On the Toxicity and Transport Mechanisms of Cisplatin in Kidney Tissues in Comparison to a Gold-based Cytotoxic Agent

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Mechanisms of toxicity and cellular transport of anticancer metallodrugs, including platinum-based agents, have not yet been fully elucidated. Here, we studied the toxic effects and accumulation mechanisms of cisplatin in healthy rat kidneys *ex vivo*, using the Precision Cut Tissue Slices (PCTS) method. In addition, for the first time, we investigated the nephrotoxic effects of an experimental anticancer cyclometallated complex $[Au(py^{b}\text{-}H)(PTA)Cl]PF_6$ (PTA = 1,3,5-triazaphosphaadamantane). The viability of the kidney slices after metallodrug treatment was evaluated by ATP content determination and histomorphology analysis. A concentration dependent decrease in viability of PCKS was observed after exposure to cisplatin or the Au(III) complex, which correlated with the increase in slice content of Pt and Au, respectively. Metal accumulation in kidney slices was analysed by ICP-MS. The involvement of OCTs and MATE transporters in the accumulation of both metal compounds in kidneys was evaluated co-incubating the tissues with cimitedine, inhibitor of OCT and MATE. Studies of mRNA expression of the markers KIM-1, villin, p53 and Bax showed that cisplatin damages proximal tubules, whereas the Au(III) complex preferentially affects the distal tubules. However, no effect of cimetidine on the toxicity or accumulation of cisplatin and the Au(III) complex was observed. The effect of temperature on metallodrug accumulation in kidneys suggests the involvement of a carrier-mediated uptake process, other than OCT2, for cisplatin; while carrier-mediated excretion was suggested in the cases of the Au(III) complex.

Introduction

Cisplatin (*cis*-diamminedichloridoplatinum(II), Figure 1) is an antineoplastic drug used in the treatment of many solid tumours, including those of the head, neck, lung, and testis. Unfortunately, severe side effects following cisplatin treatment may occur, including ototoxicity and myelosuppression,[1] with the main dose-limiting side effect being nephrotoxicity.[2] The pathophysiological basis of cisplatin nephrotoxicity has been studied for the last four decades, and the emerging picture is that the exposure of tubular cells to cisplatin activates complex signalling pathways, leading to tubular cell injury and death via both apoptosis and necrosis.[3]

Studies in rats and mice suggest that the drug undergoes metabolic activation in the kidney to a more potent toxin, a process possibly involving glutathione and mediated by gluthathione-S-transferase.[4] Activation of cisplatin to its highly reactive and toxic metabolites includes spontaneous intracellular aquation reactions, which involve the substitution of the chlorido ligands with water/hydroxide molecules.[5,6] Previously reported studies using kidney slices,[7] cultured renal epithelial cells[8] and isolated perfused proximal tubule segments[9] have provided evidence for basolateral carrier-mediated uptake of cisplatin. Moreover, it was found that cisplatin concentration within the kidneys exceeds the concentration in blood by at least five-fold, suggesting accumulation of the drug by renal parenchymal cells.[7]

At a molecular level, experimental evidence has led to the conclusion that cisplatin enters cells via two main pathways: (i) passive diffusion and (ii) facilitated uptake by a number of transport proteins,[6,10] including copper transporters (CTR) and organic cation transporters (OCT).[6,11] Pabla et al demonstrated that CTR1 is mainly expressed in both proximal and distal tubular cells in mouse kidneys, whereas cisplatin toxicity has been observed mainly in the proximal tubular cells.[11] In the same study, it was shown that down-regulation of CTR1 in human embryonic kidney cells (HEK293), by small interfering RNA or copper (Cu(I)) pre-treatment, resulted in decreased cisplatin uptake.

Various studies, demonstrating that cisplatin can be transported by OCTs in cells, are based on competition experiments with other established OCTs substrates such as tetraethylammonium (TEA) and inhibitors such as cimetidine.[12–14] OCTs belong to the solute carrier SLC22A family consisting of three sub-categories: the electrogenic transporter (OCT1-3), electroneutral organic cation/carnitine transporter (OCTN1-3) and the organic anion transporter (OATs and urate transporters, URAT-1).[15] Many transporters of the SLC22A family are found in secretory organs such as the liver and the kidneys, as well as the intestine, where they play pivotal roles in drug adsorption and excretion.[16] Moreover, different OCTs show species and tissue-specific distribution. For example, the human OCT1 is highly expressed in the sinusoidal membrane of the liver and in the apical membrane of the jejunum[17] but not in the kidney. Instead, human OCT2 is mainly expressed in the basolateral side of renal proximal tubule cells, and in the dopaminergic brain regions.[18] In order to correctly interpret translational studies, it is important to note that in rodents, both OCT1 and OCT2 show a high renal expression in the basolateral membrane of proximal tubule cells. [19–21] hOCT3 shows a much broader tissue distribution, including skeletal muscle, heart, brain, and placenta, but the distribution in the membrane and physiological role of OCT3 are not yet clearly understood.[16] The interaction of cisplatin with hOCT2 in the kidney, or hOCT1 in the liver, was investigated with the fluorescent cation 4-[4-(dimethyl-amino)styril]-methylpyridinium (ASP) in stably transfected HEK293 cells overexpressing these transporters, and in cells physiologically expressing them, such as human

proximal tubules and human hepatocyte couplets.[22] Notably, cisplatin inhibited ASP transport in hOCT2-HEK293 but not in hOCT1-HEK293. Furthermore, incubation with cisplatin induced apoptosis in hOCT2-HEK293 cells; a process that was completely suppressed by simultaneous incubation with the hOCT2 inhibitor cimetidine. Moreover, in isolated human proximal tubules, cisplatin competed with basolateral organic cation transport, whereas it had no effect in human hepatocytes.[22] Overall, these findings support the idea of the interaction of cisplatin with hOCT2 in renal proximal tubules, but not with hOCT1, possibly explaining its organ-specific toxicity.

