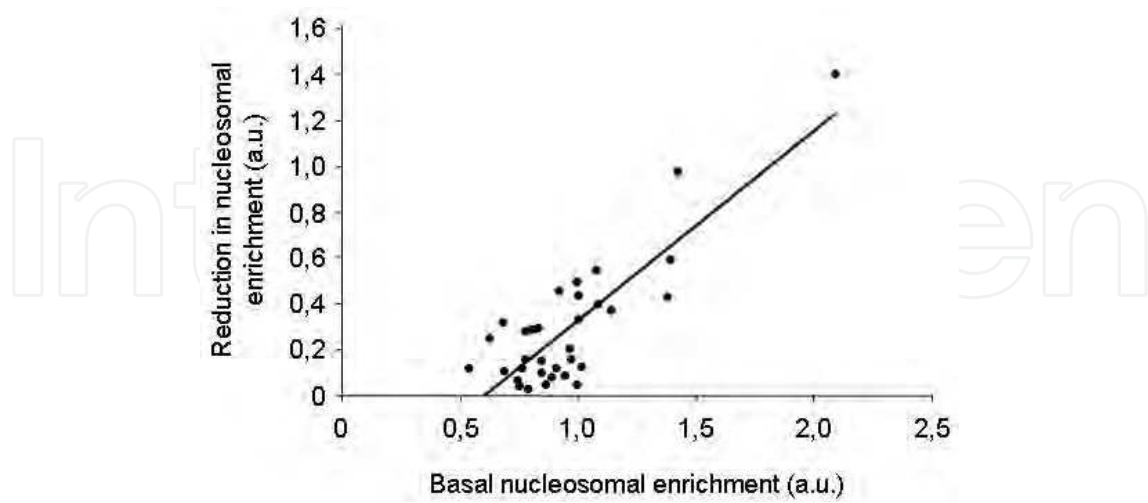


Fig. 2. Reduction in nucleosomal enrichment induced by cilastatin over basal nucleosomal enrichment induced by each toxin. Individual experimental data are provided. There is a common trend for all the data, suggesting a common behavior, with cilastatin protection being proportional to basal damage. Linear regression of “cilastatin-induced reduction” vs. “basal nucleosomal enrichment”, slope=0.82,  $r=0.839$ , adjusted  $r^2=0.695$ ,  $p < 0.0005$ .

