Cisplatin-Induced Apoptosis Is Enhanced by Hypoxia and by Inhibition of Mitochondria in Renal Collecting Duct Cells

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Cisplatin is a widely used chemotherapeutic agent. Here we show that cisplatin induces apoptosis in renal collecting duct-derived cells (MDCK-C7 cells, resembling principal cells) in a dose-dependent manner. Additionally, we studied the role of mitochondria in this process by inhibition of the mitochondrial respiratory chain, the F$_1$F$_0$-ATP synthase or by uncoupling. The role of intra- and extracellular pH in apoptosis induction was investigated. Activation of caspase-3 and DNA ladder formation were used to monitor the apoptotic response. When cells were incubated with inhibitors of the mitochondrial respiratory chain or an inhibitor of the ATP-synthase, cisplatin-induced apoptosis was markedly enhanced. Mitochondrial blockade led to enhanced production of lactic acid. Also, anoxia potentiated the cisplatin-induced caspase-3 activation. Neither intra- nor extracellular pH had an influence on caspase-3 activation at low cisplatin concentrations. Acidic conditions (pH 6.8) potentiated the caspase-3 activation when high (100 μM) cisplatin concentrations were used. We demonstrate that intact mitochondria are important to prevent cisplatin-induced apoptosis in MDCK-C7 cells and that acidic conditions can aggravate the toxic effects of cisplatin.

Key Words: cisplatin; hypoxia; collecting duct; apoptosis; mitochondria.

Cisplatin is a potent and frequently used anticancer drug, which has nephrotoxic side effects (Safirstein et al., 1986). Either the proximal tubule or the collecting duct segments of the nephron are impaired by cisplatin even though by obviously different mechanisms (Brady et al., 1993; Kroning et al., 1999). However, in both segments, proximal tubule and collecting duct, mitochondria seem to play a role in the cytotoxic action of cisplatin. Cisplatin causes apoptosis in proximal tubule cells by caspase-3-dependent and -independent mechanisms (Cummings et al., 2002; Schwerdt et al., 2003). Also, in mouse collecting duct cells, cisplatin induces apoptosis (Lee et al., 2001). Cisplatin-induced cell death is believed to be mediated by its interaction with nuclear DNA (Eastman, 1999; Pinto and Lippard, 1985). Additionally, mitochondria are thought to be a major target of cisplatin and mitochondrial DNA is heavily damaged by cisplatin (Olivero et al., 1995; Murata et al., 1990) leading to mitochondrial loss of energy production, release of a mitochondrial serin protease (Cilenti et al., 2005) with subsequent cell death.

Apoptosis is a well-described sort of cell death induced by a variety of substances. The processes in an apoptotic cell are well characterized and several reports describe that mitochondria play a crucial role (Kroemer et al., 1997; Kroemer and Reed, 2000). Release of cytochrome C and other proteins from mitochondria (Liu et al., 1996; Patterson et al., 2000) often induces a series of events which finally leads to activation of caspase-3, followed by DNA ladder formation and cell death. Besides different substances, inhibition of mitochondrial respiration was shown to lead to apoptosis induction (Wolvetang et al., 1994). Additionally, inhibition of mitochondrial respiration forces the cell to increase the anaerobic glycolysis pathway to guarantee the ATP supply of the cell. In this case, the cell produces increased amounts of lactic acid which acidify either the cell interior and/or the surrounding media.

The collecting duct cell can experience an acidic microenvironment both physiologically and pathophysiologically: physiologically, in the collecting duct the pH underlies considerable variations from alkaline conditions down to pH 4.5 (Hamm and Alpern, 1992; Sabatini and Kurtzman, 1989) and pathophysiologically hypoxic conditions with lowered extracellular pH (down to pH 6.0; Vaupe1 et al., 1989) at the basolateral side of the cells may occur. Furthermore, pH maintenance plays an important role in tumor genesis. It is well known that cancer cells produce acidification of the extracellular compartment. This is due to increased use of anaerobic, lactic acid producing glycolysis, which is the main source of energy production of cancerous cells. At the same time the mitochondria-mediated oxidative phosphorylation is diminished (Gatenby and Gawlinski, 2003; Warburg, 1956). These observations underline the importance of mitochondria and extracellular pH on cell survival. Additionally, in collecting duct cells acidic apical pH leads to increased cellular apical uptake, increased transepithelial reabsorptive transport, and increased apoptosis rates induced by other nephrotoxic substances, e.g., ochratoxin A (Dahlmann et al., 1998; Schwerdt et al., 1997, 2004; Zingerle et al., 1997).

