Protein Kinase C Mediates Cisplatin-induced Loss of Adherens Junctions Followed by Apoptosis of Renal Proximal Tubular Epithelial Cells.

Raoef Imamdi, Marjo de Graauw and Bob van de Water

Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, The Netherlands
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Address for correspondence:
Dr. B. van de Water
Division of Toxicology
Leiden/Amsterdam Center for Drug Research
Gorlaeus Laboratoria
P.O. Box 9502
2300 RA LEIDEN
The Netherlands
Phone: 31-71-5276223
Fax: 31-71-5274277
e-mail: b.water@lacdr.leidenuniv.nl

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Abbreviations: Bis I, bisindolylmaleimide I; Gö6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; Gö6983, 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide; PTC, proximal tubular epithelial cells; ECM, extra cellular matrix; PS, phosphatidylserine; DAG, diacylglycerol; PKC, protein kinase C; RPTE, renal proximal tubular epithelial cells; LDH, lactate dehydrogenase.
Abstract

Cisplatin is a commonly used anti-tumor agent in the treatment of various human cancers with nephrotoxicity as a major side effect. Cisplatin causes loss of cell-cell contacts of renal proximal tubular epithelial cells prior to the onset of apoptosis. We studied the involvement of protein kinase C in these events in the renal epithelial cell line LLC-PK1. Cisplatin caused apoptosis in LLC-PK1 cells which was directly related to the activation caspase-3 and DNA fragmentation. Apoptosis was almost completely inhibited by the protein kinase C inhibitors bisindolylmaleimide (Bis) I and Gö6983, but not by Gö6976. Also in primary cultured rat renal proximal tubular cells inhibition of PKC inhibited apoptosis. Cisplatin also caused the early loss of cell-cell adhesions which was associated with altered localization of the adherens junction-associated protein β-catenin in association with PKC-mediated phosphorylation of the actin capping protein adducin. These events preceded and were independent of caspase activation. β-Catenin did not dissociate from E-cadherin. Cisplatin-induced loss of cell-cell contacts was associated with increased formation of F-actin stress fibers which was inhibited by Bis I and Gö6983 as well as dominant negative PKC-epsilon. Also loss of cell-cell adhesions by cisplatin was prevented by Bis I and Gö6983. Activation of protein kinase C with phorbol esters promoted cisplatin-induced loss of cell-cell adhesions as well as apoptosis. In conclusion, the combined data fit a model whereby protein kinase C mediates the cisplatin-induced loss of cellular interactions. Such a loss of these interactions has a role in the onset of apoptosis.
Introduction

The chemotherapeutic drug cis-diammine-dichloro-platinum-II (cisplatin) is commonly used for treatment of several solid tumors. The use of cisplatin is limited by its nephrotoxicity associated with the onset of cell death of proximal tubular epithelial cells (PTC): either necrosis or apoptosis occurs depending on the severity of the injury (Safirstein et al., 1987; Lieberthal and Levine, 1996). Cisplatin causes various biochemical and cellular perturbations in renal cells including DNA crosslinking (Yasumasu et al., 1992); decreased capacity of several transport systems such as Na⁺/K⁺-ATPase activity (Nowak, 2002); reduced activity of antioxidant enzymes such as Mn-superoxide dismutase (SOD), glutathione peroxidase and glutathione transferase (GST) (Sadzuka et al., 1994) in association with lipid peroxidation; mitochondrial dysfunction (Kruidering et al., 1997); cytoskeletal reorganization (van de Water et al., 1994a) and changes in cell adhesion (Kruidering et al., 1998). Cisplatin-induced oxidative stress is important in necrosis of PTC. Cisplatin-induced renal cell apoptosis follows the common degradation pathway which involves activation of caspase-9 and caspase-3 followed by caspase substrate cleavage, nuclear condensation and DNA fragmentation, and is blocked by Bcl-2 (Kruidering et al., 1997; Kaushal et al., 2001). So far little is known about the more upstream cellular perturbations that link cisplatin injury to the onset of apoptosis.

There is increasing evidence that injury to PTC caused by either ischemia-related ATP depletion or nephrotoxic chemicals, results in impaired adhesion to the extracellular matrix (cell-ECM adhesion) as well as to neighboring cells (cell-cell adhesion) (Kroshian et al., 1994; Racusen et al., 1991). Both cell-ECM as well as cell-cell interactions are of vital importance to provide the renal epithelial cells with
survival cues to prevent the onset of apoptosis; these are largely mediated through the activation of phosphoinositide-3 kinase/protein kinase B pathway (King et al., 1997). Cell-ECM adhesions are mediated by integrin receptors that link the ECM to the cellular actin cytoskeleton (Schwartz, 1997; Richardson and Parsons, 1995). Cell-cell contacts are mediated by E-cadherins that form transdimers at the adherens junctions (Steinberg and McNutt, 1999). E-cadherins are indirectly linked to the cortical actin cytoskeleton through β-catenin and α-actinin. Cisplatin-induced apoptosis is also associated with changes in cell adhesion. In primary cultured pig proximal tubular epithelial cells cisplatin causes a drastic decrease of ECM expression and a reorganization of actin cytoskeletal network (Kruidering et al., 1994); yet, cell-ECM signaling seems not a major component that controls cisplatin-induced apoptosis (van de Water et al., 2000). In primary cultures of rat renal proximal tubular epithelial cells (RPTE), cisplatin causes early disruption of cell-cell interactions that are independent of caspase activity and precede the onset of apoptosis. It is not clear which molecular mechanisms are involved in this process and what the role is of the loss of these interactions in cisplatin-induced apoptosis of PTC.

The cortical actin cytoskeleton is central in the regulation of cell-cell adhesions, which is tightly regulated by various signal transduction molecules. Protein kinase C (PKC) is a family of signaling molecules that is involved in the regulation of a variety of processes including proliferation, differentiation, transformation and apoptosis (Kiley et al., 1996; Mellor and Parker, 1998). These biological effects are linked to the differential expression profile and cellular location of PKC isoenzymes. These are divided into three subclasses, i.e. classical, novel and atypical, according to their requirement of Ca\textsuperscript{2+}, phosphatidylinerine (PS) and diacylglycerol (DAG). PKCs are important in the regulation of the actin cytoskeleton. Thus, the acting capping
protein adducin was identified as a major substrate for PKC and its phosphorylation on serine residue 726 resulting in dissociation from the actin filaments and translocation to the cytosol. Previously we found that in RPTE cells adducin is phosphorylated on serine 726 after cisplatin treatment (van de Water et al., 2000). In rabbit proximal tubular cells cisplatin causes mitochondrial injury that is associated with increased phosphorylation of PKCα, indicative of activation (Nowak, 2002). So far a role for PKC in the control of cell-cell interactions in relation to cisplatin-induced apoptosis has not been investigated. In the current study we establish such a role.