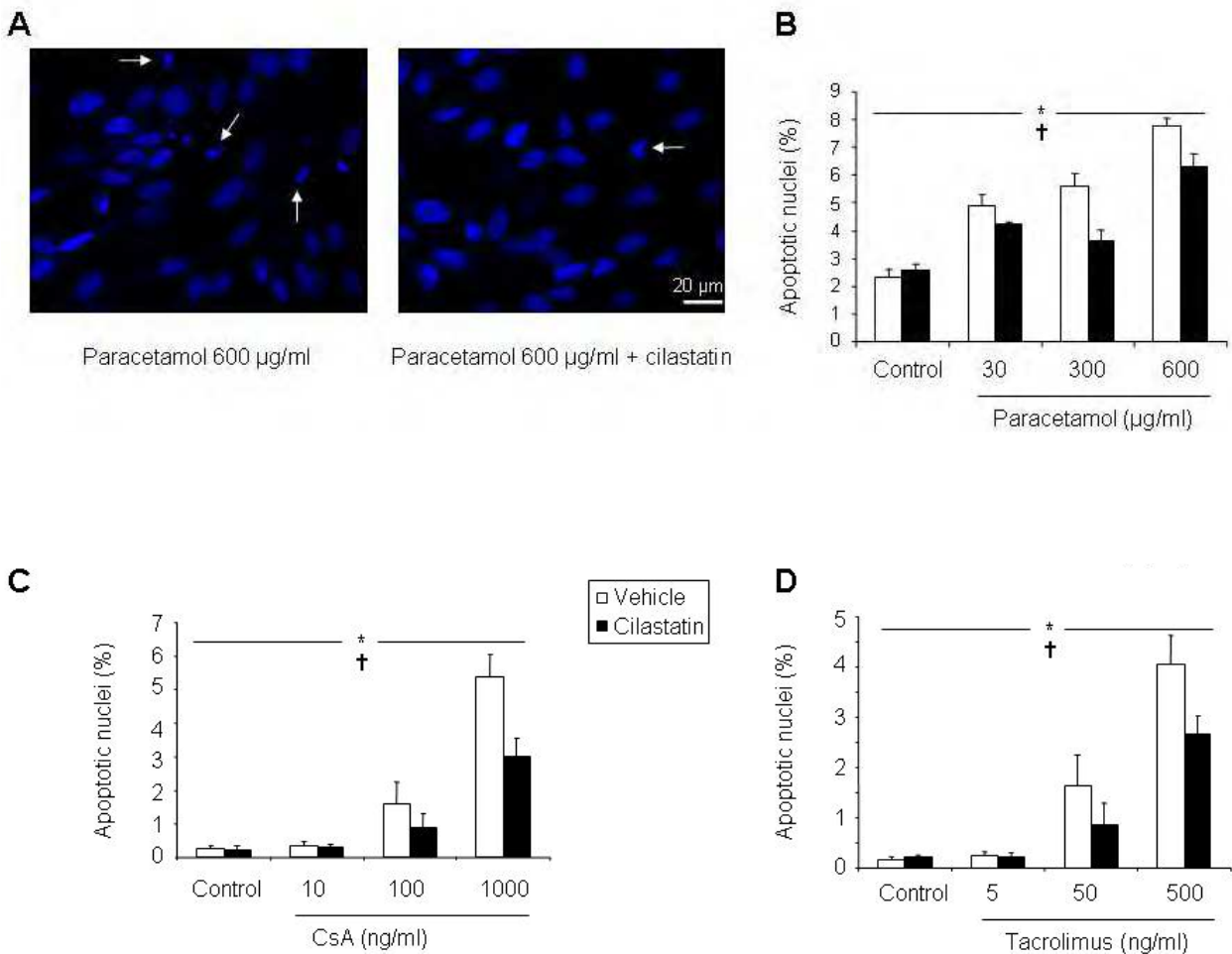


Fig. 3. Effects of cilastatin on the nuclear morphology of renal proximal tubular epithelial cells (RPTECs) during treatment with toxins. RPTECs were cultured in the presence of paracetamol (30, 300 and 600  $\mu\text{g/ml}$ ), cyclosporine (CsA, 10, 100, and 1000 ng/ml) and tacrolimus (5, 50, and 500 ng/ml) with or without cilastatin (200  $\mu\text{g/ml}$ ) for 24 hours. A, Example of nuclear staining with DAPI to determine whether an apoptotic-like nuclear morphology was present. Arrows point to fragmented, apoptotic nuclei. B, C and D, Quantitative approach to staining for paracetamol, CsA and tacrolimus, respectively. Data are represented as the mean  $\pm$  SEM of at least 3 separate experiments. ANOVA models  $p < 0.0001$ . \* cilastatin effect,  $p < 0.05$ ; † dose effect,  $p < 0.05$ .

We quantified the functional impact of CsA, tacrolimus and paracetamol treatments on cell survival by measuring the percentage of adherent cells still able to reduce MTT to formazan after exposure to increasing doses of toxins. After 24 hours of incubation with toxins, the amount of surviving cells able to reduce MTT decreases progressively as the concentrations of CsA, tacrolimus, and paracetamol increase. However, in the presence of cilastatin, all surviving cells keep their capacity to reduce MTT (Fig. 4). Cilastatin is able to counteract both the structural and functional damage induced by toxins.