In 2010, the functional effects of cisplatin treatment on kidney and hearing were studied *in vivo* in wild-type and OCT1/2 double-knockout mice.[23] No sign of ototoxicity and only mild nephrotoxicity were observed after cisplatin treatment of knockout mice, while cisplatin accumulation in the kidneys was reduced.[23] Co-medication of wild-type mice with cisplatin and the organic cation cimetidine resulted in protection against ototoxicity and partly against nephrotoxicity.[23] Moreover, experiments in rats showed that treatment with both cisplatin and cimetidine did not interfere with the antitumoral activity of the Pt drug.[24] Based on these studies and others, hOCT2 has been proposed as a target for protective therapeutic interventions in cisplatin chemotherapy.

Furthermore, membrane transporters are also involved in carrier-mediated Pt efflux pathways, including the ATP-binding cassette (ABC) multidrug transporters[6,25] and the multidrug and toxin extrusion proteins (MATEs).[26–28] MATEs belong to the SLC47 family and are also part of organic cation homeostasis. Specifically, MATEs act as H^+/organic cation antiporters, transporting protons from the extracellular side to the cytoplasm in concomitance with organic cations export to the lumen of the proximal tubule. Two isoforms are known, SLC47A1 (MATE1) and SLC47A2 (MATE2-K). Both in human and in rat, MATE1 is primarily expressed in the liver and kidney, while MATE2-K exhibits a kidney-specific expression at the brush border membrane of the tubular cells. [29] Several studies have confirmed cisplatin transport by MATEs.[13,14] Overall, these studies suggest that, in humans, the interplay between OCT2 and MATE is responsible for the net renal secretion of cisplatin, and possibly also for the net accumulation of cisplatin in the tubular cells, but further investigation is essential to fully elucidate the complex pathways of cisplatin transport and related side-effects.[30]

Within this framework, the lack of conclusive information is at least partly due to the lack of suitable models to study transport mechanisms in renal tissues. I*n vitro* models, generally 2D cell cultures, have been applied to study the mechanisms of action, metabolism and transport of metallodrugs.[30] These 2D cultured cells usually are characterized by low level of differentiation and mostly consist of one cell type, thereby lacking interactions between the different cell types as in a tissue. Therefore, a model including all cell types in their natural environment is indispensable for studying complex, multicellular organ functions and the pharmacological and toxicological response to drugs, as well as for the identification of the transport mechanisms. The precision cut tissue slices (PCTS) is such a technique, where the original cell-cell and cell-matrix contacts stay unaltered and as such is a useful technique for drug testing *ex vivo*.[31] In a PCTS model, the tissue can remain viable during culture with physiological expression and localization of enzymes and transporters. Thus, the PCTS system is uniquely suited to examine molecular responses to toxicant exposures and compare species differences, and is nowadays a FDA-approved technology. [32] Recently, our group has successfully used the PCTS technique to study the toxic effects of experimental anticancer organometallic compounds,[33–36] aminoferrocene-containing pro-drugs,[37] ruthenium-based kinase inhibitors,[38] as well as supramolecular metallacages as possible drug delivery systems.[39]

Interestingly, nephrotoxic side effects induced by cisplatin were already investigated in human and rat kidney slices and characterized morphologically, as well as in terms of gene expression and functional changes, providing evidence for the mechanisms of apoptosis induction.[40–42] Furthermore, the acute nephrosis of tubular epithelium induced by cisplatin *in vivo* was reproduced in both human and rat kidney slices *ex vivo*, while the glomerulus appeared unaffected even at high drug concentration (80 μM).[40]

However, further studies are necessary to evaluate possible transport mechanisms using the precision-cut kidney slices (PCKS) *ex vivo* model.

Here, we report on the toxicity and mechanisms of accumulation and transport of cisplatin studied in rat kidney using the PCKS technique. Tissues viability was assessed by three different methods, including ATP content, histomorphology, and mRNA determination of different biomarkers. Moreover, intracellular metal accumulation was determined by inductively coupled plasma mass spectrometry (ICP-MS). In addition, the involvement of carrier–mediated transport was investigated performing experiments in the presence of cimetidine, an inhibitor of both rat OCTs and MATE transporters, as well as varying the temperature of tissues incubation. Furthermore, we also studied the toxicity, accumulation and transport mechanisms of another metallodrug, the previously reported experimental cytotoxic cyclometallated (C^N) Au(III) complex [Au(py^b-H)(PTA)Cl]PF_6 (PTA = 1,3,5-triazaphosphaadamantane, Figure 1) featuring a relatively stable Au(III) centre.[43] Interestingly, this Au(III) complex showed promising antiproliferative effects against several cancer cell lines and inhibits the zinc-finger enzyme PARP-1 in nM concentrations.[43] For most of these new generation experimental metal-based compounds with cytotoxicity towards cancer cells, the mechanisms leading to their pharmacological and toxicological profiles are still not fully elucidated and different biological targets and transport systems have been proposed which still need validation.[30]

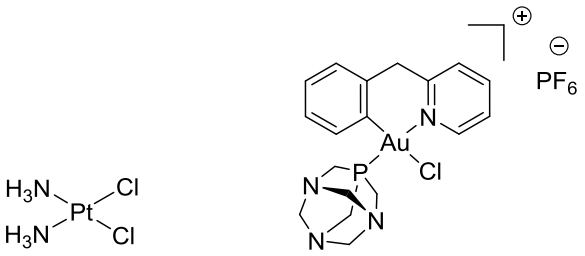

Figure 1. Structure of the anticancer metal complexes evaluated in this study.

Experimental methods

Materials

Cisplatin and cimetidine were purchased from Sigma Aldrich, and the Au(III) complex was synthesized according to the protocol previously reported.[43]

PCKS

Male Wistar rats (Charles River, France) of 250-300 g were housed under a 12 h dark/light cycle at constant humidity and temperature. Animals were permitted *ad libitum* access to tap water and standard lab chow. All experiments were approved by the committee for care and use of laboratory animals of the University of Groningen and were performed according to strict governmental and international guidelines.

