Inhibition of brush border dipeptidase with cilastatin reduces toxic accumulation of cyclosporin A in kidney proximal tubule epithelial cells

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Abstract

Background. Cilastatin reduces nephrotoxicity associated with cyclosporin A (CyA) in solid organ and bone marrow transplantation. This appears to be unrelated to changes in renal haemodynamics or CyA metabolism. How cilastatin induces this protection is unclear, but it could result from changes on accumulation of CyA proximal cells.

Methods. We investigated the effects of cilastatin on primary cultures of pig kidney proximal tubule epithelial cells (PTECs) treated with CyA and FK506. Cell membrane fluidity and membrane-bound cholesterol-rich raft (MBCR) distribution were evaluated by fluorescence microscopy, and CyA transport by radioimmunoassay. Changes in CyA- and FK506-induced apoptosis were also evaluated by electron and light microscopy, flow cytometry, and detection of cytoplasmic nucleosomes by enzyme-linked immunoabsorbent assay.

Results. CyA caused a dose-dependent reduction of cell membrane fluidity, which was prevented by pretreating PTECs with cilastatin. Cilastatin also inhibited CyA transport across membranes and reduced recovery of CyA in mitochondria and membrane-bound fractions from cilastatin-treated PTECs. This effect was not related to an altered distribution of MBCRs, which are essential for CyA transport. Cilastatin protected against CyA- and FK506-induced apoptosis.

Conclusions. Prevention of CyA-induced reduction of cell membrane fluidity and inhibition of CyA transport are features of cilastatin’s direct effects on PTECs. Unaltered distribution of MBCRs in the presence of cilastatin suggests that cilastatin binding to raft-bound dipeptidases, rather than MBCR modifications, causes interference with CyA transport. These results provide additional insight into the mechanisms and scope of cilastatin nephroprotection.

Keywords: cilastatin; cyclosporin A; dipeptidase; FK506; proximal tubule cells; raft

Introduction

Cyclosporin A (CyA) is a calcineurin inhibitor, which remains currently one of the most widely used immunosuppressive drugs for solid organ and haematopoietic stem cell transplantation, and for autoimmune disease treatment. Despite its efficacy, immunosuppression with CyA may be limited by inherent untoward effects, of which nephrotoxicity is most striking. Acutely, CyA may induce nephrotoxicity characterized by renal vasoconstriction, which is largely reversible upon dose reduction [1]. However, an irreversible decline in kidney function may also be observed after long-term CyA use [1], and is associated with chronic histopathological changes including obliteratorative arteriolopathy with deposition of proteinaceous material in necrotic arteriolar walls and tubulointerstitial damage, which are rarely reversible and potentially progressive. Similar observations have been made with another calcineurin inhibitor, FK506 [1], which shares with CyA similar pharmacological action and toxicity profile.

Although the mechanisms of CyA-induced nephrotoxicity are not fully understood, it is generally agreed that the acute haemodynamic effects of CyA may be mediated by an imbalance of different vasoconstrictors and vasodilators, including the renin–angiotensin system, prostaglandins, endothelins or nitric oxide [2,3]. For chronic CyA nephrotoxicity, histopathological studies have suggested a toxic effect of the drug on
afferent arterioles and tubular epithelial cells. Moreover, it has been hypothesized that therapeutic concentrations of CyA may induce tubular cell apoptosis in vivo [4,5]. Several investigators have performed in vitro experiments in established cell cultures of renal epithelial cells in an attempt to identify the cellular mechanisms underlying CyA damage to the kidney [4–6]. However, despite this growing evidence, the information gathered so far from the various experimental approaches cannot be structured into a unifying view of CyA-induced nephrotoxicity.

In the last few years, several clinical and experimental studies have reported that administration of the antibiotic imipenem/cilastatin reduces CyA-associated nephrotoxicity in kidney [7,8], heart [9] and bone marrow transplantation [10]. Although imipenem could be partially or totally responsible for this nephroprotective effect, this hypothesis seems unlikely since imipenem is known to cause acute proximal tubular necrosis [11]. Cilastatin has also been reported to alleviate the nephrotoxicity of other drugs, such as vancomycin and cisplatin [12].

How cilastatin induces this protection is unclear, but it could result from changes occurring at the level of the cell. The aim of the present study was to investigate the protective effects of cilastatin on primary cultures of pig kidney proximal tubule epithelial cells (PTECs) treated with CyA and FK506. Specifically, we examined the impact of cilastatin on epithelial cells in an attempt to identify the cellular mechanisms underlying CyA damage to the kidney [4–6].