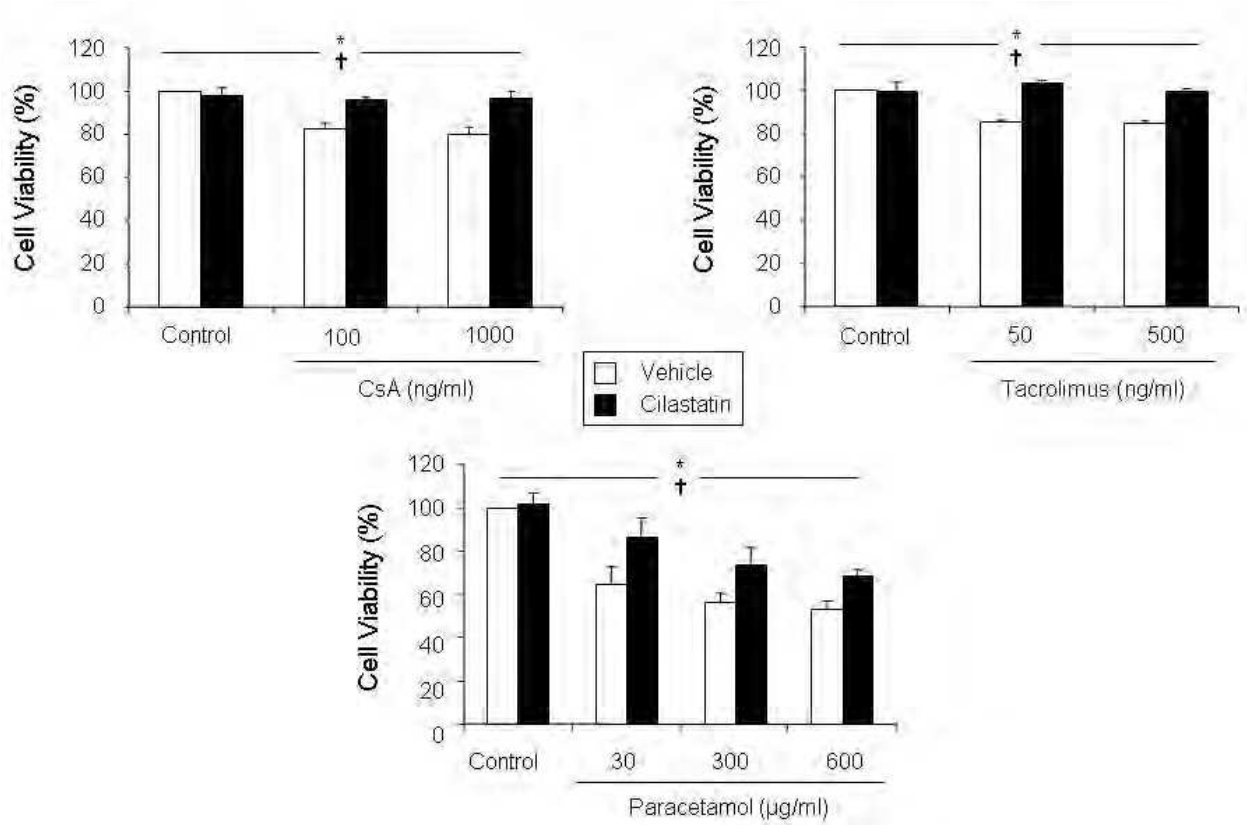


Fig. 4. Effect of cilastatin on toxin-induced loss of cell viability determined by the ability to reduce MTT (see Methods). Renal proximal tubular epithelial cells were exposed to toxins and toxins + cilastatin (200 µg/ml) for 24 hours. Results are expressed as the percentage of the value obtained relative to control (without toxins and cilastatin) of at least 3 separate experiments. ANOVA: for CsA, \*dose effect,  $p \leq 0.05$ ; †cilastatin effect,  $p < 0.05$ ; for tacrolimus, \*dose effect,  $p < 0.05$ ; †cilastatin effect,  $p \leq 0.04$ ; for paracetamol, \*dose effect,  $p \leq 0.05$ ; †cilastatin effect,  $p \leq 0.05$ .

3.2 Cilastatin prevents toxin-induced mitochondrial damage

The effect of cilastatin on mitochondria may be observed very early after CsA, tacrolimus, or paracetamol is added to cell culture plates. In Fig. 5, an inverted IX-80 microscope was fitted with a black chamber, a photomultiplier, and a spectrofluorimeter (SML Aminco) to obtain absorbance readings at specific wavelengths on single (or small groups of) cells in culture. This set-up allows real time follow-up of colorimetric in vivo reactions.

Recording the first seconds after MTT addition shows the initial kinetics of MTT reduction and formazan precipitation, thus offering a first approach to the activity of the mitochondrial chain in intact cells. Although not suitable for detailed kinetic studies, this method allows a quick check of mitochondrial oxidative activity.

RPTECs exposed to toxins showed a quick and deep depression in the reduction of MTT activity compared with controls (Fig. 5). Coincubation with cilastatin partially recovers this effect, although the effect was less visible for paracetamol. Differences are observed even during the first 5 minutes of drug additions.

