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Expression of organic anion transporter 1 or 3 in human kidney proximal tubule cells reduces cisplatin sensitivity

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OAT expression reduces proximal tubule cisplatin sensitivity

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List of nonstandard abbreviations

AKI	acute kidney injury
ASP^+	4-di-1-ASP (4-(4-(dimethylamino)styryl)-n-methylpyridinium iodide)
ciPTEC	conditionally immortalized proximal tubule epithelial cell
CM	complete medium, supplemented with 10% fetal calf serum
CTR	high affinity copper uptake protein
DMSO	dimethyl sulfoxide
eYFP	enhanced yellow fluorescent protein
HEK293	human embryonic kidney cells 293
hTERT	human telomerase reverse transcriptase

MATE	multidrug and toxin extrusion
MTT	thiazolyl blue tetrazolium bromide (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2h-tetrazolium bromide)
OAT	organic anion transporter
OCT	organic cation transporter
Pgp	P-glycoprotein
ROS	reactive oxygen species
SFM	serum free medium
SV40T	simian virus 40 large T antigen

Abstract

Cisplatin is a cytostatic drug used for treatment of solid organ tumors. The main adverse effect is organic cation transporter 2 (OCT2)-mediated nephrotoxicity, observed in 30% of patients. The contribution of other renal drug transporters is elusive. Here, cisplatin-induced toxicity was evaluated in human-derived proximal tubule epithelial cells (ciPTEC) expressing renal drug transporters, including OCT2 and organic anion transporter 1 (OAT1) or 3 (OAT3). Parent ciPTEC demonstrated OCT2-dependent cisplatin toxicity (TC_{50} 34±1 µM after 24 h exposure), as determined by cell viability. Over-expression of OAT1 and OAT3 resulted in reduced sensitivity to cisplatin (TC_{50} 45±6 µM and 64±11 µM after 24 h exposure, respectively). This effect was independent of OAT-mediated transport, as the OAT-substrates probenecid and diclofenac did not influence cytotoxicity. Decreased cisplatin sensitivity in OAT-expressing cells associated directly with a trend towards reduced intracellular cisplatin accumulation, explained by reduced OCT2 gene expression and activity. This was evaluated by V_{max} of the OCT2-model substrate ASP^+ (23.5±0.1 min^{-1}, 13.1±0.3 min^{-1} and 21.6±0.6 min^{-1} in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3, respectively). While gene expression of cisplatin efflux transporter multidrug and toxin extrusion 1 (MATE1) was 16.2±0.3 fold upregulated in ciPTEC-OAT1 and 6.1±0.7 fold in ciPTEC-OAT3, toxicity was unaffected by the MATE substrate pyrimethamine, suggesting that MATE1 does not play a role in the current experimental set-up. In conclusion, OAT expression results in reduced cisplatin sensitivity in renal proximal tubule cells, explained by reduced OCT2-mediated uptake capacity. *In vitro* drug-induced toxicity studies should consider models that express both OCT and OAT drug transporters.

Introduction

The chemotherapeutic drug cisplatin is commonly used to treat solid organ tumours located in various tissues, including the head, neck, lung, testis, ovary and bladder. Nephrotoxicity is the main adverse effect of cisplatin, causing acute kidney injury (AKI) in 30% of patients (Hartmann et al., 1999). AKI is currently the dose-limiting factor in treatment with cisplatin and is characterized by dysfunction of the proximal tubule. Cisplatin continues to be prescribed, as less toxic alternatives including oxaliplatin, are suggested to have reduced anticancer potential (Wang and Lippard, 2005; Manohar and Leung, 2017).