Subjects and methods

Chemicals

Test compounds were prepared as stock solutions using various solvents. CyA was obtained as Sandimmun Neoral®, containing ethanol as vehicle (Novartis Farmaceutica S.A., Spain). FK506 was obtained as Prograf®, containing polyoxyethylene hydrogenated ricin oil (HCO-60) and dehydrated alcohol as vehicle (Fujisawa S.A., Spain). Cilastatin powder was kindly donated by Merck Sharp & Dohme S.A., Spain, and was dissolved in 0.9% saline serum.

Experimental animals

Miniature swine [aged 3 months, with an average weight of 31.3 kg (SD, 0.7 kg)] were used as the source for primary cultures of kidney PTECs. These animals have been genetically selected to be isogenic for three loci of the major histocompatibility complex, as described previously [13]. All animal care procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources and published by the National Institutes of Health. Approval of the project was obtained from the local committee of Ethics of Research, according to Spanish regulations.

Primary cultures of kidney proximal tubule epithelial cells

The kidneys were aseptically removed from the animals and placed in a sterilized beaker containing Ham's F-12 culture medium, pH 7.4. Briefly, cortex was trimmed from kidneys and sliced with a Stadie-Riggs microtome. Cortical slices were incubated with 0.1% collagenase A (Boehringer Mannheim, Germany) in Ham's F-12 culture medium for 30 min at 37°C.

After enzymatic dissociation, the resulting suspension was filtered through a metal mesh (250 μm), washed three times with Ham's F-12 medium, and centrifuged on a Percoll gradient (45% [v/v]) at 20000 g for 30 min. Proximal tubules were recovered by aspiration with a sterile pipette of the corresponding cell fraction. Next, proximal tubules were washed three times and resuspended in Dulbecco's modified Eagle's medium/Ham's F12 at a 1:1 ratio supplemented 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 × 10−5 M hydrocortisone, 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml sodium selenite and 2% fetal bovine serum (all from Sigma, USA). Proximal tubules were then seeded on plastic 60 mm culture dishes at a density of 0.66 mg/ml. The cells were incubated at 37°C in a 95% air/5% CO2 atmosphere for 3 days. Thereafter, culture medium was renewed every 2 days. Cell viability was assessed by cell counting in a phase-contrast inverted microscope. Counts of viable cells were performed every 24 or 48 h throughout the experiment, starting on the fourth day of culture and up to 15 days after seeding. Values shown are the mean ± SEM of cell counts per cm2 and represent the average of triplicate culture dishes.

Assessment of cell membrane fluidity in response to CyA and cilastatin

Fluidity of PTEC membranes was evaluated by fluorescence anisotropy of membrane-bound dyes. Cell membrane fractions were prepared by homogenizing kidney cortical sections in ice-cold medium containing 300 mM mannitol, 1 mM EGTA, 1 mM KH2PO4, 1.74 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.2% bovine serum albumin (BSA) and 10 mM Tris–HCl buffer, pH 7.4. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 1075 g. The resulting supernatant was centrifuged at 48 000 g for 30 min in a Sorvall SS-34 rotor. The crude nuclei and cell debris were sedimented and supernatant was centrifuged at 8635 g for 10 min to sediment mitochondria. The post-mitochondrial supernatant was centrifuged at 12 000 g for 10 min. The resulting supernatant was centrifuged at 48 000 g for 30 min in an SW 70TI rotor in a Beckman L8-70 ultracentrifuge. The resulting pellet was the cell membrane fraction and was suspended in homogenization medium. The entire operation was carried out at 4°C.

Membrane fluidity was measured by dynamic fluorescence anisotropy of membranes stained with the fluidity-sensitive fluorophore 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Molecular Probes, USA). The measurements were carried out at 37°C with a spectrofluorometer (SLM AMINCO 8000).

Changes in fluorescence anisotropy were monitored continuously. At the end of the experiments, fluorescence anisotropy
was normalized by adding Triton X-100 detergent to the cell membrane preparations to a final concentration of 0.2% (v/v). Variations in fluorescence anisotropy measurements are inversely proportional to changes in cell membrane fluidity. All the fluorescence measurements were corrected for the contribution of light scattering by performing control experiments on cells without fluorescent probes added, and were repeated six times.