Kidneys were harvested (from rats anesthetized with isoflurane) and immediately placed in University of Wisconsin solution (UW, ViaSpan, 4°C) until further use. After removing fat, kidneys were cut in half lengthwise using a scalpel, and cortex cores of 5 mm diameter were made from each half perpendicular to the cut surface using disposable Biopsy Punches (KAI medical, Japan). PCKS were made as described by de Graaf et al.[31,44] The cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer, pH 7.4 saturated with carbogen (95% O_2 and 5% CO_2). Kidney slices weighing about 3 mg (~150 µm thickness), were incubated individually in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria), at 37°C in culture medium, Williams' medium E (WME, Gibco by Life Technologies, UK) with glutamax-1, supplemented with 25 mM D-glucose (Gibco) and ciprofloxacin HCl (10 µg/mL, Sigma-Aldrich, Steinheim, Germany) in an incubator (Panasonic biomedical) in an atmosphere of 80% O_2 and 5% CO_2 with shaking (90 times/min).

In order to remove debris and dead cells before the start of the experiments, PCKS were pre-incubated for 1 h in culture medium and then transferred to new plates containing fresh medium.

Evaluation of ATP content. After PCKS pre-incubation, different concentrations of cisplatin and Au(III) complex were added to the wells and the slices were incubated for 10 min, 30 min, 60 min or 24 h. After the incubation time, slices were collected for ATP and protein determination, by snap freezing in 1 ml of ethanol (70% v/v) containing 2 mM EDTA with pH=10.9. After thawing, the slices were homogenized using a mini bead beater and centrifuged. The supernatant was used for the ATP essay and the pellet was dissolved in 5N NaOH for the protein essay. ATP was measured using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) as described in the experimental section. The ATP content was corrected by the protein amount of each slice and expressed as pmol/µg protein. The protein content of the PCKS was determined by the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) using bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) for the calibration curve.

Evaluation of involvement of rOCT2/rMATE drug transporter. To evaluate the involvement of rOCT2 as uptake transporter and rMATE as efflux transporter, the slices were first incubated for 30 min with a non-toxic concentration of 100 µM cimetidine. Afterwards, cisplatin or the Au(III) complex were added in the selected concentrations. Each condition was evaluated in triplicates after 24 h incubation. Control slices were taken directly after slicing, after pre-incubation and after 24 h incubation.

Histomorphology

Kidney slices were fixated in 4% formalin for 24 h and stored in 70% ethanol at 4°C until processing for morphology studies. After dehydration, the slices were embedded in paraffin and 4 µm sections were made, which were mounted on glass slides and PAS staining was used for histopathological evaluation. Briefly, the glass slides were deparaffinised, washed with distilled water, followed by treatment with a 1% aqueous solution of periodic acid for 20 min and subsequently Schiff reagent for 20 min. Then, the slides were rinsed with tap water; finally, a counterstain with Mayer's haematoxylin (5 min) was used to visualize the nuclei.

Expression determination of kidney-injury molecule-1 (KIM-1), villin, p53 and Bax.

RNA isolation. Three precision cut kidney slices from each treatment group were pooled and snap-frozen in RNase free Eppendorf tubes. RNA was isolated with the Maxwell® 16 simplyRNA Tissue Kit (Promega, Leiden, the Netherlands). Slices were homogenised in homogenisation buffer using a minibead beater. The homogenate was diluted 1:1 with lysis buffer. The mixture was processed according to the manufacturer's protocol using the Maxwell machine. RNA concentration was quantified on a NanoDrop One UV-Vis Spectrophotometer (Thermoscientific, Wilmington, US) right before conversion to cDNA.

cDNA generation. RNA samples were diluted 0.5 µg in 8.5 µl of RNAsa free water. cDNA was generated from RNA using random primers with TaqMan Reverse Transcription Reagents Kits (Applied Biosystems, Foster City, CA). To each sample the following solutions were added: 2.5 µL 5x RT-buffer, 0.25 µL

10mM dNTP's, 0.25 µL Rnasin (10 units), 0.5 µL M-MLV Reverse Transcriptase (100 units), 0.5 µL random primers.

qPCR. Real-time quantitative PCR was used to determine relative mRNA levels of KIM-1, villin, p53 and Bax. PCR was performed using SensiMixTM SYBR Low-ROX kit (Bioline, London, UK) with the QuantStudio 7 Flex Real-Time PCR System (Thermoscientific, Wilmington, US) with 1 cycle of 10 min at 95°C, 40 cycles of 15 sec at 95°C and 25 sec at 60°C, with a final dissociation stage of 15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C. cDNA for each sample was diluted to 10 ng/µl and measured in triplicate. All primers were purchased from Sigma-Aldrich. Fold induction of each gene was calculated using the housekeeping gene GAPDH.

The primer sequences used in qPCR were:
KIM-1: 5'-GTGAGTGGACAAGGCACAC-3' (forward), and
5'-AATCCCTTGATCCATTGTTT-3' (reverse);
villin: 5'- GCTCTTTGAGTGCTCCAACC-3' (forward), and
5'-GGGGTGGGTCTTGAGGTATT' (reverse);
p53: 5'- CCCCTGAAGACTGGATAAC-3' (forward), and
5'-AACTCTGCAACATCCTGGGG-3' (reverse);
Bax: 5'- ACAGGGGCCTTTTTGTTACAG-3' (forward), and
5'-GGGGAGTCCGTGTCCACGTCA-3' (reverse);
GAPHD: 5'-CGCTGGTGCTGAGTATGTCG-3' (forward) and
5'-CTGTGGTCATGAGCCCTTCC-3' (reverse).

ICP-MS analysis

After incubation with different concentrations of cisplatin or the Au(III) compound, PCKS were washed with ice-cold Krebs-Henseleit buffer and snap-frozen and stored at -80°C until the analysis.