Using the renal LLC-PK1 we demonstrate that cisplatin causes increased formation of F-actin stress fibers in conjunction with subtle changes in the organization of adherens junctions preceded by caspase activation and apoptosis. Pharmacological inhibitors of novel PKC, but not inhibitors of classical PKC, protect against cisplatin-induced apoptosis. This is associated with protection against the reorganization of the actin cytoskeleton and loss of adherens junctions. Altogether the data indicate an important role for PKC controlled restructuring of and signaling by cell-cell adhesions in cisplatin-induced apoptosis.
Materials and Methods

Chemicals.

Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, trypsin/EDTA, and Fetal bovine serum (FBS) were from Invitrogen. Collagen (type I, rat tail) was from Upstate Biotechnology (Lake Placid, NY). Bovine serum albumin (BSA), propidium iodide (PI), RNAse A, 7-amino-4-methylcoumarin (AMC), cisplatin and bisindolylmaleimide I (GF 109203X, Bis I) were from Sigma (St. Louis, MO). 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö6976) and 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Gö6983) were from Calbiochem. Phorbol 12,13-dibutyrate (PDBu). Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was from Bachem (Bubendorf, Switzerland).

Cell culture, transfection and treatment.

LLC-PK1 cells of porcine proximal tubular origin (American Type Culture Collection, Rockville, MD), were cultured in DMEM supplemented with 10% (v/v) FBS and 50 U penicillin/L and 50 mg streptomycin/L (penicillin/streptomycin). Cultures were maintained in a humidified incubator gassed with 5% CO₂ and 95% air at 37 °C. Medium was changed every other day.

For experiments the cells were cultured at a density of 100,000 cells/ml in 24-wells dishes containing collagen coated glass coverslips, 6-well (Corning Costar, Acton, MA) or 10-cm dishes (Greiner, Germany). After 5 days experiments were performed when the cells reached 100% of confluence.
For transient transfection LLC-PK1 cells were plated overnight on collagen coated coverslips in complete medium without antibiotics. Cells were transfected with dominant negative (DN)-PKC-alpha and DN-PKC-epsilon (Dr. P.J. Parker) using Lipofectamine 2000 (Invitrogen) as indicated by the supplier. Forty hours after transfection cells were treated with cisplatin as indicated above followed by immunofluorescent staining.

Cells were treated with cisplatin in the absence or presence of the indicated concentrations of Bis I (1 mM stock in DMSO), Gö6976 (1 mM stock in DMSO), Gö6983 (1 mM stock in DMSO) or PDBu (2 mM stock in DMSO) in DMEM supplemented with 10% v/v FBS and 50 U penicillin/L and 50 mg streptomycin/L.

**Isolation and culturing of rat renal proximal tubular epithelial cells.**

RPTE were isolated by collagenase perfusion and separated by density centrifugation using Nycodenz as described (van de Water et al., 1994b). Cells were cultured on rat tail collagen (Collaborative Research, Bedford, MA) coated dishes in DME/F12 containing 1 % v/v FBS, 0.5 mg/ml BSA, 10 µg/ml insulin, 10 ng/ml EGF, 10 ng/ml cholera toxin and antibiotics as described (complete medium A; (van de Water et al., 1999)). Cells were maintained at 37°C in a humified atmosphere of 95 % air and 5 % carbon dioxide and fed every other day. RPTE cells were used after they had reached confluence, 6 to 9 days after plating. Cells were treated in complete culture medium with indicated concentrations of cisplatin in the presence or absence of inhibitors of protein kinase C, and after 24 hr apoptosis was determined by cell cycle analysis as well as determination of caspase-3-like activity. Necrosis and secondary apoptosis were determined after 24 and 48 hr by determination of the % LDH release.
Cell cycle analysis.

Apoptosis was determined by cell cycle analysis. Medium containing floating cells was collected. Adherent cells were washed twice with PBS 1 mM EDTA and trypsinized. Floating cells and adherent cells that were trypsinized were pooled, centrifuged for 10 minutes at 2000 rpm, resuspended in 100 ul PBS followed by fixation in 90% ethanol (-20 ºC). Fixed cells were centrifuged and washed once with PBS followed by resuspending in PBS-EDTA containing 7.5 mM PI and 10 µg/ml RNase A. After 30 minutes at room temperature cells were analysed by flow cytometry (FACS-Calibur, Beckton Dickenson). The amount of cells in subG0/G1 was calculated using the Cellquest software (Beckton Dickenson).

Determination of lactate dehydrogenase (LDH)-release.

Cell death was measured by the release of LDH from cells in the culture medium as described (Chen et al., 1990). The percentage cell death was calculated from the amount of LDH release caused by treatment with toxicants relative to the amount to that released by 0.1 % (w/v) Triton X-100, i.e. 100 % release.

Caspase activity measurement

Cells were scraped in medium and collected by centrifugation together with floating cells. The cell pellet was taken up in lysis buffer (10 mM HEPES, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl2, and 5 mM EGTA) and subjected to 3 cycles of freezing and thawing in liquid nitrogen. The suspension was centrifuged at 13,000 rpm in a microfuge for 30 minutes. The supernatant was collected and used to determine the protein concentration using the Bradford protein assay using IgG as a
standard. Equal amounts of cell protein (10 µg) were used for measuring caspase activity using Ac-DEVD-AMC as a substrate (25 µM). AMC fluorescence was followed in time using a fluorescence plate reader (HTS 7000 Bio assay reader; Perkin Elmer). Caspase activity was calculated as pmol/mg of cell protein/minute using AMC as a standard.

**Para-cellular transport of fluorescein-labeled dextran.**

LLC-PK1 cells were cultured on 12-well trans-well dishes with a pore size of 0.4 µm (Costar). Cells were allowed to form a tight monolayer for seven days. Thereafter cells were treated with cisplatin in complete medium both in the apical (0.5 ml) and basolateral (1.5 ml) compartment. After 8 hr of treatment cisplatin was removed from both compartments and cells were incubated for an additional period with fluorescein-labeled dextran with a molecular weight of 4 kD (FD4; 200 µg/ml; Sigma) dissolved in Hank’s/HEPES buffer in the basolateral compartment. After 4 hr 100 µL samples were taken from both the basolateral and the apical compartment and the FD4 was analyzed using a fluorescent platereader (HTS 7000 Bio assay reader; Perkin Elmer).