**Measurement of CyA transport and accumulation in PTEC primary cultures**

CyA concentrations in PTEC cultures were determined by a validated radiolabelling assay (Immunotech®, Beckman Coulter, UK). The technique was performed in accordance with the instructions provided by the manufacturer. Calibrators and controls supplied with each kit were used. Briefly, duplicated 100 μl samples obtained from PTEC cultures exposed to 1 μg/ml CyA and 200 μg/ml cilastatin, 10 μM filipin (Sigma, USA) or 1 mM 2-hydroxy-β-cyclodextrin (Research Plus, USA) and standards were incubated for 30 min with 125I-labelled CyA in polystyrene tubes coated with anti-CyA monoclonal antibody. Thereafter, the supernatants of the tubes were discarded and the bound radioactivity of calibrators, controls and samples was measured using a gamma scintillation counter. Cilastatin was expected to inhibit dipeptidase activity; filipin and cyclodextrin were expected to inactivate cholesterol rafts. Experimental samples were handled in a blinded fashion. Each experiment was carried out in duplicate.

In order to determine CyA concentrations in specific cell fractions of PTEC cultures, a monoclonal anti-CyA antibody-based fluorescence polarization immunoassay system was used on a TDX analyser (Abbot Laboratories, USA). Again, the technique was performed in accordance with the instructions provided by the manufacturer. Calibrators and controls supplied with each kit were used. In brief, PTECs were incubated for 24 h at 4°C in the presence of 500–1000 ng/ml CyA and 200 μg/ml cilastatin. Subcellular fractions were obtained by differential centrifugation using standard procedures. PTEC cultures were lysed and centrifuged to obtain the nuclei, mitochondria and cytosol subcellular fractions, sedimenting, respectively, at 1075 (10 min), 8635 (20 min) and 110 000 g (35 min). For each experimental condition, duplicate 150 μl samples were prepared. Calibration curves showed linearity for concentrations of CyA up to 1000 ng/ml. Preliminary experiments were performed to demonstrate that measurements of CyA concentrations using this technique were unaffected by the use of Ham’s F-12 medium in primary cultures of PTECs. Similarly, the type of subcellular fraction tested did not influence the results. Each measurement was carried out in duplicate.

**Changes in cell membrane morphology and membrane-bound cholesterol-rich raft distribution**

PTECs cultured on glass coverslips were washed two or three times with phosphate-buffered saline (PBS), incubated with 4% paraformaldehyde for 10 min at room temperature, and for 15 min with 1% BSA in PBS at room temperature. Cells were then stained with 10 μg/ml fluorescein isothiocyanate (FITC)-labelled cholera toxin B (CTB) subunit at 37°C and mounted with DAKO® Fluorescent Mounting Medium. The images of the distribution of FITC–CTB across membranes were obtained with an Olympus IX70 inverted microscope.

Morphological changes were also assessed by scanning and transmission electron microscopy. Briefly, PTECs were seeded in culture dishes fitted with glass coverslips. Before reaching confluence, PTEC cultures were rinsed twice with cold PBS and were fixed by immersion of the coverslips in 1% glutaraldehyde in PBS for 1 h. After several washes with PBS, the coverslips were next post-fixed in buffered 1% OsO4 for 24 h at 4°C and dehydrated in graded acetone. Finally, culture specimens were sputter coated with gold before being examined on a JEOL JSM-T300 scanning electron microscope. For transmission electron microscope assays, cultured cells were harvested by trypsinization and washed at 2000 r.p.m. for 5 min. Cell pellets were fixed by immersion for 1 h in 1% glutaraldehyde. After three washes, the samples were post-fixed for 24 h at 4°C in OsO4 (1%), dehydrated through ascending grades of alcohol, and embedded in resin (EPOXI-SPURR). Semithin (1 mm) sections were stained with the Richardson method. Ultrathin (800 Å) sections were examined, with uranyl acetate and lead citrate staining, using a JEOL 100 SX electron microscope.

**Detection of apoptosis**

**Fluorescence microscopy.** In order to quantify the presence of apoptotic cells in PTEC cultures, direct staining of cultures with acridine orange (Sigma, USA) was carried out. At appropriate times, culture medium was removed from culture dishes and, without any washing, cells were stained directly with 13 μM acridine orange for 15 min at 37°C. Next, the cells were washed twice with PBS and visualized immediately under a fluorescence microscope (Olympus IX70) with a peak excitation wavelength of 490 nm.