Sample preparation. The tissue samples were digested with 100 µL nitric acid overnight, all samples were completely dissolved. 100 µL hydrochloric acid and 800 µL milliQ were added to produce a volume of 1 mL. Prior to analysis the samples were diluted 20 times with 0.65% HNO_3/0.1% HCl.

Pt and Au determination. The Pt and Au contents were quantitated applying a Perkin Elmer (Waltham, MA, USA) Sciex Elan DRC-e ICP-MS instrument, equipped with a Cetac ASX-110FR autosampler, a 0.2 mL min-1 MicroMist U-series pneumatic concentric nebulizer (Glass Expansion, West Melbourne Vic, Australia) and a PC3 cyclonic spray chamber (Elemental Scientific Inc., Omaha, NE, USA). ICP-MS RF power, lens voltage and nebulizer gas and flow were optimized on a daily basis and other settings were: 1 sweep/reading, 25 readings/replicate, 5 replicates, 50 ms dwell time. The $^{197}Au^+$, $^{195}Pt^+$, and $^{194}Pt^+$ isotopes were monitored. Pt and Au concentrations were determined by external calibration (0-20 ppb Pt and Au). LODs were 0.1 and 0.2 µg L^{-1} for Pt and Au, (3*SD on blank, n=10) and the spike recovery were 102% and 99% for Pt and Au (n = 3), respectively. Pt and Au single element PlasmaCAL standards (SCP Science, Quebéc, Canada) were used and the standards were prepared in a mixture of 0.1% HCl and 0.65% subboiled HNO_3 in MilliQ water. This mixture was furthermore used to dilute samples after digestion and as blank solution.

Temperature dependency. For the evaluation of temperature dependency, after the pre-incubation at 37°C for 1 h, rat kidney slices were incubated for 10, 30 and 60 min with the metal complexes at 37°C or 4°C, and were subsequently washed with ice-cold Krebs-Henseleit buffer and snap-frozen as described above.

Statistics

A minimum of three independent experiments were performed using slices in triplicates from each rat kidney. The TC_{50} values were calculated as the concentration reducing the viability of the slices by 50%, relative to the untreated samples using a nonlinear fitting of log(concentration compound) vs response and is presented as a mean (± SD) of at least three independent experiments. Statistical testing was performed with repeated measures ANOVA and Bonferroni as post hoc test to compare the treated samples with the untreated controls. A p-value of ≤ 0.05 was considered to be significant. In all graphs and tables, the mean values and standard deviation (SD) are shown.

Results and discussion

Toxicity evaluation

ATP content determination. Initially, to determine the toxicity of the evaluated compounds, PCKS were incubated with different concentrations of cisplatin and Au(III) complex for 24 h. In addition, another set of kidney slices of the same rat were co-incubated with 100 µM cimetidine to assess its effect on the toxicity of cisplatin and Au(III) complex in the PCKS. It is hypothesized that this inhibitor for OCTs and MATE transporters might reduce the accumulation of cisplatin and thereby protects against toxicity. The viability of the kidney slices was determined by measuring the ATP/protein content. The obtained results are presented in Figure 2. Both compounds show concentration-dependent reduction of viability. The Au(III) complex showed a higher toxicity than cisplatin, with TC_{50} values of 4.3 ± 0.2 µM and 17 ± 2.0 µM, respectively (Table 1). Co-incubation of cisplatin or Au(III) complex with 100 µM cimetidine did not result in any significant change of toxicity in the rat kidney slices. These results are in contrast with previously reported studies in *in vitro* cellular models. [22,23] It should be noted that cimetidine inhibits not only the uptake transporter OCT2, which is supposed to reduce cellular accumulation, but also the active efflux transporter MATE, which may result in higher accumulation in the slices and thereby increasing toxicity.[45] Thus, our toxicity evaluation results suggest that cisplatin and Au(III) complex accumulation is not dependent on rat OCTs or MATEs.

Table 1. TC_{50} values for PCKS treated with cisplatin or Au(III) complex, in the absence and presence of cimetidine, for 24 h.

Compound	TC_{50}	
	No cimetidine	+ 100 µM cimetidine
cisplatin	17.0 ± 2.0	14.7 ± 3.5
Au(III) complex	4.3 ± 0.2	4.4 ± 0.9

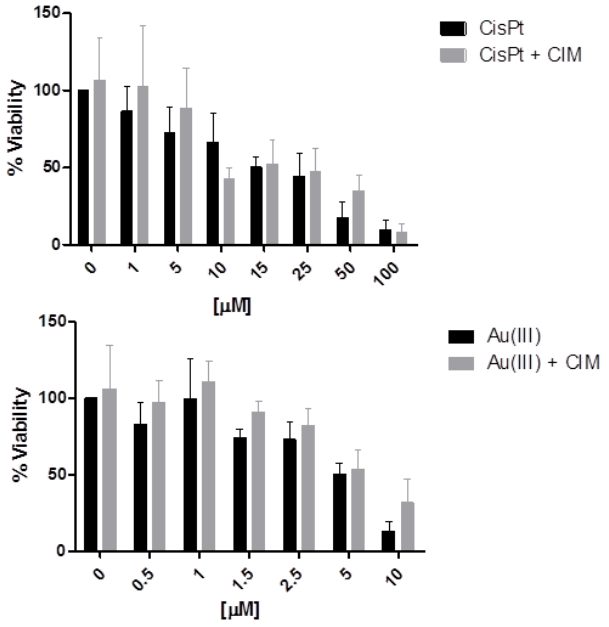

Figure 2. Viability of PCKS treated for 24 h with different concentrations of cisplatin (top) and of Au(III) complex (bottom), without cimetidine (black bars) and co-incubated with cimetidine (grey bars, indicated as: + CIM). The error bars show the standard deviation of at least three independent experiments.