Intact mitochondria are thus a prerequisite for a cell to fulfill its responsibilities in an organism. Therefore, to investigate the role of mitochondria in cisplatin-induced apoptosis, we studied the effects of inhibition of mitochondria or of hypoxia on...
cisplatin-induced apoptosis in epithelial collecting duct cells. Additionally, the effects of extracellular pH and the role of the intracellular pH on cell survival after cisplatin administration of MDCK-C7 cells were studied.

MATERIALS AND METHODS

Cell culture. Madin-Darby canine kidney cells, clone C7 (MDCK-C7 cells), which resemble the principal cells of the collecting duct (Gekle et al., 1994) were seeded in plastic culture dishes (9.62 cm² culture area) in 1 ml of minimum essential medium (MEM) with Earle’s salts, nonessential amino acids, and L-glutamine (Biochrom KG, Berlin, Germany), and cultured under standard culture conditions (37°C, 5% CO₂). MEM was supplemented with 10% fetal calf serum (Biochrom KG) and 24 mM NaHCO₃. Media were changed three times a week and cells were subcultivated once a week. Cells were exposed to cisplatin or other substances for 24 h if not indicated otherwise. Hypoxic conditions were maintained by incubation at 37°C in a water-saturated atmosphere containing 95% nitrogen and 5% CO₂. Measured fractional oxygen content was below 0.01.

Caspase-3 activity assay. Before incubation with cisplatin or other substances, cells were incubated in serum-free medium for 24 h in Petri dishes (9.62 cm² culture area, 104 µl media/cm²). Caspase-3 activity was measured according to the manufacturer’s instructions (Clontech Laboratories GmbH, Heidelberg, Germany) with slight modifications: cells were washed once with PBS buffer (4°C) and incubated with 150 µl cell lysis buffer (Clontech) for 10 min on ice, harvested, and centrifuged at 16,000 × g for 10 min at 4°C. Sixty µl of the supernatant was incubated with 50 µM DEVD-AFC (end-concentration) for 60 min at 37°C, and fluorescence of the cleaved product, 7-amino-4-trifluoromethylcoumarin (AFC), was measured at 400 nm excitation and 505 nm emission wavelength using a multwell-multilabel counter (Victor², Wallac, Turku, Finland). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. As control, cell extracts were incubated as described above but in the presence of caspase-3 inhibitor zDEVD-CHO. No activity could be found under these conditions. Protein content was determined with bicinechonic acid assay (Pierce) using bovine serum albumin as standard.

LDH activity assay. Activity of LDH in media and cell lysates was determined in an automatic analyzer (Cobas-Mira, Roche Diagnostics, Mannheim, Germany), using standard protocol (Bergmeyer and Bernt, 1974).

DNA ladder formation assay. DNA ladder was visualized as described previously (Schwerdt et al., 1999). Briefly, cells in culture medium were collected by short centrifugation. Adherent cells on the Petri dish were harvested in cell lysis buffer (5 mM Tris, 20 mM EDTA pH 8.0, 0.5% Triton X-100), briefly incubated on ice and together with the previous cell pellet centrifuged (20 min, 16,000 × g, 4°C), 50 µg/ml proteinase K and 40 µg/ml RNase A were added to the supernatant and incubated for 60 min at 37°C. DNA was extracted by adding the same volume phenol/chloroform/isoamylalcohol (25:24:1 in TE buffer [10 mM Tris, 1 mM EDTA, pH 8.0]). After shaking and centrifugation (30 min, 3420 g, 4°C), the upper phase was collected. One tenth volume 3 M sodium acetate pH 5.2 and two volumes ice cold (−20°C) ethanol were added and samples were left overnight at −20°C. After centrifugation (30 min, 16,000 × g, 4°C), pellet was washed with 70% ice cold ethanol and dried. DNA was dissolved in water. DNA concentration was measured at 260 nm in a photometer. DNA ladder was visualized in 1.5% agarose gel.