**Flow cytometry.** Following exposure of PTEC cultures to CyA or FK506 in the presence or absence of cilastatin, cells were harvested and resuspended at a density of 2 × 10⁶ cells/ml in 2% paraformaldehyde in PBS for 30 min at 4°C. The cells were next washed with PBS and permeabilized with PBS–Triton 0.5% for 15 min at room temperature. A volume of 100 ml containing 1 × 10⁶ cells was incubated with 250 μg/ml RNase and 40 μg/ml propidium iodide (Sigma, USA) for 45 min at room temperature. Next, assay samples containing no less than 10 000 cells each were analysed on a FACScan equipped with a single argon ion laser (Becton Dickinson, UK) using standard Lysis II software. Appropriate gating on forward-angle light scatter vs side-angle light scatter dot plots before fluorescence was performed. The percentage of hypodiploid cells was used to quantitate apoptosis.

**DNA fragmentation.** To evaluate DNA fragmentation in the context of apoptosis, PTEC cultures were lysed and centrifuged at 200 g for 10 min to remove nuclei and mitochondria. The DNA and histones present in the soluble fraction were analysed using an enzyme-linked immunosorbent assay (ELISA) cell death kit (Boehringer Mannheim, Germany). This technique is based on the detection of histone-associated DNA fragments by a sandwich-ELISA using horseradish peroxidase-coupled anti-histone and anti-DNA antibodies. With this approach, apoptosis may be
quantitated by measuring horseradish peroxidase activity at 405 nm and calculating the enrichment factor, i.e. the ratio of enzyme activity in treated vs control cells.

Statistics

Data are reported as mean ± SEM. PTEC cultures obtained from one miniature swine were considered an n of 1. For comparisons between control conditions and treatment conditions, a one-way factorial model of ANOVA has been used. If more than one factor was considered, a general factorial statistical model was applied, in which case the significance of the general model, of every factor and of the possible interactions are presented in the figure legends. When some of the factors included in the one-way or in the general model had more than two levels (concentration, time of culture, etc.), contrast between levels was explored by means of post hoc tests. In general, the test of least significant differences (LSD) has been used to analyse these contrasts.

When data distribution showed a homogeneous trend, a study of linear regression has been applied. In that case, the significance of the ANOVA model, slope and standard deviation of the regression line, significance of the general model, of every factor and of the possible interactions were analyzed. In case of significant results, post hoc multiple comparison tests were performed using the least significant difference (LSD) method.

Results

Effects of CyA and cilastatin on kidney PTEC cultures

In the absence of CyA, addition of cilastatin, ranging from 50 to 500 μg/ml, did not influence cell survival of PTEC cultures (Figure 1A). When renal PTEC cultures were grown in the presence of concentrations of CyA ranging from 1 to 10 μg/ml for up to 15 days post-seeding, cell growth was reduced in a dose-dependent way. A CyA concentration of 1 μg/ml was selected as the peak value of plasma CyA obtained in human clinics after a single dose of CyA (see Discussion for details). A CyA concentration of 10 mg/ml was selected to ensure CyA toxicity, as the aim of the study was to prove potential protection of cilastatin against CyA-induced toxicity. The observed dose-dependent inhibitory effect of CyA on cell growth is illustrated in Figure 1B. This anti-proliferative effect became statistically evident after 6 days of exposure to CyA. PTEC cultures exposed to CyA did not attain confluence. Interestingly, co-incubation of kidney PTECs with CyA (1 and 10 μg/ml) and 200 μg/ml cilastatin totally or partially prevented the anti-proliferative effect associated with exposure to CyA. Differences became statistically significant after 11 days of co-treatment.

When CyA was added to primary cultures of PTEC after reaching confluence, i.e. in the absence of active cell replication, cell survival was significantly reduced in a dose-dependent way and cilastatin was also found to prevent CyA-induced cell death of quiescent primary cultures of kidney PTECs (data not shown).

When PTEC cultures were treated with CyA (1 μg/ml), the observed reduction in cell viability induced by CyA was associated with varying degrees of ultrastructural abnormalities (Figure 2A–C) consistent with apoptosis induction, condensed cytoplasm, and development of translucent cytoplasmic vacuoles and apoptotic bodies or ‘blebbs’ (Figure 2A). The mitochondrial matrix often became darker. There were also signs of altered cell shape and adherence (Figure 2B), with loss of intercellular tight junctions resulting in lack of cell confluence, changes in cell polarity and induction of cell death.