Histomorphology

The differential effects of cisplatin and Au(III) complex on PCKS viability, in the absence or presence of cimetidine, were further assessed by histomorphology. Specifically, Periodic Acid-Schiff staining (PAS) was used to evaluate slice integrity and particularly to visualize the basal membranes and epithelial brush border in the proximal tubule. After 24 h incubation, the untreated kidney slices show minor morphological changes, indicated by occasional pyknosis and swelling of tubular cells (Figure 3A). The kidney slices co-incubated with cimetidine (100 µM) showed similar characteristics of integrity as the untreated controls (Figure 3A and 3B). However, the cell swelling is more evident in the samples treated with cimetidine, including slight Bowman's space dilatation (Figure 3B).

As expected, exposure of PCKS to cisplatin (Figure 3) and to the Au(III) complex (Figure 4) results in damage in several of the cellular structures in the cortex. In the case of cisplatin, at 5 µM concentration (Figure 3C-D) there is evidence of damage on the distal and proximal tubular cells as well as some dilatation of the Bowman's space in the glomerulus, which seems more prominent in the samples treated with cimetidine. With the increase of the concentration of cisplatin to 10 and 25 µM (Figure 3E-H) the damage intensifies and disruption of the brush borders of the proximal tubules becomes more evident. Furthermore, cimetidine treatment did not reduce the kidney damage induced by cisplatin, and slices resulted to be equally affected by the metallodrug as those incubated without cimetidine.

The tubular damage found in our study for cisplatin is in line with the results of Vickers et al. in rat PCKS. [40] However, in our study we observed that cisplatin also affects the glomerulus structure. This difference in toxicity profiles might be caused by the different culture media used, especially the presence of serum in the study of Vickers might be the cause of the difference between these results. In conclusion, our data shows tubular damage by cisplatin, which is not influenced by cimetidine, in line with the ATP viability data.

For the samples exposed to the Au(III) complex, the increase in drug concentration does not produce any major differences with respect to the lower tested concentration. In fact, in all cases, extensive damage is observed mainly in the distal tubular cells, with the structure of the brush border in the proximal tubule almost intact (Figure 4 C-H). No difference was observed in the absence (Fig. 4, left column) or presence (Fig. 4, right column) of cimetidine.

While cisplatin generates more damage towards the glomeruli and the proximal tubular cells, the Au(III) complex displayed selective damage of the distal tubular cells, which was also previously described for anticancer Au(I) complexes.[36]

Overall, the presented histomorphology and ATP results show that there is no evidence of reduced toxicity in the slices exposed to either cisplatin or Au(III) complex co-incubated with cimetidine (Figures 3 and 4, right columns).

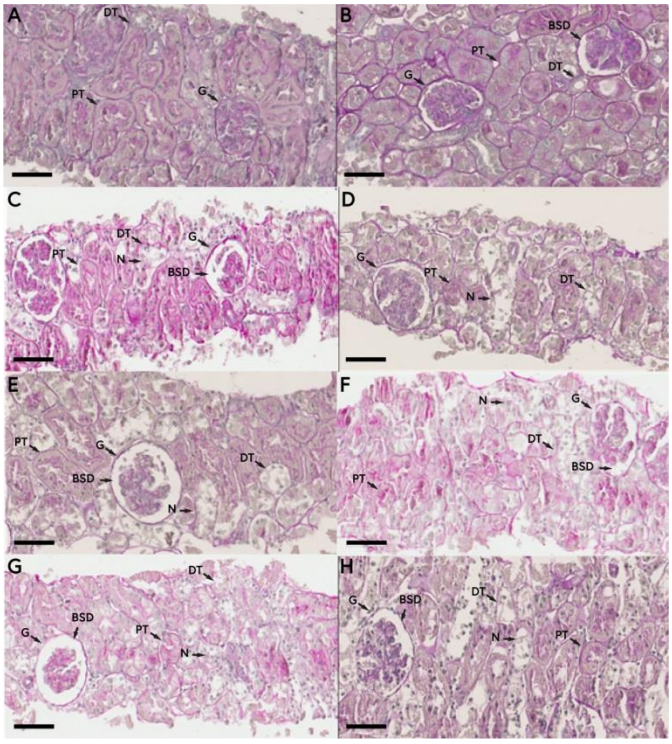

Figure 3. Morphology of rat precision cut kidney slices exposed to different concentrations of cisplatin for 24 h. Left column: absence of cimetidine; right column: co-incubation with cimetidine. A and B: 24 h control incubation; C and D: 5 µM; D and E: 10 µM; F and G: 25 µM h. PT: proximal tubule, DT: distal tubule, G: glomerulus, BSD: Bowman's space dilatation, N: Necrotic areas. Scale bar indicates 50 µm.

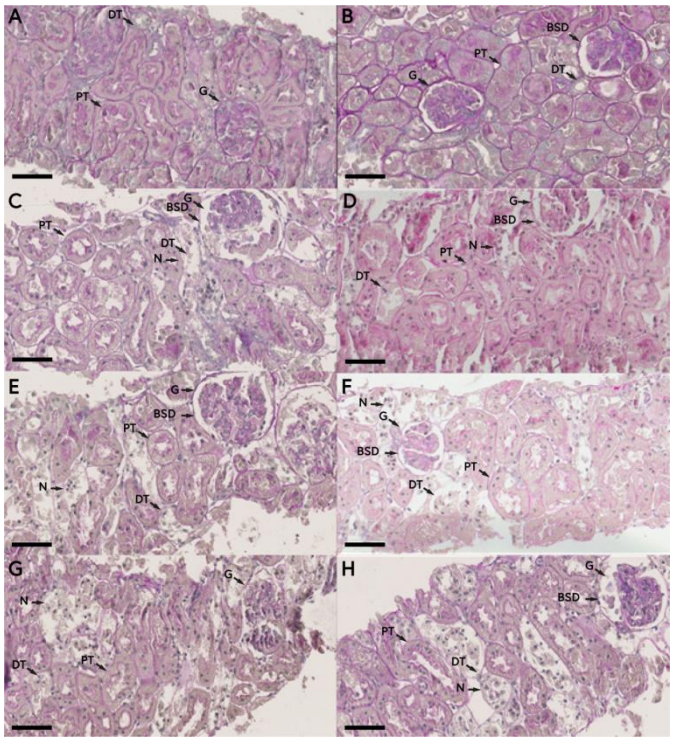

Figure 4. Morphology of rat precision kidney slices exposed to different concentrations of Au(III) complex for 24 h. Left column: absence of cimetidine; right column: co-incubation with cimetidine. A and B: 24 h control incubation; C and D: 1 µM; E and F: 2.5 µM; G and H: 5 µM. PT: proximal tubule, DT: distal tubule, G: glomerulus, BSD: Bowman's space dilatation, N: Necrotic areas. Scale bar indicates 50 µm.