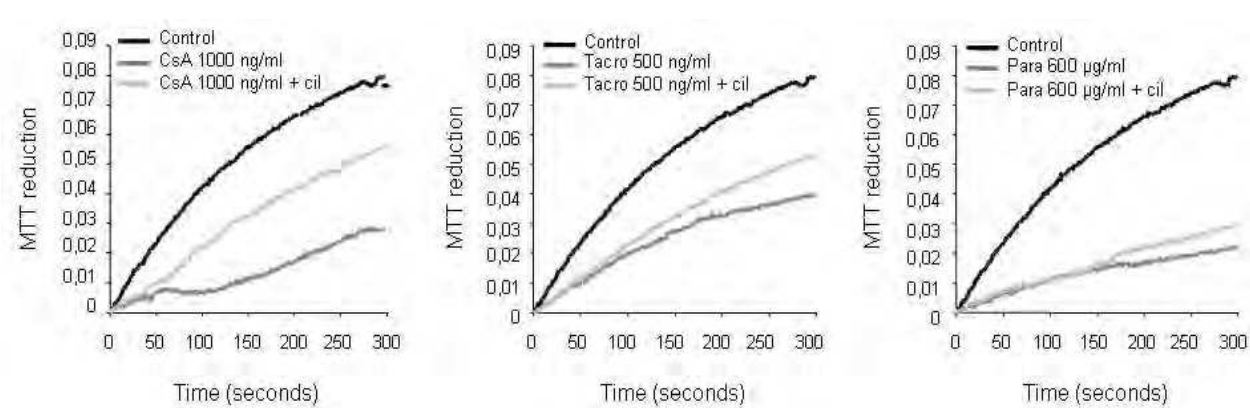


Fig. 5. Effect of cilastatin on toxin-induced mitochondrial damage. Changes in the mitochondrial oxidative capacity of RPTECs were assessed by MTT reduction at 570 nm. The graphs show formation of formazan as detected in isolated cells in real time with no treatment (control) and CsA (cyclosporin, 1000 ng/ml), tacro (tacrolimus, 500 ng/ml) and para (paracetamol, 600 µg/ml) with or without 200 µg/ml cilastatin, after the incubation times on the X-axis.

**3.3 Cilastatin improves long-term recovery and viability of RPTECs after exposure to CsA, tacrolimus, and paracetamol**

To know the long-term viability of surviving RPTECs after 24 hours of exposure to CsA, tacrolimus, or paracetamol, we tested the ability of those cells to proliferate into new cell colonies. Colony-forming units (CFUs) were quantified as specified in Methods. The CFUs count decreased after 24 hours of treatment with CsA, tacrolimus, and paracetamol, and this decrease was clearly dose-dependent (Fig. 6). If the cells were exposed to toxins in the presence of cilastatin, the number of CFUs was significantly greater after 7 days of recovery for every CsA, tacrolimus, and paracetamol concentration studied. The intracellular dye was extracted, and absorbance was quantified at 595 nm (Fig. 6B, C and D).

**3.4 Cilastatin reduces intracellular accumulation of CsA, tacrolimus, and paracetamol**

In many cases, nephrotoxicity is largely dependent on the intracellular concentration of drug reached. As cilastatin is a ligand of the brush border membrane, we investigated whether it affected toxin uptake by RPTECs. To test this hypothesis, we measured the intracellular content of CsA, tacrolimus, and paracetamol by TDX analysis, as described in Methods. Cellular CsA, tacrolimus and paracetamol content increased progressively in a dose-dependent manner when RPTECs were incubated for 24 hours in the presence of different concentrations of toxins (Fig. 7). Coincubation with cilastatin consistently reduced accumulation of CsA, tacrolimus and paracetamol in the cells for every concentration studied (Fig. 7). These results confirm that adding cilastatin to primary cultures of proximal cells decreases cellular toxin accumulation. This effect may be involved in the reduced impact of CsA, tacrolimus, and paracetamol on damage to and survival and death of RPTECs.