Fig. 1. CyA-induced cytotoxicity in pig kidney PTEC cultures. Cell number was determined by cell counting in a phase-contrast decenter microscope. Counts of viable cells were performed every 48 h throughout the experiment, starting on the fourth day of culture and up to 15 days after seeding. (A) Effect of cilastatin 0, 50, 200 and 500 μg/ml on cell viability. Values shown are the mean ± SEM of cell counts per cm². (B) Effect of CyA 1(●) and 10 (▲) μg/ml in the absence or presence of cilastatin (□ CyA 1 μg/ml + cilastatin 200 μg/ml; Δ CyA 10 μg/ml + cilastatin 200 μg/ml) on cell viability. Values shown are the mean ± SEM of cell counts per cm². The dark solid line (●) represents PTEC growth under control conditions. Factorial ANOVA: main combined effects, P < 0.0001; factor ‘treatment’, P < 0.0001; factor ‘days of culture’, P < 0.0001; interaction term ‘treatment × days of culture’, P = 0.004. Post hoc contrast analysis (LSD) showed a significant decrease of cell culture growth induced by CyA compared with control from day 8 (both 1 and 10 μg/ml), and significant cell growth recovery by cilastatin from day 11. *CyA vs ‘control’, P < 0.05; ”cilastatin + CyA’ vs ‘control’, P < 0.05; #cilastatin + CyA’ vs ‘CyA’, P < 0.05.
of dilated, isometrically vacuolated endoplasmic reticulum (Figure 2C). Similar changes were detected in PTEC cultures exposed to FK506 (data not shown).

When PTEC cultures were co-treated with CyA (1 µg/ml) and cilastatin (200 µg/ml), cells preserved an almost normal appearance, unlike cells treated with CyA only (Figure 2C, D and F). Some degree of vacuolization of intracellular structures could be observed, yet to a much lesser extent than in PTEC cultures treated with CyA only. Also, some apoptosis-like ‘blebbing’ could also be seen. However, the epithelial appearance of cultures and cell polarization were better preserved. Cilastatin by itself did not cause any visible alteration of PTEC morphology.

Fig. 2. CyA-induced changes of the morphology of primary cultures of pig kidney PTECs. PTECs were treated for 24 h with CyA 1 µg/ml (A, B and E) or CyA 1 µg/ml + cilastatin 200 µg/ml (C, D and F), and cell morphology was assessed by scanning and transmission electron microscopy. CyA-treated cells presented epithelial disruption, cytoplasmic condensation, vacuolization and ‘blebbing’. The same doses of CyA in the presence of cilastatin still presented some ‘blebbing’ formation, but restoration of epithelial appearance was evident.

Changes in cell membrane fluidity, transport of CyA across membranes and MBCR raft distribution

The DPH fluorescence anisotropy measurements showed that addition of CyA to isolated cell membranes of the renal cortex resulted in a significant reduction of the baseline cell membrane fluidity (Figure 3A). This effect was dose dependent (data not shown). Subsequent addition of cilastatin did not reverse the changes in cell membrane fluidity induced by CyA. CyA-induced membrane fluidity reduction was reversed by addition of detergent Triton X-100. In contrast to this, pre-treating the renal cortex cell membranes with cilastatin prevented reductions in cell membrane fluidity associated with later exposure to CyA (Figure 3B). Cilastatin alone did not modify cell membrane fluidity.

We investigated whether cilastatin would counter the effects of CyA by interfering with its transmembrane transport. Cilastatin was found to inhibit effectively CyA transport into cultured PTECs (Figure 4). The rate of CyA transport into PTECs was significantly reduced when co-incubated with cilastatin 200 µg/ml. This effect became statistically significant shortly after exposure to cilastatin (5 min); a steady-state inhibitory effect was reached within 60 min of addition of 200 µg/ml cilastatin to PTECs cultured in the presence of 1 µg/ml CyA (Figure 4A). The inhibition of CyA transport by cilastatin was dose dependent at doses of cilastatin ranging from 20 to 2000 µg/ml (Figure 4B). Figure 4B shows the statistically significant dependence
of intracellular CyA concentration on the logarithm of the cilastatin concentration.