**Preparation of cytoskeletal and soluble cellular fractions**

To obtain soluble and cytoskeletal fractions, the Triton X-100 extraction method was used (Dong et al., 1995). Briefly, RPTE cells cultured in 10-cm dishes were treated with cisplatin as above. The medium was removed, and adherent cells were washed twice with PBS and a final wash with microtubule stabilization buffer (MSB; 100 mM PIPES, 2 M glycerol, 1 mM EGTA, 1 mM magnesium acetate, pH 6.9). Adherent cells were scraped in 500 µl of MSB buffer containing protease and phosphatase inhibitors and 0.2% (w/v) Triton X-100 (MSB Plus). Floating cells were
collected from the pooled washes by centrifugation for 5 min at 500 x g. Pelleted cells were mixed with the adherent cells in MSB buffer. Cells were extracted for 4 min at room temperature. The homogenate was centrifuged at 20,000 x g for 20 min at 15 °C. The supernatant (soluble fraction) was removed, and the pellet (cytoskeleton fraction) was resuspended in MSB Plus. Equal amounts of protein were separated by SDS-PAGE followed by Western blotting.

**Gel electrophoresis and immunoblotting**

Medium with floating cells was collected and centrifuged (5 min, 500 x g, 4 °C). Adherent cells were kept on ice, washed twice in ice-cold PBS and once in TSE (10 mM Tris-HCl, 250 mM sucrose, 1 mM EGTA, pH 7.4). Cells were then scraped and resuspended in TSE containing 1 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonylfluoride. Floating cells in pellet were pooled with scraped cells. Cells were then sonicated and the protein concentration was determined using the Bradford protein assay using IgG as a standard. Equal amounts of total cellular protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were blocked for 1 h in blocking buffer, 5% (w/v) BSA in Tris-buffered saline-Tween (TBS-T; 0.5 M NaCl, 20 mM Tris-HCl, 0.05% (v/v) Tween 20, pH 7.4). Primary antibody incubation was performed overnight at 4 °C for active caspase-3 (CM-1 kindly provided by Dr. A. Srinivasan), β-catenin (clone 14, Transduction Laboratories), pan-phospho-Ser660-PKC (Cell Signaling), phospho-Ser729-PKC-ε (Biosource), PKC-α (clone M1, Upstate Biotechnology Inc.), PKC-ε (clone 21, BD Biosciences), adducin and phospho-adducin (Fowler *et al.*, 1998) (kindly provided by Dr. S. Jaken), α-tubulin (Sigma) and protein disulfide isomerase
Secondary antibody incubation was performed using either horseradish peroxidase-coupled secondary antibodies or Cy-5 labeled secondary antibodies. Visualization was performed with the ECL Plus reagent (Amersham Pharmacia Biotech) for the horseradish peroxidase coupled antibodies or directly with the Cy-5 labeled antibodies, followed by imaging with the Typhoon 9400 multilabel imager (Amersham Biosciences).

**Immunofluorescence and fluorescence microscopy.**

Cells were cultured on 12 mm collagen-coated glass coverslips and fixated in 3.7% (w/v) formaldehyde in PBS. Coverslips were blocked in TBP (0.5% (w/v) BSA and 0.1% (v/v) Triton-X100 in PBS; pH 7.4) (1 h, RT) and subsequently incubated with primary antibody in TBP (O/N, 4 °C). Coverslips were washed three times in TBP and incubated with Alexa-488, Cy3 or Cy5-conjugated secondary antibodies or 0.2 units/ml rhodamine phalloidin (Molecular Probes, Eugene, OR) in TBP (1 h, RT). After washing with TBP, coverslips were incubated with 2 µg/ml Hoechst 33258 in PBS (15 min, RT), washed in PBS and mounted in Aqua Polymount (Polysciences, Warrington, PA). Cells were viewed using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope and a 60x Nikon objective. Images were processed with Paint Shop Pro 7.02.

**Statistical analysis.**

Student’s t test was used to determine if there was a significant difference between two means (p<0.05); statistical differences are indicated with an asterisk. When multiple means were compared, significance was determined by one-way
analysis of variance (ANOVA; p<0.05). For ANOVA analysis, letter designations are used to indicate significant differences: common letters means not different; different letters means statistically different.
Results

Cisplatin-induced loss of cell-cell adhesion precedes apoptosis.

To determine a concentration of cisplatin which selectively causes apoptosis of LLC-PK1 cells, we exposed these cells to various concentrations of cisplatin. Apoptosis was determined by evaluating the percentage of cells with hypodiploid DNA content (i.e. subG₀/G₁). Cisplatin caused apoptosis in a time- and concentration-dependent manner (Fig 1A). The minimal concentration of cisplatin required to induce a substantial amount of apoptosis within 24 hours was 25 µM; at 50 µM too much apoptosis was observed that would make pharmacological intervention to block apoptosis more difficult; at 100 µM also morphological signs of necrosis were observed as previously reported by others (Lieberthal et al., 1996) (data not shown). At 25 µM the process of apoptosis started after 8 hours. This apoptosis was associated with a time- and concentration-dependent increase in the enzyme activity of caspase-3 with a maximal activity after 24 hours; this was associated with the formation of the active form of caspase-3 as well as the formation of cleavage products of caspase-3 substrates such as β-catenin as determined by Western blotting (Fig 1C). Importantly, the general caspase inhibitor zVADfmk blocked all of the events typical of apoptosis, indicating the requirement for caspase activity (Fig. 1C). Since we wanted to study the effect of cisplatin on processes that take place before the cells become apoptotic, a concentration of 25 µM was chosen throughout our studies.