Measurement of lactic acid in the medium. Lactic acid concentration in the media was measured using the lactic acid determination kit from Sigma (Deisenhofen, Germany) following their instructions with slight modifications. In brief, samples were incubated in a 96-well plate for 30 min at 37°C in 200 µl of 200 mM glycine/hydrazine buffer, pH 9.2, containing 1.2 mM NAD⁺ and 8.3 U/ml lactate-dehydrogenase and absorbance was measured in a multilabel counter at 334 nm. Lactic acid concentrations in the samples were calculated from calibration curve of lactic acid standard solutions.

Determination of intracellular pH. Intracellular pH of MDCK-C7 cells was measured using the pH sensitive fluorescent dye 2′-7′ bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) according to Weiner and Hamm (1989). Cells were incubated on coverslips with the media of interest at 37°C and 5% CO₂. After the desired time 2 µM BCECF (final concentration) was added. After 15 min the medium on the coverslips was exchanged against BCECF free medium from cells grown in parallel under the same conditions. Coverslips were transferred to the stage of an inverted Axiovert 100 TV microscope (Zeiss, Oberkochen, Germany) and kept in an aerated and heated chamber under 5% CO₂ and 37°C. Excitation wavelengths were 460 and 488 nm. The emitted light was filtered through a bandpass filter (515–565 nm). The data acquisition rate was one fluorescence intensity ratio every 10 s using an ICCD camera (Hamamatsu Deutschland, Herrsching, Germany). Images were digitized online using video-imaging software (Aquaacosmos image acquisition and analysis system, Hamamatsu). After background subtraction fluorescence intensity ratios were calculated. pH calibration was performed after each determination by the nigericin technique, using two calibration solutions (132 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES) adjusted to pH 6.8 and 7.6.

Statistics. Experiments were done in triplicate with three Petri dishes used each time if not indicated otherwise in the text or figure legends. All data are given as mean values ± SEM. Columns represent the means and error bars the SEM. The significance of difference was determined by the unpaired Student’s t-test, p < 0.05 was considered to be statistically significant. To compare the dependent data an analysis of variance with appropriate post-hoc analysis was made (ANOVA-test, ProPhet 5.0 software). Expected mean results for the effect of two substances were calculated as follows:

\[
\text{mean}_{\text{expected}} = \text{mean}_{\text{substance 1}} + \text{mean}_{\text{substance 2}} - 100\%.
\]

The expected mean SEM was calculated as follows:

\[
\text{SEM}_{\text{expected}} = \sqrt{\left(\frac{\text{SEM}_{\text{substance 1}}}{\text{SEM}_{\text{substance 2}}}\right)^2 + 1}
\]

The significance of difference was calculated using an unpaired t-test and appropriate tables according to the following equation:

\[
t-test-value = \frac{\text{mean}_{\text{control}} - \text{mean}_{\text{expected}}}{\frac{\text{SEM}_{\text{measured}}}{\sqrt{2}} + \frac{\text{SEM}_{\text{expected}}}{\sqrt{2}}}
\]
decreased, which is due to increased necrosis rates (20 μM cisplatin: 1.75-fold increase of LDH released into media; 50 μM cisplatin: 5.39 and 100 μM cisplatin: 6.03-fold increase of LDH released into media, n = 3).

Inhibition of Respiratory Chain of Mitochondria but Not Uncoupling Multiplies the Caspase-3 Activating Effects of Cisplatin

As demonstrated above, 20 μM cisplatin increased caspase-3 activity only slightly compared with higher concentrations. Therefore, to determine activating effects of inhibition of mitochondria this low concentration was used in the further experiments. Inhibition of the mitochondrial respiratory chain by rotenone led to no significant increase in caspase-3 activity as compared to control cells. Also, inhibition of the F1F0-ATP synthase by oligomycin did not increase caspase-3 activity. In contrast, incubation with 5 μM antimycin led to 4.9-fold increase of caspase-3 activity. In the presence of 20 μM cisplatin caspase-3 activity was potentiated significantly as shown in Figure 2A [cisplatin plus rotenone: expected (exp.) 2.3-fold increase of control caspase-3 activity, was 20.7-fold increase; cisplatin plus antimycin: exp. 6.04-fold increase, was 8.6-fold; cisplatin plus oligomycin: exp. 1.74-fold increase, was 15.6-fold increase]. Mitochondrial uncoupling by carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) already led to an increase of caspase-3 activity (6.7-fold). This already high effect was not potentiated by co-exposure to 20 μM cisplatin (exp. 6.7-fold increase, was 5.3-fold). These results show that inhibition of the respiratory chain of mitochondria but not treatment with CCCP significantly potentiates the increase of caspase-3 activity following cisplatin exposure. Also necrosis rates measured by LDH released into media were potentiated by simultaneous exposure of cisplatin and rotenone or oligomycin but not by uncoupling, similar to the apoptotic behavior (Fig. 2B).