In another series of experiments, following exposure of primary cultures of PTECs to CyA (1 µg/ml) for 24 h, the amount of CyA recovered in mitochondrial, cytosol, nuclei and membrane-bound subcellular fractions was compared with that of PTEC cultures simultaneously exposed to CyA 1 µg/ml and cilastatin 200 µg/ml (Figure 5). CyA concentration in terms of ng of CyA/mg protein was significantly different between each subcellular fraction, being higher in the cytosol and membrane-bound fractions. Treatment with cilastatin had different effects on every fraction, reducing the CyA concentration in the cytosol and membrane-bound fractions. For the global experiment, cilastatin was found to significantly reduce the intracellular CyA concentration (see Figure 5 legend for details on factorial ANOVA analysis).

To investigate MBCR implication on CyA transport in kidney PTECs, the effect of filipin, a sterol-binding agent that disrupts MBCRs and similar structures, on the uptake of CyA was examined. When CyA uptake was measured, a significant reduction was detected in cells continuously exposed to 10 µM filipin compared with untreated cells (Figure 6). These results were similar to those obtained with cilastatin. Similar
experiments were performed using another sterol-binding agent, 2-hydroxy-β-cyclodextrin (1 mM), which has been shown specifically to remove cholesterol from the plasma membrane. Results were more relevant than those observed with filipin treatment (Figure 6).

Interestingly, cilastatin did not affect the distribution of MBCRs along the brush border of kidney PTECs (data not shown). In control PTEC cultures, staining of cell membranes with FITC–CTB, which specifically binds MBCR-bound ganglioside GM₁, revealed regions of fluorescence condensation all along the cell surface corresponding to GM₁ clusters present in detergent-insoluble glycolipid rafts and in non-coated cholesterol-rich plasma membrane invaginations named caveolae.

When PTEC cultures were treated with doses of cilastatin ranging from 2 to 2000 μg/ml, no significant changes in FITC–CTB staining patterns were observed, suggesting that cilastatin inhibition of CyA was not mediated by modifications of MBCRs along the brush border. Of interest, FITC–CTB (10 μg/ml) did not inhibit CyA transport into PTECs by itself (Figure 6).

As expected, evidence of apoptotic cell death was detected in PTEC cultures exposed to the calcineurin inhibitors CyA (1 μg/ml) and FK506 (50 ng/ml).

A hallmark of apoptosis is the DNA degradation into oligonucleosomal sized fragments subsequent to activation of endonucleases. Detecting cytosolic histone-associated DNA fragments with immunoassays using anti-histone and anti-DNA antibodies may be used to monitor DNA fragmentation and release from nuclei. Nucleosomal enrichment was determined on PTEC cultures treated for 48 h with CyA 1 μg/ml or FK506 50 ng/ml (Figure 7). Similarly, other PTEC cultures were treated for 48 h with CyA 1 μg/ml + cilastatin 200 μg/ml or FK506 50 ng/ml + cilastatin 200 μg/ml. ANOVA factorial analysis showed relatively higher induction of nucleosomal formation with FK506 than CyA. Cilastatin (200 μg/ml) was able to reduce significantly the magnitude of DNA degradation induced by both calcineurin inhibitors (Figure 7). This observation was in agreement with the ultrastructural modifications observed with CyA (Figure 2).

**Effect of cilastatin on apoptotic cell death induced by calcineurin inhibitors**

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Additional evidence came from the analysis of the frequencies of apoptotic cells by fluorescence-activated cell sorting (FACS). Figure 8 revealed that the number of hypodiploid particles released to the supernatant of PTEC cultures after 48 h exposure to CyA (1–1000 ng/ml) or FK506 (5–500 ng/ml) increased in a dose-dependent way when compared with control cultures. Linear regression of ‘supernatant % hypodiploid cells’ vs ‘log [immunosuppressor]’ showed increases of $3.9 \pm 1.8$ and $6.3 \pm 2.2$ points for every increment in 1 log unit of [CyA] or [FK506], respectively. However, simultaneous addition of cilastatin at concentrations ranging from 50 to 1000 µg/ml to PTEC cultures treated with either CyA (1000 ng/ml) or FK506 (50 ng/ml) was associated with a significantly lower frequency of release of apoptotic cells than controls (Figure 9).

**Discussion**

Recent evidence suggests that tubular injury is probably more relevant than ischaemic injury in CyA-induced nephrotoxicity. In this study, we investigated the effects of cilastatin on primary cultures of pig kidney PTECs treated with the calcineurin inhibitors CyA and FK506.