Renal proximal tubular epithelial cells are the most important targets for cisplatin toxicity within the kidney. In most cases of nephrotoxicity, kidney damage is caused by an impaired adhesion of proximal tubular epithelial cells. Since cell-cell interactions are essential to provide cells with survival signals and suppress apoptosis,
we hypothesized that perturbations of such interactions could be observed after cisplatin treatment. Therefore we studied the process of cisplatin-induced cell detachment in LLC-PK1 cells. Confluent monolayers of LLC-PK1 cells form so-called domes (Fig 2). The formation of these domes is dependent on well-formed cell-cell interactions and consequently loss of these interactions results in loss of domes. To study the effect of cisplatin on dome formation, LLC-PK1 cells were exposed to 25 µM cisplatin and the domes were counted at different time points. A decrease in dome size and quantity started after 8 hr of treatment (Fig. 2). After 16 hours and 24 hours of exposure to cisplatin domes were no longer seen. In fact many cells were now detached and floating in the medium. These floating cells represent apoptotic cells, which is consistent with the apoptosis results (Fig. 1). Although it was apparent by light microscopy that cells loose cell-cell interactions, we wanted to verify that loss of domes was indeed related to loss of cell-cell adhesion structures, and not to perturbations of trans-cellular water transport. Since tight cell-cell interactions in epithelial cell monolayers prevent para-cellular transport/diffusion, we reasoned that if cisplatin causes the loss of cell-cell interactions para-cellular diffusion would be facilitated. To test this we performed transport studies with fluorescein-labeled 4 kD dextran (4FD) molecules using trans-well culture dishes. While hardly any 4FD was transported from the basolateral site to the apical site of the transwell under control conditions, treatment with cisplatin causes a collapse of the barrier and allowed the para-cellular diffusion of 4FD (Table 1). Since various caspase-3 substrates control cellular adhesions (in)directly, including β-catenin and E-cadherin (Van de Water et al., 1999; Schmeiser and Grand, 1999), the possibility existed that the loss of domes is purely related to cisplatin-induced caspase activity. To investigate this we exposed LLC-PK1 cells to cisplatin in combination with zVAD-fmk. Although zVAD-fmk
prevented cisplatin-induced apoptosis and β-catenin cleavage (Fig. 1), it did not block the loss of domes after cisplatin treatment (Fig. 2).

Since the early loss of domes suggested changes in cell-cell interactions organization, we studied these interactions in more detail. The most important cell-cell contacts are formed at the adherens junctions by intercellular E-cadherin homodimer formation. E-cadherin is linked to the actin cytoskeletal network through β-catenin, α-catenin and actinin. Since changes in both the localization of these molecules as well as the actin cytoskeletal network are indicative for changes in the cell-cell interactions their localization and organization was studied using immunofluorescent staining and confocal laser scan microscopy (Fig. 3). After 8 hours cells exposed to cisplatin showed a subtle but clear reorganization of β-catenin-containing adherens junctions from large plaques to thinner structures (Fig. 3). In addition, the F-actin network shifted from a more intense cortical actin cytoskeletal network with less stress fibers, to a condition with a less intense cortical actin and more stress fibers at the basement membrane plane after cisplatin treatment (Fig. 3). After 24 hours of cisplatin treatment most of the β-catenin had disappeared from the sharp cell-cell boundaries. Since at this time-point apoptosis is already present and since β-catenin is a caspase-3 substrate, we wanted to make sure that this effect was not related to increased caspase-3 activity. Therefore, cells were also immuno-stained for active caspase-3. Importantly, those cells that had lost β-catenin from the membrane did not yet have activated caspase-3. Treatment with the pan-caspase inhibitor zVADfmk, which inhibited cisplatin-induced β-catenin cleavage (Fig. 1), did not affect cisplatin-induced β-catenin translocation (Fig. 3). Together these data indicate that reorganization of β-catenin at the cell membrane is not caused by caspase-3-mediated cleavage of β-catenin.
Critical role of PKC in cisplatin-induced apoptosis of renal epithelial cells

PKC is known to phosphorylate and thereby regulate several cytoskeletal associated proteins. Previously we showed that in RPTE phosphorylation of the actin-capping protein adducin is preceded by its cleavage (Dong et al., 1995; van de Water et al., 2000). Also in primary rabbit proximal tubular epithelial cells cisplatin exposure leads to the phosphorylation of PKC-α, indicative of activation (Nowak, 2002). Therefore PKC might be a good candidate involved in the weakening of the cellular adhesion. Such a weakening of the cell adhesion may facilitate the onset of apoptosis. First we determined the effect of cisplatin on PKC. PKC function is regulated in a complex manner by multiple phosphorylation sites in the protein. Thus the serine/threonine residue homologues to serine 660 in PKC-betaII is a critical autophosphorylation site that is present in several PKC isoforms and modulates the activity/localization/turnover of PKCs (Keranen et al., 1995; Feng and Hannun, 1998). We used a phospho-state-specific antibody that binds the phosphorylated form of this residue present in various PKC isoforms, including α, β, δ and ε. In LLC-PK1 after 8 hr no direct effect of cisplatin (either 25 or 100 uM) was observed on phosphorylation of PKC using either pan-phospho-PKC antibody or phospho-PKC-epsilon antibodies (Fig. 4A). Since PKC activity is also associated with translocation, we also determined the effect of cisplatin on the localization of phosphorylated PKC. Cisplatin (25 uM) caused an increased localization of phosphorylated PKC at cell-cell junction, indicative for modulation of PKC (Fig. 4B). To further study a role for PKC in cisplatin-induced apoptosis we used several pharmacological inhibitors of PKC: Gö6976, a selective inhibitor of the Ca2+ dependent PKC-isozymes α and βI; and BisI and Gö6983, two more general PKC inhibitor (Kiley et al., 1999; Way et al., 2000).
Dose finding experiments indicated that the most optimal concentration of these inhibitors for inhibition of cytotoxicity was 1 µM. At this concentration BisI and Gö6983 both significantly reduced the cisplatin-induced apoptotic cell death after 24 hrs (Fig. 5A and B). In contrast, Gö6976 did not affect the cisplatin-induced apoptosis (Fig. 5C). To verify whether this protection against apoptosis was related to inhibition of caspase-3 activation, we also measured the caspase-3 enzyme activity. Indeed, both BisI and Gö6983, but not Gö6976, inhibited the activation of caspase-3 caused by cisplatin (Fig. 5D, E and F). The combined data suggest that PKC plays an important role in the cisplatin-induced apoptotic pathway. We reasoned that if this is indeed the case, then activation of PKC by the addition of phorbol esters would promote cisplatin-induced apoptosis. Indeed, co-treatment of LLC-PK1 cells with PDBu (200 nM) increased both the time point of the onset as well as the percentage of apoptosis caused by cisplatin, further supporting a role of PKC in the regulation of cisplatin-induced apoptosis (Fig. 6A and B).