DNA Ladder Formation after Inhibition of Mitochondria

One consequence of caspase-3 activation is the activation of caspase-dependent DNases. These DNases cut the DNA at the
linker region between the histones leading to a characteristic fragment pattern visible in an agarose gel. To test whether not only a key enzyme in the apoptotic process was activated but also the execution of apoptosis was done when mitochondria were blocked, we isolated the DNA from cells exposed to cisplatin and inhibitors of mitochondria. As shown in Figure 3, DNA ladder formation is clearly visible after co-exposure of cisplatin and inhibitors. Exposure to 20 μM cisplatin alone led to no clearly visible ladder formation as in control cells. Only treatment with CCCP led to visible DNA ladder formation. This result shows that apoptosis is really aggravated when mitochondria were blocked in the presence of cisplatin.

**Lactic Acid Formation after Exposure to Cisplatin and Mitochondrial Inhibitors**

Co-exposure of cisplatin (20 μM) with rotenone, antimycin, or oligomycin led to a significant potentiation of lactic acid formation whereas cisplatin exposure alone did not alter lactic acid formation (Fig. 4). Only treatment with CCCP did not potentiate the effect of cisplatin because exposure to CCCP alone already led to marked increase of lactic acid formation. This demonstrates that the energy demand of the cell is maintained at least partially by increased use of anaerobic glycolysis when cisplatin and mitochondrial inhibitors are present. This increased formation of lactic acid consequently leads to either acidification of the cytosol and/or of the extracellular environment.

**Severe Hypoxia Potentiates the Caspase-3 Activating Effect of Cisplatin**

As demonstrated, cisplatin-induced apoptosis was potentiated after artificial inhibition of mitochondria. Also under oxygen deprivation mitochondria are inhibited and glycolysis becomes more and more important as is the case after artificial blocking of the mitochondria. To compare these artificially induced blockings of mitochondria with more (patho-)physiological conditions, we incubated the cells under severe hypoxic conditions for 14 h (i.e., 5% CO2 and 95% N2; PO2 < 0.05 mmHg). As demonstrated in Figure 5, the strong hypoxia per se led to a slight but significant increase of caspase-3 activity (1.7-fold, compared with control cells) after 14 h exposure and to an extracellular pH (pHeX) of 7.22 due to...
increased lactic acid production. Cisplatin exposure (20 μM) led to 1.83-fold increase of caspase-3 activity under normoxic conditions after 14 h exposure. However, under hypoxic conditions the cisplatin-induced caspase-3 activity was increased 4.59-fold compared to control cells under normoxic conditions and 2.82-fold compared with cells grown under hypoxic conditions. This shows that not only artificial blockage but also a (patho)-physiological turn off of mitochondria leads to potentiation of cisplatin-induced apoptosis. Also, the lactic acid content in the media was increased by hypoxia, proving the increased recruitment of the glycolysis pathway under hypoxic conditions (not shown).

Effect of Extracellular Acidification or Alkalization on Cisplatin-Induced Caspase-3 Activation

Increased glycolysis led to increased lactic acid formation which acidified the media (which also occurred during severe hypoxia). The apical environment of collecting duct cells can change in a remarkable range from acidic to alkaline conditions. Therefore, we measured, in the presence of cisplatin, the effect of artificial changes of extracellular pH on caspase-3 activity by adding either HCl or NaOH (extracellular pH between 6.8 and 7.68). As shown in Figure 6, moderate artificial extracellular acidification (pH 7.18) did not influence the caspase-3 activating effect of either 20 μM cisplatin or of a much higher dose (100 μM). Conversely, extracellular alkalization led to a significant slight decrease of cisplatin-induced caspase-3 activity in the presence of 100 μM cisplatin. The effect of 20 μM cisplatin was not affected by extracellular alkalization. At more acidic conditions (pH 6.8), caspase-3 activity was increased compared to pH 7.4 when 100 μM cisplatin was used but not when 20 μM cisplatin was used. This shows that extracellular acidification dose-dependently increases the caspase-3 activating effect of cisplatin.