Expression of kidney-injury molecule-1 (KIM-1), villin, p53 and Bax.

The variation of the expression levels of different markers were evaluated in PCKS after incubation with the Pt(II) and the Au(III) metallodrugs. Specifically, kidney injury molecule-1 (KIM-1) and villin were chosen as proximal tubule damage specific biomarkers. An increase of KIM-1 fold expression is expected in the presence of tubular damage, [46,47] whereas decrease in the expression levels of villin is considered a sign of brush border damage.[48–50] Additionally, p53 and Bax were studied to determine the role of apoptosis in the toxicity of PCKS upon exposure to cisplatin and to the Au(III) complex. In fact, it has been proposed that cisplatin induced nephrotoxicity consists of activation of multiple stress pathways, including p53 mediated responses and intrinsic and extrinsic apoptosis pathways (with Bax playing an important role in the intrinsic ones).[51–54] Thus, KIM-1, villin, p53 and Bax mRNA expression was evaluated in PCKS after 3 h, 5 h and 24 h exposure with cisplatin and Au(III) complex at concentrations close to their TC_{25} and TC_{50}. The obtained results are shown in Figure 5.

In the untreated control samples, the KIM-1 expression showed a tendency to increase over time compared with the 0 h controls reaching a peak after 24 h, ca. 100-fold increase, (Figure 5A); these results suggest the tubular cells are *per se* undergoing damage by the slicing and/or culturing process. Interestingly, KIM-1 expression decreased with increasing cisplatin concentration compared to the time-matched controls; even the lowest concentration of cisplatin and the shortest period of incubation (7.5 µM – 3 h) resulted in a decreased KIM-1 expression compared to untreated controls. However, due to the high variation, this decrease reached significance only after 24 h exposure to 15 µM cisplatin. Interestingly, upon exposure to the Au(III) complex, no change in KIM-1 expression was observed. From these findings, it can be hypothesized that when slices are exposed to high concentrations of cisplatin, the cellular machinery of the proximal tubular cells is too damaged to be able to produce KIM-1 mRNA.

As shown by the morphological studies, the gold compound seems to target more specifically the distal tubular cells, with reduced damage to the proximal tubular cells, explaining the lack of effect on KIM-1. Concerning villin mRNA expression (Figure 5B), a reduction up to 40% was observed in the controls after incubation for 24 h, indicating some damage of the brush border of the proximal tubular cells. However, no dose-dependent effect of cisplatin was observed, while the Au(III) compound slightly reduced the villin expression, which only reached significance after 5 h at 2 µM.

The assessment of the expression patterns of p53 (Figure 5C) displayed a slight, but not significant increase of the p53 expression during incubation of the control slices. Conversely, upon treatment with cisplatin after 5 h at 15 µM, a decrease of p53 expression is observed, as well as 24 h at 7.5 and 15 µM compared to the untreated samples at each time point. These results differ from the findings in the previously mentioned study of Vickers et al,[40] where the expression of p53 increased in rat PCKS after treatment with cisplatin at 20 and 40 µM for 24 and 48 h, respectively.[40] Exposure to the Au(III) complex had no effect on p53 expression (Figure 5C, right panel).

Finally, no significant difference in Bax expression levels was observed during incubation of the control samples (Figure 5D). Bax expression increased slightly but significantly after 24 h exposure to the highest concentration of both compounds. These results indicate possible activation of the intrinsic apoptotic pathway depending mainly on the mitochondrial integrity. This finding is in line with previous reports that indicate the possibility of induction of apoptosis independent of p53 after treatment with cisplatin of human and mouse cancer cell lines.[54–59]

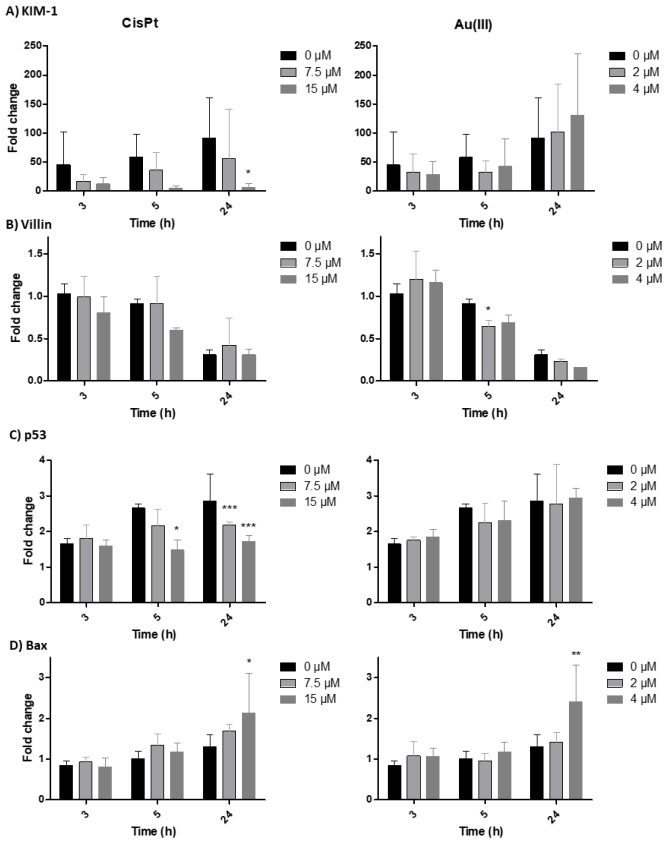

Figure 5. Fold change of KIM-1 (A), villin (B) p53 (C) and Bax (D) after exposure to cisplatin (left) and to the Au(III) complex (right) during 3 h, 5 h and 24 h. The untreated control (0 h) was set as 1 to calculate the relative fold induction (not shown). The error bars show the standard deviation of at least three independent experiments. Statistical significance was determined by repeated measures ANOVA and Bonferroni as post hoc test to compare the treated samples with the untreated controls for each time point (*: p<0.05, **: p<0.01, ***: p<0.001).