In our previous work we demonstrated that in primary cultured RPTE cisplatin causes the phosphorylation of the PKC substrate adducin before the onset of apoptosis (van de Water et al., 2000). We first determined the effect of cisplatin on PKC phosphorylation and localization in RPTE cells. Cisplatin caused a time-dependent increase of phosphorylated PKC in RPTE cells, with a maximum increase after 16 hr (Fig. 4C). After 24 hr phosphorylated PKC was decreased. To verify that the phosphorylation was related to activation, cells were also treated with an activator of PKC, PDBu. PDBu caused an increase in the phosphorylation of PKC after 30 min which already decreased after 1 hr and remained lower up till 8 hr (Fig. 4C). This implies a rapid turnover of phosphorylated PKC upon activation. Next we also determined the localization of phosphorylated PKC in RPTE cells after cisplatin.
treatment. While in untreated cells phosphorylated PKC was located primarily in the cytosol and the nucleus, after cisplatin treatment phosphorylated PKC was also associated with cell-cell junctions in a similar fashion as observed for LLC-PK1 cells (data not shown). To verify whether similar findings were observed with another phospho-PKC antibody, we also used a phospho-state specific antibody that specifically recognizes PKC-ε when phosphorylated on serine 729. Cisplatin also caused a time dependent phosphorylation of PKC-ε in a similar fashion as for the pan-phospho-PKC antibody. To check whether PKC is also involved in the regulation of cisplatin-induced apoptosis in primary cultured RPTE, these cells were treated with cisplatin in combination with the PKC inhibitors Bis I, Gö6983 and Gö6976. Bis I, Gö6983, Gö6976 all inhibited apoptosis of RPTE as determined by cell cycle analysis and caspase-3 activity measurement (Fig. 7). Since apoptosis is generally followed by secondary necrosis, which is associated with leakage of plasma membrane, we also measured the release of lactate dehydrogenase (LDH) from the cells in the medium. At 24 hr no significant difference in the levels of LDH release were observed between controls (data not shown). After 48 hr cisplatin caused the release of LDH, indicative of (secondary) necrosis, which was prevented by both Bis I, Gö6983 and Gö6976 (Fig. 8). In RPTE cells activation of PKC by PDBu also caused an increase in cisplatin-induced cell death (Fig. 8). Finally, we wanted to exclude a possible required co-operation of growth factor present in the medium for the cisplatin effects. Therefore, in an other set of experiments we treated RTPE cells with 25, 50 or 100 µM in Hank’s/HEPES buffer for 8 hr followed by a recovery in complete medium till 24 hrs in the presence of the above inhibitors of PKC. Under these conditions also protection against cisplatin-induced cell death was observed by inhibition of PKC
(data not shown). Altogether, the combined data indicate a critical role for PKC in the control of cisplatin-induced apoptosis of renal proximal tubular epithelial cells.

**PKC is involved in cisplatin-induced loss of cell-cell contacts and F-actin reorganization.**

The above data indicated that cisplatin causes an early loss of domes in association with a reorganization of adherens junctions preceding the onset of apoptosis. The fact that PKC is important in the regulation of cell-cell adhesions prompted us to investigate whether PKC also regulates the cisplatin-induced loss of cell-cell adhesion. Both Bis I and Gö6983 inhibited the rapid loss of domes caused by cisplatin (Fig. 9A and B). Importantly, even after 24 hours there were still domes detected, albeit that the domes were somewhat smaller than in the control situation. Gö6976 did again not protect against the rapid cisplatin-induced loss of domes. In conjunction with the apoptosis data, the combined treatment of cisplatin and PDBu accelerated loss of domes (data not shown).

Since PKC mediates the phosphorylation of cytoskeletal components that on their turn control, amongst others, the cortical actin cytoskeletal network organization/turnover, it is most likely that the inhibition of PKC also protected against a reorganization of the cortical actin network and/or localization of adherens junction components, such as E-cadherin and β-catenin. When LLC-PK1 cells were treated with Bis I and Gö6983 in combination with cisplatin, F-actin stress fiber formation was inhibited and the localization of β-catenin was maintained at the site of the cell membrane (Fig. 10A). However, Gö6976 did not protect against the translocation of β-catenin and the formation of F-actin stress fibers (Fig. 10B). When cells were treated with PDBu and cisplatin, the organization of adherens junctions was
lost already after 8 hr and β-catenin staining was drastically decreased (Fig. 8B). This was independent of caspase activity since zVADfmk did not block this effect of PDBu (data not shown). Since cisplatin-induced perturbations of cellular interactions were associated with increased actin stress fiber formation, we wanted to determine the relationship between PKC activity and F-actin formation after cisplatin treatment. While cisplatin itself caused a pronounced increase in the number of actin filaments at the basement membrane (i.e. see Fig. 3), less stress fibers were detected when PKC was inhibited by either Gö6983 or BisI (Fig. 10A). As an alternative approach to determine the role of PKC in cisplatin-induced cytoskeletal reorganization, we also transfected LLC-PK1 cells with dominant negative acting PKC-α and PKC-ε, followed by analysis of F-actin rearrangement in transfected cells. Both DN-PKC-α and DN-PKC-ε positive cells were clearly distinguishable (Fig. 11). DN-PKC-ε overexpression inhibited F-actin stress fiber formation caused by cisplatin, compared to untransfected cells. This effect was not observed for DN-PKC-α. Neither DN-PKC-α nor DN-PKC-ε affected the actin cytoskeleton in untreated cells (Fig. 11). Altogether the data indicate that PKC is involved in cisplatin-induced loss of the cell-cell contacts in direct relation to increased formation of actin stress fibers.

**Cisplatin increases phosphorylation of adducin without affecting cytoskeletal association of β-catenin.**

The above data indicate that cisplatin affects the localization of β-catenin as determined by immunofluorescence. In RPTE cisplatin causes a phosphorylation of the acting capping protein adducin. Therefore, we next investigated whether also in LLC-PK1 cells α-adducin is phosphorylated after cisplatin treatment and whether this is associated with a translocation of β-catenin from the cytoskeleton to the cytosol.
For this purpose LLC-PK1 cells were exposed to cisplatin for different time points and cytoskeletal and soluble cellular fractions were prepared using Triton-X100 extraction. The endoplasmatic reticulin associated protein protein disulfide isomerase (PDI) was used as a control for the extraction procedure (Fig. 12A). Cisplatin caused an increase in phosphorylation of the serine residue 726 of both the α- and γ-adducin isoform. This increased phosphorylated adducin was primarily observed in the cytoskeleton fraction; after 8 hours no increased phosphorylated adducin was present in the cytosol (Fig. 12A). Despite the changes in adducin phosphorylation and F-actin organization after cisplatin treatment, no obvious change in the association of β-catenin with the cytoskeleton or its location in the cytosol was observed. Next we evaluated the effect of both Bis I and Gö6983 on the phosphorylation status of adducin and β-catenin localization in LLC-PK1 cells. Both Bis I and Gö6983 reduced the phosphorylation of adducin, indicating that PKC is involved in this process. These two PKC inhibitors did not affect the association of β-catenin with the cytoskeleton.