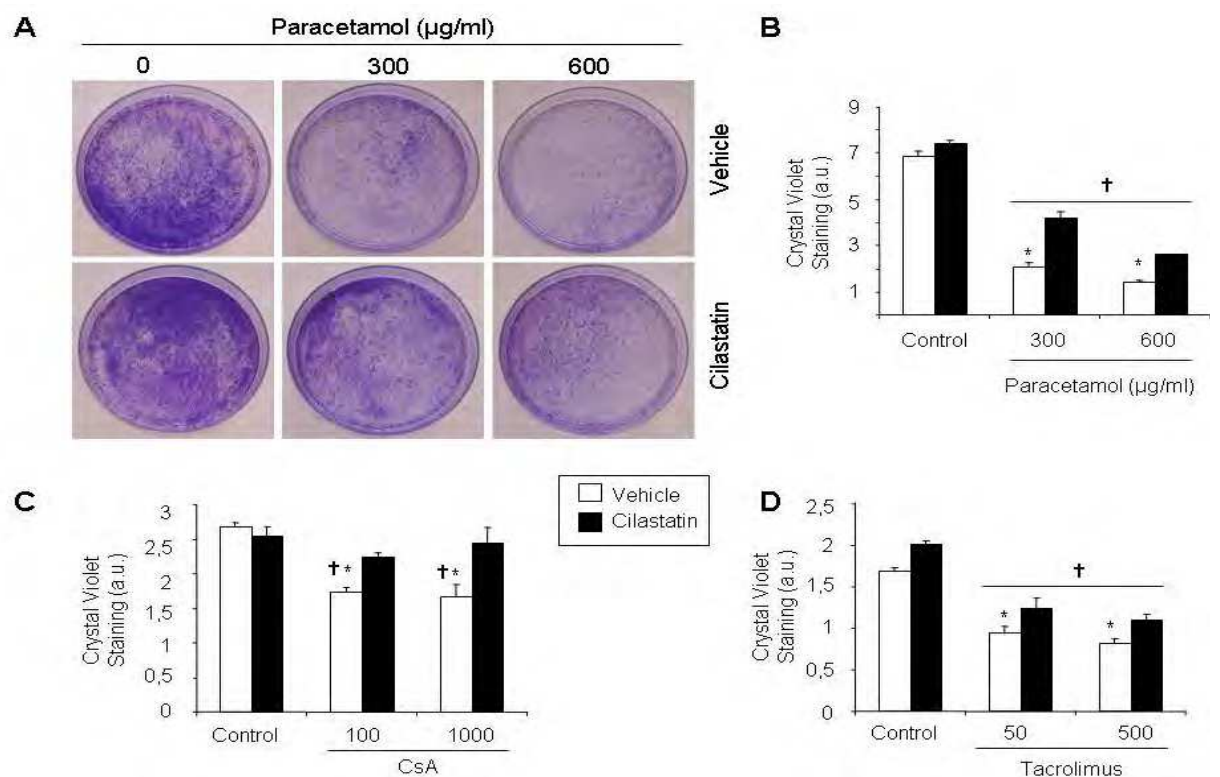


Fig. 6. Cilastatin preserves long-term recovery of toxin-treated RPTECs. A, RPTECs were incubated with paracetamol, CsA (cyclosporin), or tacrolimus in the presence or absence of 200 µg/ml cilastatin for 24 hours. The number of colony-forming units was determined by staining with crystal violet after 7 days (the figure shows the experiment with paracetamol). B, C, and D, Quantification of crystal violet staining for paracetamol, CsA and tacrolimus, respectively. Data are expressed as mean ± SEM; of 3 separate experiments. ANOVA model,  $p<0.0001$ . † $p<0.05$  vs. control; \* $p\leq0.05$  vs. same data with cilastatin.