Uptake studies

Metal content determination by ICP-MS. In order to assess the intracellular accumulation of the tested compounds and to evaluate the relation between toxicity and intracellular metal content, we determined the Pt and Au content of PCKS exposed to cisplatin and to the Au(III) complex by ICP-MS. Thus, PCKS were incubated for 24 h in the same conditions as for the ATP determination. The concentrations of cisplatin and Au(III) complex used were below or around their TC_{50}. As can be seen in Figure 6, the Pt and Au contents increase as a function of the compounds' initial concentration (up to 110.3 ng Pt per slice in the case of slices treated with 10 µM cisplatin, and up to 84 ng Au per slice treated with 5 µM of Au(III) complex). However, the obtained results did not show a significant difference between the samples treated with cimetidine or without it, which is in line with the viability and histomorphology studies above, suggesting that OCT and MATE are not involved in the transport of the compounds at the tested concentrations. Moreover, it is worth mentioning that the Au(III) complex appears to be more efficiently accumulated into PCKS than cisplatin: the amount of Au and Pt after 24 h of incubation is approximately the same (~28 ng) for the samples treated with cisplatin at 3 µM concentration or Au(III) complex at 1 µM. Additionally, from the obtained results, it can be calculated that the accumulation of cisplatin and of the Au(III) complex results in approximately 20-fold and 30-fold increased concentration of the metals in the slices compared to the medium, respectively. This is in line with the reported accumulation of cisplatin in kidneys *in vivo*,[40] indicating either high binding or metabolism in the cells, or the involvement of active uptake transporters.

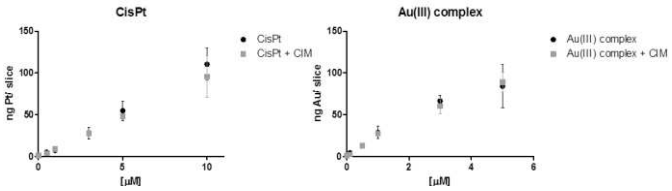

Figure 6. Total metal content determined by ICP-MS in PCKS treated with cisplatin or with the Au(III) complex at different concentrations, without and with cimetidine (indicated as: + CIM), for 24 h. The error bars show the standard deviation of at least three independent experiments.

Effect of temperature on uptake in PCKS. To evaluate if the uptake of cisplatin and of the Au(III) complex is by passive diffusion or carrier-mediated transport, PCKS were incubated with the selected compounds at three different concentrations at 4°C or 37 °C over a period of 60 min. Slices were collected after 0, 10, 30 and 60 min incubation with the metallodrugs, washed with ice-cold Krebs Henseleit buffer and their metal content was evaluated by ICP-MS to assess the effect of different temperatures on the uptake of the drugs.

Slices treated with cisplatin at 5 µM and 25 µM incubated at 4°C or 37°C showed an initial rapid uptake phase followed by a slower accumulation, indicating sequestration by excretion. No significant differences in the Pt content were seen between the two temperatures (Figure 7), suggesting that only passive uptake mechanisms play a role at these tested concentrations. However, slices treated with cisplatin at 100 µM showed significant differences at 30 and 60 min, with a lower Pt content in the slices incubated at 4°C compared to 37°C, indicating that carrier-mediated uptake mechanisms are implicated in cisplatin accumulation in cells at this high concentration. Apparently, both passive and carrier-mediated mechanisms are involved with cisplatin uptake at low concentrations, while carrier-mediated transport is only significantly involved in Pt accumulation at higher concentrations, indicating a low affinity for the transporter.[6,26,30]

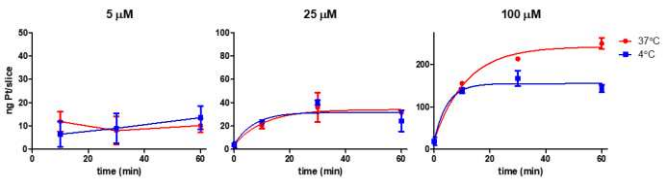

Figure 7. Pt content (ng per slice) in PCKS treated with cisplatin at 5 µM, 25 µM, 100 µM. Incubated at 37°C and 4°C and collected at three different time points (10, 30 and 60 min). t=0 value for the lowest concentration was not determined due to limitations in the amount of tissue, but is estimated to be 0.8 ng Pt/slice based on the values found for the two higher concentrations. The error bars show the standard deviation of at least three independent experiments.

Remarkably, evaluation of the Au content in rat kidney slices upon treatment with the Au(III) complex showed a significant higher Au accumulation at 4°C compared to 37°C at all concentrations and time points (Figure 8). A fast initial uptake rate is followed by a slower uptake rate, which is observed for all concentrations during 60 min. The higher Au content found in the slices incubated at 4°C could be due to the inhibition of active excretion mechanisms such as an efflux transporter, other than MATE, at this low temperature.