Intracellular pH after Inhibition of Mitochondria or Variation of the Extracellular pH

As demonstrated, moderate changes in pH\textsubscript{ex} have no significant effects on caspase-3 activation at low cisplatin concentrations. However, the resulting intracellular pH (pH\textsubscript{in}) after inhibition of mitochondria or artificial manipulation of the extracellular pH remained unknown. Additionally, pH\textsubscript{in} may correlate with caspase-3 activity as is the case for another nephrotoxin, ochratoxin A (Schwerdt\textit{ et al.}, 2004). Therefore, we determined the intracellular pH after 24 h exposure to the various substances as demonstrated in Figure 7. We measured pH\textsubscript{in} in MDCK-C7 cells maintained in the media in which cells have been cultivated and exposed to cisplatin and/or other substances and not after transfer to a freshly buffered solution with a fixed pH.

pH\textsubscript{in} in control cells was 7.00 ± 0.032 (n = 138) 24 h after incubation in cisplatin-free medium. Cisplatin-exposed cells (20 μM) showed a slightly significant higher pH\textsubscript{in} of 7.10 ± 0.025 (n = 56). Although cells produce more lactic acid when exposed to cisplatin and mitochondrial inhibitors, the pH\textsubscript{in} was not significantly different between cells exposed to cisplatin and mitochondrial inhibitors. Artificial extracellular acidification (pH\textsubscript{ex} = 7.18) reduced pH\textsubscript{in} to 6.87 ± 0.039 (n = 69). In the presence of 20 μM cisplatin, the pH\textsubscript{in} was 6.83 ± 0.026 (n = 67). Alkalization of the extracellular medium (pH\textsubscript{ex} = 7.68) led to an increase of pH\textsubscript{in} to 7.44 ± 0.033 (n = 76) and in the presence of 20 μM cisplatin to a significant decrease to 7.24 ± 0.022 (n = 71) compared with NaOH exposure.
Therefore, no correlation between the intracellular pH and caspase-3 activity could be detected, neither in the presence nor in the absence of cisplatin. This shows that the increased activation of caspase-3 after inhibition of mitochondria is independent of the intracellular pH.

DISCUSSION

Cisplatin is a commonly used drug in chemotherapy. Besides its undisputed beneficial action on cancer cells it has some not ignorable side effects. One organ affected by these side effects is the kidney (Safirstein et al., 1986). Here cisplatin can impair both, either the proximal tubule or the collecting duct (Brady et al., 1993).

Because mitochondria often play a major role in apoptotic processes and mitochondria seem to be one target of cisplatin (Liang and Ulyatt, 1998; Melendez-Zajgla et al., 1999; Murata et al., 1990) we investigated the effects of inhibition of mitochondrial function on caspase-3 activity in the presence of cisplatin. We observed that blocking of the respiratory chain by rotenone or the F$_{1}$F$_{0}$-ATP synthase was not enough to increase caspase-3 activity or to induce DNA ladder formation. Cisplatin significantly increased the effect of inhibition of the respiratory chain or blockade of F$_{1}$F$_{0}$-ATP synthase cisplatin on caspase-3 activity. Therefore, functional mitochondria seem not to be necessary for apoptosis or rather caspase-3 activation as reported by others (Li et al., 2003; Wolvetang et al., 1994). Only after treatment of cells with CCCP caspase-3 activity and DNA ladder formation were not significantly increased by cisplatin due to the already high caspase-3 activity after treatment of the cells with CCCP. This difference in the effect which the inhibitors of mitochondrial function exert are explainable by the observation that (1) CCCP alone already can induce apoptosis (de Graaf et al., 2004) and (2) the action of CCCP is not only restricted to mitochondria but also to other cellular targets such as endosomes or the cell membrane (Zhang et al., 2000). Necrotic cell death induced by cisplatin and inhibition of mitochondria showed the same pattern as apoptotic cell death. These observations demonstrate that a disturbance of the mitochondria caused by a single substance, which itself may lead to only mild perturbations can be potentiated when cisplatin is present. Notably, this occurs already at cisplatin concentrations which itself do not lead to increased cell death. Specific inhibition of mitochondria therefore shifts the threshold concentration of cisplatin-induced cell death down to lower cisplatin concentrations.