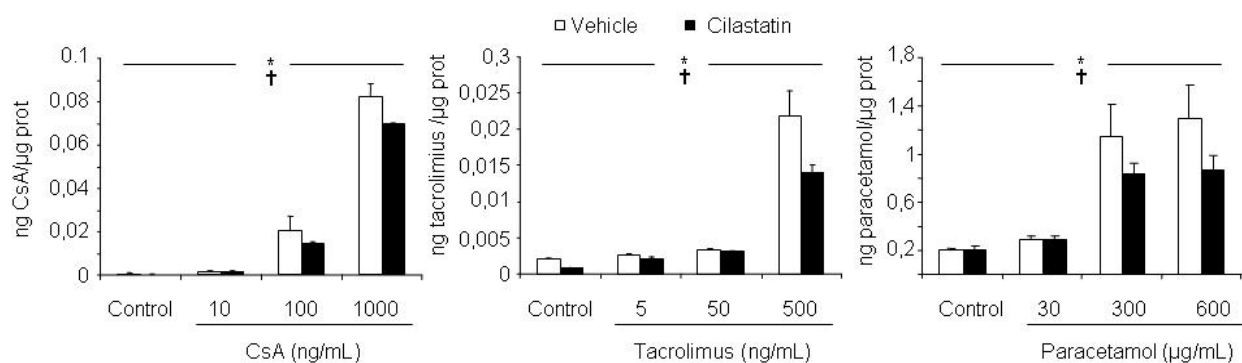


Fig. 7. Effects of cilastatin on accumulation of toxins by RPTECs. Intracellular accumulation was measured in the lysates of RPTECs treated with nephrotoxins for 24 hours, in the presence or absence of cilastatin (200 µg/ml), using a specific fluorescence polarization immunoassay (TDX). Cilastatin was shown to prevent entry of all nephrotoxins into RPTECs. Values were expressed as means ± SEM of drug concentrations (n=4 different experiments). ANOVA model,  $p<0.0001$ ; \*, cilastatin effect  $p<0.05$ ; †, dose effect  $p<0.05$ .

### 3.5 Effect of cilastatin on lipid rafts distribution

According to these results, which suggest that cilastatin interferes with intracellular administration of the nephrotoxins tested, cilastatin appears to be able to inhibit an intracellular nephrotoxin accumulation pathway as a result of its binding to renal DHP-I. We explored the possibility that cilastatin, through its interaction with DHP-I and when anchored to cholesterol lipid rafts by a glycosyl-phosphate-inositol (GPI) group (Adachi et al., 1990; Parkin et al., 2001), could block transport through lipid rafts or interfere with the cholesterol lipid raft-dependent endocytic pathway. The expression and cell membrane localization of cholera toxin, which specifically binds to its ganglioside GM1 receptor present in cholesterol lipid rafts, were assessed using confocal microscopy in RPTECs treated for very short periods. In Fig. 8, cholera toxin is identified on the cell surface after 15 minutes incubation, but it disappeared from the membrane after 1 hour (top) and accumulated in a perinuclear position. In the presence of 200  $\mu\text{g}/\text{ml}$  cilastatin and after 1 hour of treatment, cholera toxin was still attached to the membrane, suggesting interference with the cholera toxin internalization site. No significant changes in FITC-cholera toxin staining patterns were observed at 2.5 hours in the presence of cilastatin.

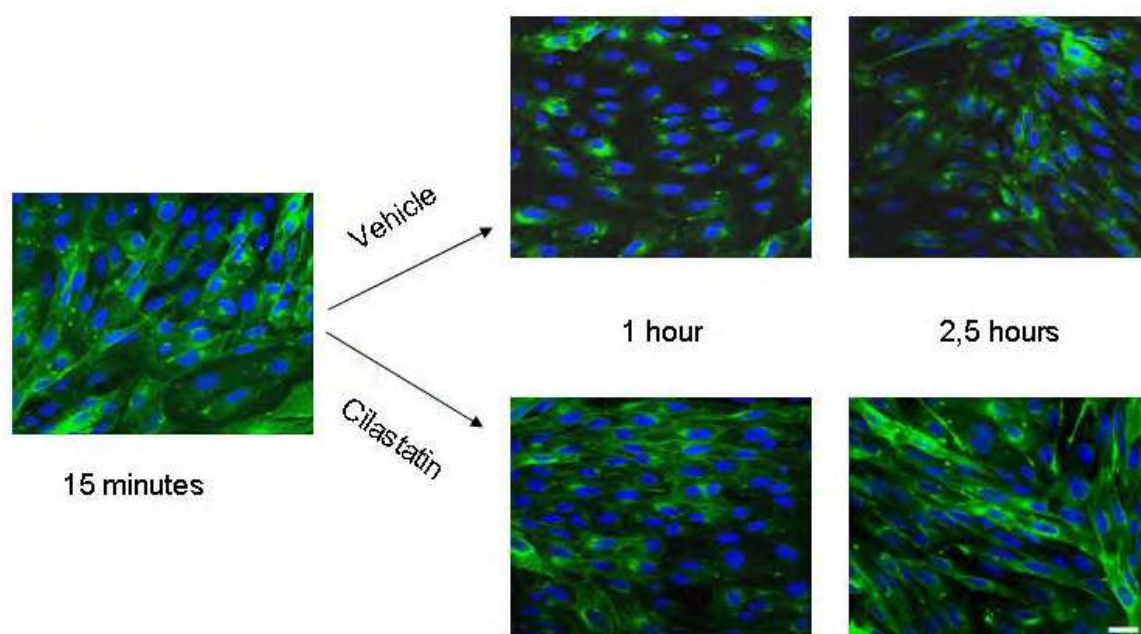


Fig. 8. Blockade of circulation of cholesterol rafts by cilastatin. This picture shows the change in cholera toxin fluorescence internalization over time in control cells and cells incubated in the presence of cilastatin (200  $\mu\text{g}/\text{ml}$ ). Bar, 20  $\mu\text{m}$ .