As expected, CyA had a direct anti-proliferative effect on kidney PTECs and also induced cell death on quiescent PTEC cultures. This observation is of interest, since in adult kidneys renal tubule cells are mostly in a quiescent state and divide at a very slow rate. Moreover, when non-confluent cultures of PTECs were exposed to CyA, a dose-dependent inhibition of cell growth was observed. This inhibitory effect could be responsible to some extent for the delayed recovery from acute tubular necrosis attributable to cold preservation injury observed in kidney transplant recipients immunosuppressed with CyA; CyA could be interfering with the proliferation of proximal tubule cells, which has been shown to play a key role in cell repair following toxic or ischaemic injury [6].

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**Fig. 7.** Effect of cilastatin (200 µg/ml) on CyA (1 µg/ml) and FK506 (50 ng/ml) induction of nucleosomal enrichment in cultured pig kidney PTECs. Oligonucleosomal DNA fragmentation was detected by an ELISA kit using horseradish peroxidase-coupled anti-histone and anti-DNA antibodies. For each experimental condition, the nucleosomal enrichment factor was determined as the ratio of horseradish peroxidase enzyme activity in treated cells compared with control cells. Factorial ANOVA: main combined effects, $P = 0.018$, factor ‘treatment’ (levels: CyA and FK506) $P = 0.055$ (NS); and factor ‘cilastatin’ $P = 0.011$; interaction ‘treatment × cilastatin’, $P = 0.485$ (NS), i.e. cilastatin reduces nucleosomal release in PTECs, and this effect is not different between CyA- or FK506-treated PTECs.

**Fig. 8.** Effect of CyA and FK506 on apoptosis of pig kidney PTECs as assessed by flow cytometric analysis (FACScan). PTEC cultures were exposed to increasing concentrations of the calcineurin inhibitors CyA (1–1000 ng/ml) and FK506 (5–500 ng/ml) in a dose-dependent way when compared with control cultures. Linear regression of ‘supernatant % hypodiploid cells’ vs ‘log [immunosuppressor]’ showed increases of $3.9 \pm 1.8$ and $6.3 \pm 2.2$ points for every increment in 1 log unit of [CyA] or [FK506], respectively. However, simultaneous addition of cilastatin at concentrations ranging from 50 to 1000 µg/ml to PTEC cultures treated with either CyA (1000 ng/ml) or FK506 (50 ng/ml) was associated with a significantly lower frequency of release of apoptotic cells than controls (Figure 9).
In agreement with other investigators [4,5], in our study both CyA and FK506 appeared to induce apoptosis in cultured PTECs, as evidenced by morphology of nuclei, detection of blebbing and nucleosomal formation, and quantitation of hypodiploid cells by FACScan analysis.

We decided to use a CyA concentration of 1000 ng/ml in our in vitro experiences. The usual value of 200 ng/ml is the blood concentration of CyA in the ‘trough’ of its kinetic profile after a single dose. For most people between 20 and 57 years old, the peak in plasma volumes is reached between 1 and 3 h after ingestion, and CyA concentration is as high as 1000 ng/ml [19]. Recently, it has been proposed to use C2 levels (blood levels after 2 h of ingestion) instead of pre-dose values to monitor CyA dosage. Normal blood values for C2 are ≥800 ng/ml. The peak value for FK506 after a single dose is more variable because of the variable absorption and biodisponibility (from 3 to 77%), with individual follow-up being recommended. However, from published data, the blood concentration peak is observed between 1 and 2 h after ingestion, and reaches 30–40 ng/ml [20].

Finally, we performed several in vitro experiments, treating isogenic minipigs with oral doses of CyA at 10 mg/kg/day or FK506 at 5 mg/kg/day. After 10–12 days of treatment, blood levels observed were 1369.8 ± 242.3 ng/ml for CyA and 10.05 ± 0.80 ng/ml for FK506.

On the other hand, the dose response presented for CyA and FK506 induction of hypodiploid populations seems to suggest that aggression of FK506 or CyA does not have a clear threshold for apoptosis induction, but may be present at very low doses (see also [5]). Only when this aggression is evident do we call it ‘clinical toxicity’.

CyA at 10 000 ng/ml and FK506 at 500 ng/ml are concentrations extremely different from what is expected in clinics, but were included because we were looking for reductions of this toxicity with cilastatin.