Under ischemic conditions or oxygen deprivation the oxygen-dependent mitochondria do not participate in energy supply. As the cisplatin-induced caspase-3 activity is increased after artificial blockade of mitochondria we tested the effect of a turn off of mitochondria by pronounced hypoxic conditions. Similar to the artificial blockade of mitochondria by rotenone or other inhibitors, the cisplatin-induced caspase-3 activity was increased under hypoxic conditions. This demonstrates that respiring mitochondria are necessary to avoid cisplatin-induced apoptotic events in MDCK-C7 cells and that an increased glycolysis rate leads to increased cisplatin-induced apoptosis.

Cellular ATP levels may be diminished after blockade of mitochondria although increased glycolysis may compensate partially the impending ATP fall. Under these conditions, ATP-fueled export of cisplatin by transporters such as p-gp (multidrug resistance protein, MDR1) or MRP2 (multidrug resistance associated protein) may be limited. This would lead to increased intracellular cisplatin levels which could explain the observed toxic effects. However, there is no evidence of p-gp expression in MDCK cells and in MDCK cells transfected with p-gp no transport of cisplatin was observed (Pastan et al., 1988). Additionally, we were not able to measure a MRP2 transport activity in MDCK-C7 cells (not shown). This shows that limited export of cisplatin cannot explain the effects of blocked mitochondria on cisplatin-induced cell death (at least for these two transporters).

Concomitant with an increase in caspase-3 activity, an acidification of the extracellular medium was observed when mitochondria were blocked or after hypoxic conditions. This acidification was due to enhanced production of lactic acid under conditions of blocked mitochondria. Mitochondrial inhibitors when administered alone led to increased lactate formation, but in the presence of cisplatin this lactate formation was significantly increased. This demonstrates an increased use of anaerobic glycolysis to fulfill the cellular energy demand when cells are exposed to cisplatin and mitochondrial inhibitors.

Because the extracellular media were acidified during simultaneous inhibition of mitochondria and cisplatin exposure, we investigated the effect of extracellular pH on caspase-3 activity. A slight acidification or alkalization led to no significant potentiation or antagonisation of the effect which cisplatin at low dose (20 µM) had on caspase-3 activity. Only the effect of high cisplatin concentration (100 µM) was reduced by extracellular alkalization. At more acidic conditions (pH 6.8) the effect of cisplatin (100 µM) on caspase-3 activity was potentiated significantly. Thus, the results presented here show that the extracellular pH plays an important role on cell survival in the presence of cisplatin. It is important to remember that acidic pH occurs daily in the collecting duct under physiologic conditions. But also under pathophysiologic conditions acidic pH occurs. Hypoxic conditions leading to acidic cell surroundings are not unusual in tumor tissues and an aggravation of cell death may be desirable in that case.

We also found that the intracellular pH is not a decisive factor for apoptosis induction in the presence of cisplatin. Even though many studies have demonstrated that apoptosis is associated with a decrease in cytosolic pH (Matsuyama et al., 2000; Segal and Beem, 2001), no correlation between caspase-3 activity and intracellular pH was observed. For example, 24 h exposure to rotenone led to a pH$_{in}$ of 6.94 and caspase-3
activity was close to the activity of control cells (126% compared to control). Under more alkaline conditions, with pH_{1H} of 7.435, the caspase-3 activity was nearly the same as with rotenone (121% compared to control).

In conclusion, we show that inhibition of mitochondria or severe hypoxic conditions can significantly increase the damaging effect of cisplatin in collecting duct-derived cells. This effect is not dependent on the intracellular pH but dependent on extracellular pH and on functional respiring mitochondria. Blocked or because of hypoxia not-used mitochondria increase the apoptosis-inducing effect of cisplatin. This suggests avoiding hypoxia or acidic conditions in the collecting duct when cisplatin is administered.

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REFERENCES