Finally we also checked whether the cisplatin-induced reorganization of β-catenin was associated with changes in the binding of β-catenin and E-cadherin using immuno precipitation. Cisplatin did not lead to any major change in the interaction between β-catenin and E-cadherin after either 8 or 12 hours (Fig. 12B). Together this suggests a role for PKC in the reorganization of the actin cytoskeleton through phosphorylation of substrates that regulate the actin cytoskeleton.
Discussion

Cisplatin chemotherapy is still associated with nephrotoxicity, with proximal tubular epithelial cells as the primary target. In our previous studies we determined that F-actin reorganization is an important event in cisplatin-induced cell death of PTC (Kruidering et al., 1998) and that this is associated with an early phosphorylation of the PKC substrate α-adducin (van de Water et al., 2000). In the present study we found that i. PKC activity is essential for the induction of apoptosis of PTC by cisplatin; ii. cisplatin causes an early formation of F-actin stress fibers and loss of cell-cell interactions; and iii. PKC is directly involved in the cisplatin-induced cytoskeletal reorganization and loss of adherens junctions. The early reorganization of the cytoskeletal network and relocation of β-catenin from cell-cell contacts occurred prior to the activation of caspases; moreover, inhibition of caspases did not affect these changes caused by cisplatin. Therefore, we propose that an early PKC-mediated reorganization of the actin cytoskeletal network is involved in the reorganization of adherens junctions and decreased cell-cell adhesion. This may provide a condition that resembles an anoikis status and, hence, facilitates the activation of the apoptotic machinery.

Previous studies have identified various cell adhesion molecules that by themselves are a target for proteolytic cleavage by the group of executioner caspases. These include both cell-matrix adhesion (cytoskeletal)-components such as focal adhesion kinase and proteins that are involved in the regulation of cell-cell contacts such as β-catenin (Wijnhoven et al., 2000) and α-adducin (van de Water et al., 2000). Our data in LLC-PK1 cells indicate that the rearrangement of the actin cytoskeletal network is independent of caspase activity. Thus, caspase-3 activation caused by cisplatin could be blocked by zVAD-fmk. Despite this blockade and protection
against β-catenin cleavage, zVADfmk did not prevent the loss of β-catenin from the sites of cell-cell contacts, indicating that this process is independent of caspase-3 activation. Furthermore cisplatin increased the formation of actin stress fibers in the cells in a caspase-3 independent fashion. Together these data indicate that other mechanisms that are activated after exposure to cisplatin induced the reorganization of the cytoskeleton and adherens junctions that by itself may directly be involved in the onset of apoptosis.

Our findings indicate that PKC activity is critical for the induction of apoptosis by cisplatin both in LLC-PK1 cells and primary cultured RPTE. Thus, BisI, a more general inhibitor of PKC isozymes, significant down-regulated cisplatin-induced apoptosis in LLC-PK1 cells as well as RPTE. In addition, increased activation of PKC with PDBu potentiated the cisplatin-induced caspase activation and cell death. Since BisI is a general PKC inhibitor we used other more PKC isozyme specific inhibitors, Gö6983 and Gö6976, to check whether inhibition of apoptosis is mediated through certain isozymes of PKC. Gö6983, which is selective for novel PKC isozymes, showed significant inhibition of apoptosis comparable to BisI in LLC-PK1 cells. In contrast, such an effect was not observed in these cells with Gö6976, which is more specific for PKC-α (Kiley et al., 1999; Way et al., 2000; Nowak, 2002); this suggests that PKC-α has no direct role in the regulation of apoptosis caused by cisplatin in LLC-PK1 cells. In contrast, in RPTE Gö6976 protected against cisplatin-induced apoptosis, which was comparable to the effect of Gö6976 reported by Nowak. This discrepancy between LLC-PK1 and primary cultured rat and rabbit PTC may be related to different species. Since LLC-PK1 are derived from pig kidneys which more closely resemble human physiology than rat and rabbit kidney, the question remains whether cisplatin-induced nephrotoxicity in human PTC is inhibited
by either Gö6976 or Gö6983. Alternatively, the difference may be explained by the fact that RPTE cultures already have a dense F-actin stress fiber network and relatively little cortical actin under control conditions (van de Water et al., 1999), while LLC-PK1 cells have a strong cortical F-actin with little stress fibers under control conditions. Regardless of the differences, the combined studies clearly indicate an essential role of PKC in cisplatin-induced cytotoxicity. Such a role for PKC may also bear relevance for the in vivo situation: cisplatin treatment of rats causes elevated activity of PKC in proximal tubulus cells, while nephrotoxicity was inhibited by H-7, a very general inhibitor of PKC as well as other serine/threonine protein kinases (Ikeda et al., 1999). It will be important to determine next whether other, more specific inhibitors of PKC, also inhibit cisplatin-induced renal failure.

Inhibition of cisplatin-induced apoptosis by Bis I and Gö6983 was associated with almost complete protection against loss of cell-cell interactions in LLC-PK1 cells. These interactions are tightly controlled by the organization of the actin network, suggesting that PKC may directly act on this network after cisplatin treatment. This would be in line with the work of Jaken and co-workers, who identified various cytoskeletal-associated proteins that are substrates of and interact with PKC (so-called STICKS; substrates that interact with C kinases); these include α-adducin, γ-adducin, MARCKS and desmoyokin (Dong et al., 1995). Interestingly, herein and in our previous study we identified that one of these STICKS, α-adducin has an increased phosphorylation on serine residue 726 after treatment of both LLC-PK1 cells as well as primary cultured rat renal proximal tubular cells with cisplatin (Fig. 12). α-Adducin is an actin-capping protein that has a role in regulation of the cortical actin cytoskeleton and is located at cell-cell contacts. Phosphorylation of adducin leads to weakening of its interaction with the actin cytoskeleton and may
thereby affect its capping activity and allow for changes in the actin cytoskeletal turnover. This is consistent with our data that cisplatin causes increased stress fiber formation. Both BisI and Gö6983 reduced cisplatin-induced phosphorylation of α-adducin in the LLC-PK1 cells which was associated with inhibition of stress fiber formation and maintenance of proper cell-cell interactions. Together this suggests that an increased phosphorylation of adducin may be related to a more active actin cytoskeletal turnover process at the cortical cytoskeleton after cisplatin treatment. Adducin as well as potential other proteins are a direct target for PKC-mediated regulation of the actin cytoskeletal network.