The presence of CyA receptor, cyclophilin D, in the mitochondria and the role that it plays in mitochondrial permeability transition pore regulation have been strongly implicated in apoptosis induced by CyA and FK506 [5]. The direct toxic effect observed is dose dependent, but no therapeutic assay to reduce intracellular renal accumulation of CyA has been reported to date. This is probably because such a reduction was not predictable without simultaneous reductions on intralymphocyte levels of CyA, and increased risk of graft rejection. Nevertheless the experimental studies of Sido et al. and Hammer et al. [8,14] and the clinical studies of Markewitz in heart [9], Gruss et al. in bone marrow [10], Baghaie et al. in heart/lung [15] and Carmellini et al. in kidney transplant recipients [7] suggest a possible differential effect of imipenem/cilastatin on CyA action in renal cells and lymphocytes. The systematic analysis of these works (meta-analysis) [16] clearly shows a significant enhancement of renal function in patients subjected to CyA/imipenem/cilastatin combined treatment and a decrease in the risk of ultimate development of acute renal failure.

Cilastatin, designed as an inhibitor of brush border dipeptidase, has been demonstrated to decrease the renal degradation, transport and toxicity of imipenem in the proximal cell [14]. In the same way, the nephrotoxicity of vancomycin and cisplatin is also reduced when they are co-administered with imipenem/cilastatin [12]. Therefore, the protective effect of imipenem/cilastatin seems to be related to cilastatin and to be non-specific and probably dependent on its activity on dipeptidase.
MBCRs are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. They appear to be small in size, yet they may constitute a relatively large fraction of the plasma membrane. In general, MBCRs have been shown to be responsible for signal transduction to and from the cell, for protein sorting and for membrane endocytic trafficking of a variety of molecules such as lipids, toxins, glycosyl-phosphatidylinositol (GPI)-anchored proteins and other transmembrane proteins [17]. GPI-anchored protein membrane dipeptidases have been located in lipid rafts isolated from porcine kidney microvillar membranes [18]. By binding GPI-anchored renal dipeptidases present in MBCRs, cilastatin could regulate the uptake of substances, such as CyA, through the brush border of the proximal tubule. Since CyA is not a substrate for the catalytic activity of renal dipeptidases, it could be hypothesized that cilastatin’s interference with CyA transport across membranes is probably related to structural modifications of MBCRs due to steric interactions or conformational changes induced by cilastatin’s binding to GPI-anchored renal dipeptidases.

Our studies strongly suggest that cilastatin could reduce CyA (and FK506) toxicity by inhibiting their transcellular transport and, consequently, their accumulation in the cell. The selective reduction of CyA concentration in the membrane fraction and the changes in membrane fluidity attributable to CyA suggest that the interaction takes place during the transmembrane diffusion process of the lipophilic CyA compound. Apparently, CyA uses these MBCRs for its diffusive transport through the cell membrane. Other MBCR ligands such as cholera toxin do not modify CyA transport in the cell. MBCR disruption with filipin or cyclodextrin interferes with CyA transport in PTECs, but may cause cell detachment and alterations in the distribution of MBCRs. However, when cilastatin binds MBCR-anchored dipeptidase, it interferes with CyA transport but it does not cause any change in MBCR distribution along the membrane, or in cell survival. MBCRs would be considered as preferred diffusive transport pathways (‘cholesterol holes’) by different xenobiotics such as imipenem, CyA, FK506, vancomycin or cisplatin. The absence of a brush border in CD4 cells would explain the lack of interference of cilastatin with the immunosupressor capacity of CyA or FK506.

In conclusion, the results of this study are in agreement with previous clinical and experimental evidence [7–14], and provide additional insight into the mechanisms underlying the protective role of cilastatin against renal toxicity associated with the use of calcineurin inhibitors, such as CyA. Due to the lack of specificity of the proposed mechanism, which is based on cilastatin binding to renal MBCR-bound dipeptidases resulting in structural changes of MBCRs, the usefulness of cilastatin as a broad renal protector is a possibility that deserves further attention.

Acknowledgements. This research was supported by grants C.M. 8.3/008.1/01 from the Madrid government and FIS-00/0013-03 from the Spanish National Institute of Health. A Merck Sharp & Dohme of Spain grant funded A.T.

Conflict of interest statement. This research was supported by grants from the Madrid local government # C.M. 8.3/008.1/01 and from the Spanish National Institute of Health # FIS-00/0013-. Merck Sharp & Dohme of Spain provided free cilastatin under a compromise of investigative purposes only. A.T. received a Medical School Grant from Merck Sharp & Dohme, USA, during part of her stay at the laboratory.

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Cilastatin reduces toxic accumulation of cyclosporin


*Received for publication: 19.1.04
Accepted in revised form: 9.6.04*