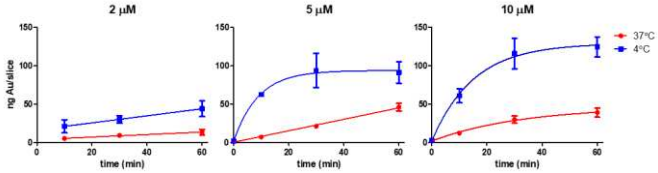

Figure 8. Au content (ng per slice) in PCKS treated with 1 at 2 µM (A), 5 µM (B), 10 µM (C). Incubated at 37°C and 4°C and collected at three different time points (10, 30 and 60 minutes). t=0 value for the lowest concentration was not determined due to limitations in the amount of tissue, but is estimated to be 0.5 ng Au/slice. The error bars show the standard deviation of at least three independent experiments.

Conclusions

In the past decades, several studies have been carried out to elucidate the mechanisms of uptake and efflux of cisplatin on kidney cells, related to the nephrotoxic effects of this extensively used anticancer drug, and various *in vitro* assays were conducted. Nonetheless, such transport mechanisms are not yet fully understood.[30] Moreover, the mechanisms leading to toxicity and accumulation of new generation anticancer gold complexes have not been fully elucidated. Even less is known on the transport of organometallic gold complexes in the kidney.

Therefore, we investigated and compared the toxicity and the accumulation of cisplatin and a cytotoxic experimental organometallic Au(III) complex in rat kidney tissues using the PCKS technology. Additionally, we evaluated the involvement of rOCTs and rMATE transporters using their inhibitor cimetidine, in competition experiments. Furthermore, passive or active transport mechanisms were assessed by measuring metal uptake by ICP-MS in PCKS at 37°C or 4°C, respectively.

As expected, a concentration dependent decrease in viability of PCKS was observed after 24 h exposure to both compounds, which correlated with the increase in PCKS content of platinum and gold after treatment. The gold complex seems to be more toxic for the kidney slices than cisplatin based on the TC50's being 4.3 ± 0.2 µM and 17 ± 2.0 µM respectively. Interestingly, the histomorphological changes after treatment suggest that the Au(III) compound exerts its toxicity towards different target cells than cisplatin, showing extensive damage of the distal tubular cells, whereas cisplatin is more toxic towards the proximal tubular cells. The latter results are in line with previously reported studies.[36,40] However, at variance with other studies present in the literature using cell cultures or isolated human tubuli,[22–24] in our *ex vivo* model no effect of co-incubation with cimetidine on the toxicity or accumulation of cisplatin and Au(III) complex was found. Based on these results we conclude that rOCTs and rMATE transporters do not play a prominent role in cisplatin or Au(III) complex accumulation in the rat kidney slices at the tested cimetidine concentration. Alternatively, cimetidine may inhibit MATE transporters with higher efficacy than OCT, thereby reducing its renoprotective effect.

Furthermore, KIM-1 and villin mRNA expression were studied as markers of proximal tubular damage. KIM-1 expression decreased with increasing cisplatin concentrations, whereas the Au(III) complex induced no change in KIM-1 expression at increasing concentrations. These findings are in agreement with the different localization of the damage in the tissue. In contrast, villin expression was not affected by cisplatin, but was slightly reduced by the Au(III) complex. It could be important to evaluate other markers of damage including markers of structures other than proximal tubular cells to assess kidney injury induced by metallodrugs in a more comprehensive way. Surprisingly, p53 overexpression was not induced in PCKS exposed to cisplatin as previously reported.[40] However, as mentioned before, the culture conditions, and specifically the serum protein content of the medium, and the concentrations of cisplatin used were different and these circumstances can lead to substantial differences in the obtained results. Moreover, Bax mRNA expression increased over time in control slices indicating possible activation of intrinsic apoptotic pathways. Nevertheless, the increment is higher when PCKS were treated during 24 h with the highest concentration of cisplatin or gold compound as evidence of further stress compared to the controls.

To get insight into the specific toxic mechanisms activated after treatment with cisplatin or other metallodrugs, it is imperative to consider the species and tissue distinct gene expression profiles during incubation without and with drugs. Specifically, cisplatin is known to activate several stress pathways, but it is dependent on the concentration, cell type and culture conditions whether the cells die by apoptosis, necrosis or both.

Our *ex vivo* studies to evaluate the passive or active character of the transport of cisplatin and the Au(III) complex revealed that both passive and active processes might play a role. Moreover, the uptake of cisplatin is achieved by both passive and active mechanisms but this becomes evident only at higher concentrations, indicating a low affinity for the active transporters. On the other hand, the results obtained for the Au(III) complex suggested an important role of carrier-mediated excretion, shown by the increased Au content in the slices incubated at 4°C compared to 37°C.

Further studies to explore the role of different transporters are needed to better understand the concentration dependent and organ-specific toxicity of our metallodrugs, which is valuable to design new experimental metallodrugs with reduced side effects in specific tissues. Certainly, the use of PCKS offers good opportunities to evaluate toxicity, uptake and

accumulation of metallodrugs in different organs and species, and finally to get insight into the effect in human tissues derived from patients. However, optimization of the experimental set-up to reduce the damage of the PCKS induced by culturing is still necessary to exclude possible interference on the obtained results.

Furthermore, new advanced approaches, such as the CRISPR-Cas9 genome editing, should be applied to validate both transport and intracellular trafficking mechanisms for metallodrugs. This technology has been recently applied to individually knock out the human copper transporters CTR1 and CTR2 and the copper chaperones ATOX1 and CCS in cells, *in vitro*.[60] The obtained results suggest that these proteins are not essential for the mechanism by which cisplatin enters human embryonic kidney cells (HEK293T) and ovarian carcinoma OVCAR8 cell lines and is transported to the nucleus, contradicting numerous previously reported studies in the field. Overall, new investigational efforts should be spent to elucidate the complex mechanism of toxicity of metallodrugs and the role of different transport pathways in tissues.

Acknowledgements

The authors thank the Department of Sciences, Technology, and Innovation COLCIENCIAS (Colombia) for a PhD fellowship to N.E.O. The University of Groningen and the University of British Columbia are acknowledged for cofounding the PhD fellowship of S.S. Authors wish to thank Marjolijn Merema, Eduard Post and Marina de Jager for training and assistance during the *ex vivo* experiments.

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