Our studies show that cisplatin exposure leads to an increase in stress fiber formation at the basement membrane of the cells, which was inhibited, but not completely prevented, by Bis I and Gö6983, but not Gö6976. Activation of PKC with PDBu resulted in increased F-actin stress fiber formation already after 4 hours when treated with cisplatin. Together these data strongly suggest the involvement of PKC in the formation of the stress fibers. The PKC-ε isoyme can bind actin and co-localizes in some cells with F-actin filaments. In RPTE we found that cisplatin causes an increased mobility phosphorylation of PKC-ε (Fig. 4). Since both Bis I (general PKC inhibitor) and Gö6983 (inhibitor of PKC-δ, ε), but not Gö6976 (inhibitor of PKC-α and β), inhibited F-actin reorganization, the combined observations suggest a possible role for PKC-ε in the increased formation of the F-actin network after cisplatin treatment. This is supported by our findings that a DN-PKC-ε inhibits the formation of F-actin stress fibers after cisplatin treatment, while DN-PKC-α does not (Fig. 11). In rabbit PTC, inhibition of PKC with Gö6976 was associated with protection against mitochondrial dysfunctioning after cisplatin treatment; no protective effect was described at earlier timepoints (Nowak, 2002). Therefore, it can not be excluded that
the late protection against mitochondrial dysfunctioning is merely correlative with the observed cytoprotection against apoptosis. We would propose that the early PKC-dependent cytoskeletal rearrangement and loss of cell-cell contacts causes disturbances in the cellular homeostasis that by itself may lead to mitochondrial dysfunctioning and/or accelerate the mitochondrial dysfunctioning that will be caused by cisplatin itself (Kruidering et al., 1997).

Our data indicate that pharmacological inhibition of PKC inhibits cisplatin-induced cytotoxicity at clinical relevant concentration of cisplatin. In the event that such inhibitors would be used for renal protection, PKC inhibition should not affect the effect of anticancer reagents on the tumors. Importantly, we recently showed that inhibition of PKC with either Gö6983 or Gö6976 strongly potentiated the cytotoxicity of the anticancer agent doxorubicin in the mammary adenomacarcinoma cell line MTLn3 (Huigsloot et al., 2003). This would indicate that inhibition of PKC has the potential on the one hand to potentiate the anticancer effects of cytostatics and on the other hand prevent the unwanted cytotoxicity on renal cells.

In conclusion, we have identified that cisplatin-induced renal cell apoptosis is related to a PKC-dependent loss of cell-cell interactions. Pharmacological inhibitors of PKC prevent both the loss of cellular interactions as well as the onset of apoptosis. Future studies should further evaluate the possibilities to modulate PKC activity in in vivo conditions thereby not only preventing cisplatin-induced renal failure but also enhancing the cytostatic action of the anticancer drugs.
Acknowledgement

All members of the Division of Toxicology of the LACDR are appreciated for their helpful suggestions throughout this work. DN-PKC-α and DN-PKC-ε constructs were kindly provided by Dr. Peter Parker.
References


Footnotes

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Figure legends

**Figure 1:** Cisplatin causes apoptosis in LLC-PK1 cells after 12 hours of exposure. LLC-PK1 cells were treated with various concentrations of cisplatin for indicated time periods, after which samples were taken for further analysis. Apoptosis was determined by cell cycle analysis (A) as described in the method section. Ac-Asp-Glu-Val-Asp-AMC cleavage activity (DEVDase activity) (B) was determined in cell extracts using Ac-DEVD-AMC as a substrate and expressed as pmol/min/mg cell protein. The presence of active caspase-3 as well as β-catenin cleavage was determined in total cell homogenates treated with 25 µM of cisplatin with or without zVAD-fmk by Western blotting (C). Data shown are mean ± S.E.M. of three independent experiment (n=3). Western blots are representative for three independent experiments.

**Figure 2:** Cisplatin-induced loss of cell-cell contacts is not inhibited by zVADfmk. LLC-PK1 cells were treated with 25 µM cisplatin with or without zVADfmk (100 µM) for the indicated time periods. Phase/contrast images of the cell monolayer were taken using an inverted microscope and a digital camera (A). The size and shape of the domes are circled in white. Loss of cell-cell contacts was determined by counting domes per cm² (B) as described in the methods section. Data shown are mean ± SEM of three independent experiments (n=3). Different letters within time-period treatment groups indicates significantly differences.
**Figure 3:** Effect of zVADfmk on cisplatin-induced weakening of cell-cell contacts and actin stress fiber formation. LLC-PK1 cells grown on collagen coated glass coverslips were exposed to 25 µM of cisplatin in the absence or presence of zVADfmk (100 µM). β-catenin localization and F-actin formation were analysed by immunofluorescence staining followed by CLSM imaging as described in the Material and Methods section. Images shown are representative for three independent experiments.

**Figure 4:** Effect of cisplatin on PKC phosphorylation and localization. (A) LLC-PK1 cells were treated with cisplatin (25 or 100 µM) for 8 hr followed by analysis of PKC phosphorylation using pan-phospho-PKC and phospho-PKC-ε antibodies. α-tubulin was used as a loading control. (B) LLC-PK1 cells were treated with cisplatin (25 µM) for 8 hr followed by fixation; the localization of phosphorylated PKC was determined by immunofluorescent staining using the pan-phospho-PKC antibody followed by CLSM analysis. (C) RPTE cells were treated with cisplatin (25 µM) or PDBu (200 nM) for indicated time periods followed by analysis of PKC phosphorylation the same as in panel A. Data shown are representative for three different experiments.
**Figure 5:** Cisplatin induced apoptosis of LLC-PK1 cells is inhibited by PKC inhibitors BisI and Gö6983, but not Gö6976. LLC-PK1 cells were exposed to 25 µM of cisplatin for 8h, 16h and 24h with or without PKC inhibitors BisI (1 µM), Gö6983 (1 µM) or Gö6976 (1 µM). Apoptosis was determined by cell cycle analysis (A, B and C) as described in figure 1. Caspase-3-like activity (DEVDase activity; D, E and F) was determined in cell extracts using Ac-DEVD-AMC as a substrate and expressed as pmol/min/mg protein. Data shown are mean ± S.E.M. of three independent experiments (n=3). Different letters indicate significantly differences within one time point group.

**Figure 6:** PDBu potentiates cisplatin-induced apoptosis. LLC-PK1 cells were treated with 25 µM of cisplatin for 8h, 16h and 24h with or without PDBu (200 nM) and the effect on apoptosis (A) and caspase-3-like activity was determined as described in figure 1. Data shown are mean ± S.E.M. of three independent experiments (n=3). Different letters indicate significantly differences within one time point group.
**Figure 7: Effect of BisI, Gö6983 and Gö6976 on cisplatin-induced apoptosis of RPTE cells.** Primary cultured RPTE were treated with cisplatin (25 µM) in the presence or absence of BisI (1 µM), Gö6983 (1 µM) or Gö6976 (1 µM) and after 24 hr apoptosis was determined by analysis of caspase-3 activity using DEVD-AMC as a substrate (A) or determination of the percentage of apoptotic cells using flow cytometric analysis of the percentage of subG₀/G₁ cells (B). Caspase-3 activity and percentage of apoptosis are expressed as the increase compared to untreated control. Caspase-3 activity in control was 54 ± 10 pmol AMC/min/mg protein and apoptosis in control was 14.9 ± 2.1 %. Data shown are the mean ± S.E.M. of three independent experiments (n=3). Asterisk indicates significantly different from cisplatin alone.