## 4. Conclusion

We report that cilastatin, a powerful and specific inhibitor of DPH-I, is able to reduce both intracellular accumulation and induction of apoptosis by antibiotic, cytotoxic, anti-inflammatory, antiretroviral, anesthetic, and immunosuppressive drugs. These findings expand our previous results with cisplatin (Camano et al., 2010) and CsA (Perez et al., 2004; Tejedor et al., 2007).

Cilastatin inhibits the activity of DPH-I, but not of DPH-4, in the brush border of renal RPTECs (Fig. 9).

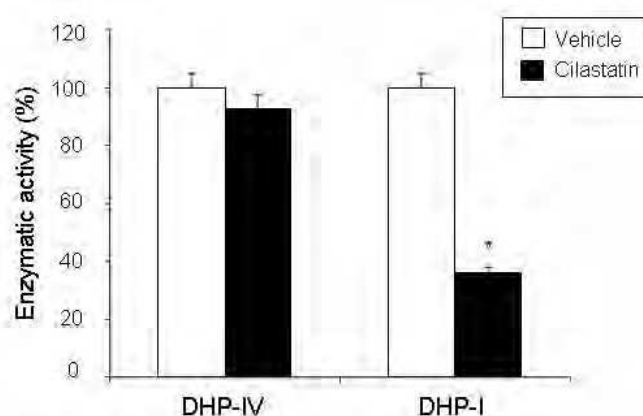


Fig. 9. Effect of cilastatin on the activity of dehydropeptidase I and IV. Activities were determined by the hydrolysis of specific substrates. Results are expressed as a percentage of enzyme activity compared to untreated controls (100% activity) and as the mean  $\pm$  SEM of 3 experiments. ANOVA model,  $p < 0.0001$ . \*  $p < 0.01$  vs. the same data without cilastatin.

Although this inhibition is probably irrelevant in the degree of nephroprotection observed – none of the nephrotoxins studied have a chemical structure that could potentially be affected by dipeptidase activity – binding to DPH-I may partially explain this protection.

DPH-I is anchored to brush border lipid rafts (Pang et al., 2004; Parkin et al., 2001). Binding of FITC-labelled B-cholera toxin to lipid rafts leads to their rapid internalization. However, internalization does not occur in the presence of cilastatin.

This mechanism is probably behind the reduction observed in the intracellular concentration of the different drugs analyzed.

We previously showed that cilastatin modifies brush border membrane fluidity by interfering with membrane-bound cholesterol (Perez et al., 2004).

The drugs tested in Fig. 1 have many different chemical structures, and their mechanisms of cell permeation are not well established in some cases. However, for all those drugs, intracellular concentrations were measured and cilastatin always reduced intracellular accumulation. By inhibiting lipid raft-dependent vesicle circulation, cilastatin seems able to reduce luminal entry of drugs, even if they are not substrates for DPH-I activity.

This interference with drug entry may explain the almost instantaneous protection observed in the real-time experiments of MTT reduction. MTT reduction relies on mitochondrial oxidative chain integrity. When single cell oxidative capacity is recorded in real time, addition of the toxin inhibits MTT reduction activity relative to the single control cell, and this is evident from the first seconds. Cilastatin partially protects against this effect. The quick time course of the effect strongly suggests that a mechanism of cilastatin inhibits drug intake by the cell.

However, other mechanisms may be implicated in the broad renal protection observed. We recently published that, when exposed to toxic concentrations of cisplatin, RPTECs increase

expression of Fas and Fas L. Fas targets brush border lipid rafts (Dimanche-Boitrel et al., 2005), binds its ligand, and triggers the extrinsic pathway of apoptosis. Internalization of Fas/Fas L seems a necessary step (Camano et al., 2010).