**Figure 8: Involvement of PKC in cisplatin-induced secondary necrosis of RPTE.** RPTE were exposed to cisplatin with the indicated concentrations in the absence or presence of either BisI (1 µM), Gö6983 (1 µM), Gö6976 (1 µM) or PDBu (200 nM) and the effect on cell death was determined by either determining the percentage of LDH-release. Data shown are mean ± S.E.M. of at least three independent experiments (n=3).

**Figure 9: Effect of BisI, Gö6983 and Gö6976 on cisplatin-induced loss of cellular interactions.** LLC-PK1 cells were treated with cisplatin in the presence of BisI (1 µM), Gö6983 (1 µM) or Gö6976 (1 µM). Dome formation was analysed as described in figure 2. Shown are phase contrast images of domes (A) and the amount of domes per cm² (B). Photographs of inhibitors alone were comparable to untreated control as in figure 2. Data are the mean ± S.E.M. of at least three independent experiments (n=3). Different letters indicate significantly differences within one time point group.
**Figure 10:** Effect of BisI, Gö6983, Gö6976 and PDBu on cisplatin-induced loss of β-catenin translocation and F-actin formation. LLC-PK1 cells were cultured on collagen coated coverslips and treated for the indicated time periods with 25 µM of cisplatin in the presence of BisI (1 µM) and Gö6983 (1 µM) (A) or Gö6976 (1 µM) or PDBu (200 nM) (B). Localization of β-catenin and F-actin formation were analyzed by immunofluorescence and CLSM. Images are representative for results obtained in three independent experiments. For control treatments see figure 3.

**Fig. 11:** Effect of DN-PKC-α and DN-PKC-ε on cisplatin-induced F-actin stress fiber formation. LLC-PK1 cells were transiently transfected with either DN-PKC-α or DN-PKC-ε followed by treatment with cisplatin (25 µM) as described in Material and Methods. After 8 hr cells were fixed followed by immunofluorescent analysis of PKC-α and PKC-ε and F-actin cytoskeletal organization using CLSM with a 60X objective and zoom 2.0. Images shown are representative fields of transfected cells observed in three different transfection experiments.
Figure 12: Cisplatin exposure leads to an increased phosphorylation of adducin and β-catenin translocation. A, LLC-PK1 cells were treated with cisplatin in the presence of BisI (1 µM) or Gö6983 (1 µM). Cytoskeletal and soluble fractions were prepared by Triton-X100 extraction as described under “Experimental Procedures.” Equal amounts of protein were separated by SDS-PAGE followed by Western blotting for phosphoSer726-adducin, α-adducin, γ-adducin and β-catenin. Protein disulfide isomerase (PDI) was used as a marker for the separation of the cytoskeletal and soluble fractions. B, LLC-PK1 cells were treated with cisplatin for 8 and 12 hours. 500 µg of total cell homogenate was immunoprecipitated (IP) with anti-β-catenin antibody, separated by SDS-PAGE and analyzed for E-cadherin by Western blotting. Data are representative for three independent experiments.
**Table 1: Effect of cisplatin on para-cellular diffusion of fluorescein 4kD dextran in LLC-PK1 cells.**

<table>
<thead>
<tr>
<th>Direction</th>
<th>% FD4 diffused³</th>
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<tr>
<td>control</td>
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<tr>
<td>A → B</td>
<td>13.2 ± 3.0⁴</td>
</tr>
<tr>
<td>B → A</td>
<td>1.9 ± 0.2</td>
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<tr>
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<td>A → B</td>
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</tr>
<tr>
<td>B → A</td>
<td>12.5 ± 0.4⁵</td>
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¹ Transport of FD4 across LLC-PK1 cell monolayers was performed as described in Material and Methods. LLC-PK1 cells were treated with cisplatin (25 µM) in both the apical and basolateral compartment.

² FD4 (200 µg/ml) was applied either from the apical (A) site (0.5 mL) or basolateral (B) site (1.5 mL).

³ Diffusion of FD4 is expressed as the percentage of total FD4 added to either the apical or basolateral compartment.

⁴ Data shown are the mean ± SEM of three experiments.

⁵ Asterisk indicates significantly different from treatment control.
Fig. 1

A

% Apoptosis

0 μM Cisplatin
25 μM Cisplatin
50 μM Cisplatin
75 μM Cisplatin
100 μM Cisplatin

Time (hrs)

B

Caspase activity (pmol/min/mg)

Time (hrs)

C

(hr) 4 8 12 16 24
active caspase-3

β-catenin

case

control

cisplatin

cisplatin+zVADfmk

Full length
Fragment
Fig. 2A

(A)

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<th>Cisplatin + zVAD-fmk</th>
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</table>
Fig. 5

Caspase-3 activity (pmol/min/mg) and % Apoptosis over time (hr) for different treatments:

- **Bis I**
  - Control
  - Cisplatin
  - Inhibitor
  - Cisplatin + Inhibitor

- **Gö6983**
  - A
  - B

- **Gö6976**
  - A
  - B

Time (hr): 8, 16, 24.
**Fig. 8**

The graphs depict the percentage of LDH release in response to varying concentrations of cisplatin. Each graph shows the effect of different compounds on LDH release:

- **Panel A**: Control and Bis in the presence of cisplatin at 0, 15, 20, and 25 μM.
- **Panel B**: Control and G6983 in the presence of cisplatin at 0, 15, 20, and 25 μM.
- **Panel C**: Control and G6976 in the presence of cisplatin at 0, 15, 20, and 25 μM.
- **Panel D**: Control and PDBu in the presence of cisplatin at 0, 15, 20, and 25 μM.

Significant differences are indicated by an asterisk (*) for each concentration level.
Fig. 9A
Fig. 10A

<table>
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<th>F-actin</th>
<th>β-catenin</th>
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<tr>
<td>Cisplatin + Bis I</td>
<td></td>
<td>Cisplatin + Gö6983</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 10B
Fig. 11
**Fig. 12B**

- **IP: β-catenin**
  - Cisplatin: - , +, - , +
  - Lysate: - , +, - , +

**IB: E-cadherin**
- 8 hr, 12 hr

**IB: β-catenin**
- 8 hr, 12 hr

kD

- 83