Cilastatin reduced cisplatin-induced cell apoptosis but not cell necrosis (Camano et al., 2010). When the extrinsic apoptosis pathway was checked, the initial step blocked by cilastatin was Fas L/Fas internalization (Camano et al., 2010).

Cilastatin reduces apoptosis (nuclear damage, nucleosome formation, MTT reduction capacity) and ameliorates surviving cell recovery. Both reductions in drug intake by proximal cells and blockade of lipid raft internalization are probably involved in these protective actions (Fig. 10).

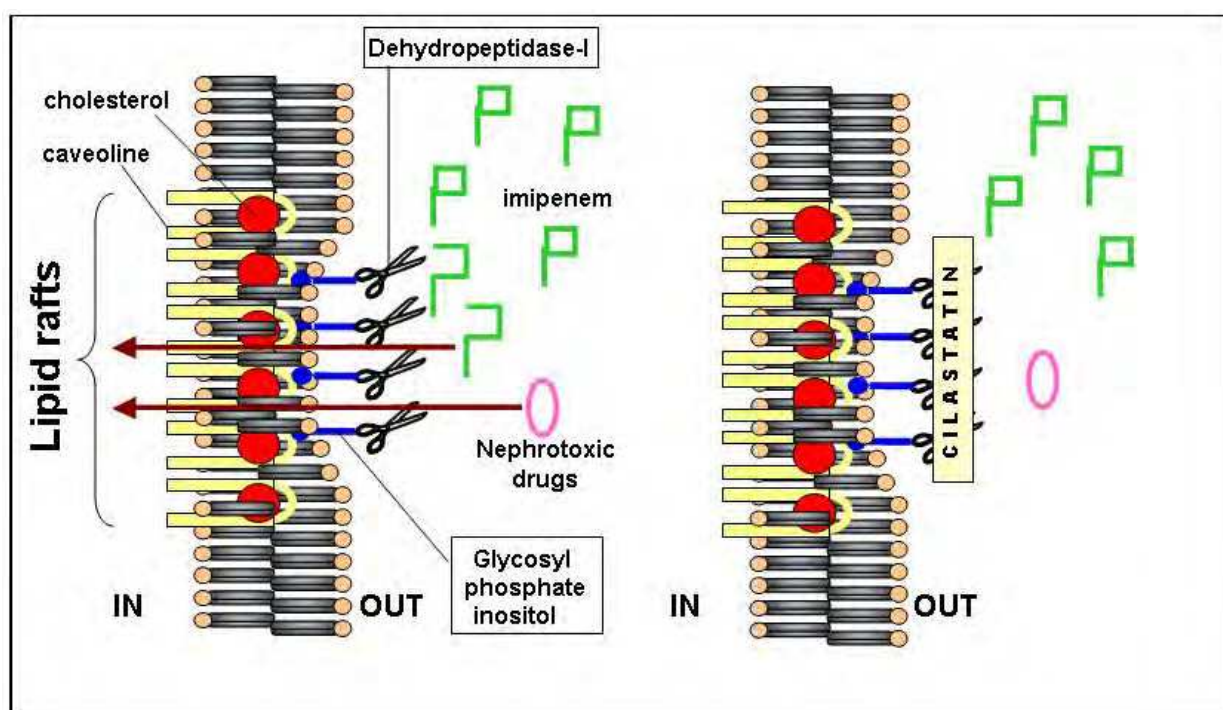


Fig. 10. Diagram of the possible protective mechanism of cilastatin. Cilastatin is a dehydropeptidase-I inhibitor used in human clinical practice combined with imipenem. Dehydropeptidase inhibition affects the structure of lipid rafts by preventing hydrolysis of the lactam ring and inhibits the absorption of imipenem and other nephrotoxic drugs, thus reducing their renal toxicity.

Protection by cilastatin depends on its interaction with DPH-I, an enzyme that is found almost exclusively in proximal tubules. Therefore, cilastatin-induced nephroprotection is specific for tissue and cell type, but not for the drug tested.

More research is necessary to confirm the mechanism of protection, the ability to protect in animal models of acute renal failure, and the absence of an effect on the pharmacological targets of tested drugs. Nevertheless, cilastatin offers a new protective strategy, as it is a tissue-specific designed drug, with unexpected tissue-specific antiapoptotic actions.

## 5. Acknowledgments

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