Proximal tubule epithelial cells mediate active excretion of xenobiotics and metabolites, and reabsorb low molecular weight proteins, such as β2-microglubulin, and glucose and amino acids filtered by the glomerulus. To this end, proximal tubule cells express dedicated membrane spanning proteins (Nigam et al., 2015). Influx of cisplatin in proximal tubule cells is critically mediated by organic cation transporter 2 (OCT2, *SLC22A2*) (Ciarimboli et al., 2005; Filipski et al., 2009; Ciarimboli et al., 2010) and ubiquitously expressed copper transporter 1 (CTR1, *SLC31A1*) (Pabla et al., 2009), both located in the proximal tubule epithelium at the basolateral membrane. Cisplatin is a substrate for efflux transporters multidrug and toxin extrusion 1 (MATE1, *SLC47A1*), and to a lesser extent MATE2-k (*SLC47A2*), both located at the apical membrane (Yonezawa et al., 2006; Nakamura et al., 2010). The combined activity of influx and efflux transporters determines the amount of cisplatin accumulation, hence correlates to the drug's nephrotoxicity (Li et al., 2013). Recent studies indicate that drug-drug interactions and interindividual genetic differences regarding OCT2 and MATE transporters may contribute to the nephrotoxic potential of cisplatin, underlining their concerted action is essential to maintain active clearance and avoid proximal tubule accumulation (Sauzay et al., 2016; Chang et al., 2017).

Organic anion transporter 1 (OAT1, *SLC22A6*) and 3 (OAT3, *SLC22A8*) in proximal tubule cells play a main role in clearance of organic anions, including endogenous uremic toxins and a wide range of drugs (Nigam et al., 2015). Recently, renal adverse effects of cisplatin were demonstrated to be reduced in an Oat1 knockout mouse model, while disposition of this drug is strongly associated with

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cation transporters (Hu et al., 2017). The mechanism is suggested to involve extra renal metabolism, yielding glutathione and cysteine conjugates that act as substrates for OAT transporters. Moreover, clinical studies demonstrated that cisplatin clearance was reduced upon co-treatment with OAT-inhibitor probenecid, consistent with a complete absence of renal adverse effects (Jacobs et al., 1984; Jacobs et al., 1991). As cisplatin transport is neither mediated by OAT1 nor OAT3, their role in cisplatin-induced nephrotoxicity remains to be elucidated.

The present study was designed to investigate the role of OATs in cisplatin-induced toxicity in a human-derived renal cell line manifesting proximal tubule cell characteristics (ciPTEC) (Wilmer et al., 2010; Nieskens et al., 2016). In this cell line, we previously demonstrated functional apical absorption of albumin and phosphate, expression of OCT2, P-glycoprotein (Pgp, *ABCB1*), multidrug resistance-associated protein 4 (MRP4, *ABCC4*), breast cancer resistance protein (BCRP, *ABCG2*), and after stable transductions either OAT1 or OAT3 were reintroduced (Wilmer et al., 2010; Schophuizen et al., 2013; Jansen et al., 2014; Nieskens et al., 2016). In addition, ciPTEC express several UDP-glucuronosyltransferases and consume oxygen, suggesting intact mitochondrial energy production (Mutsaers et al., 2013; Schirris et al., 2017). Therefore, ciPTEC is a relevant proximal tubule cell model with concerted action of different transport mechanisms, suitable for *in vitro* evaluation of drug-induced toxicity. The results of the present study show CTR1- and OCT2-dependent cisplatin-mediated toxicity in ciPTEC, which is alleviated in presence of active OAT1 or OAT3. Reduced sensitivity in OAT1- or OAT3-expressing cells was associated with a trend towards reduced cisplatin accumulation due to lower OCT2 uptake capacity. Since expression of OATs affects toxicity induced by drugs that are typically imported by OCTs, simultaneous expression of basolateral solute carrier transport proteins is important for *in vitro* drug-induced toxicity evaluation.

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Materials and methods

Cell culture

Conditionally immortalized proximal tubule epithelial cells (ciPTEC-parent) were developed as described by Wilmer et al. with informed consent of the donors in accordance with the approved guidelines of the Radboud Institutional Review Board (Wilmer et al., 2010). ciPTEC-OAT1 and ciPTEC-OAT3 were derived from ciPTEC-parent cells and demonstrate constitutive expression of organic anion transporter 1 (OAT1) or 3 (OAT3), respectively, as described by Nieskens et al. (Nieskens et al., 2016). ciPTEC constitutively expressing eYFP (ciPTEC-eYFP) were developed by transduction of eYFP from the COX8-eYFP vector (Koopman et al., 2006) into the same pLenti4/V5-DEST vector as used for the OAT constructs, by LR recombinant reaction using Gateway Cloning Technology (Invitrogen, Carlsbad, USA) and resulting in expression vector pLenti4/V5-EX-CMV-TetO2-eYFP. Upon transduction as described for ciPTEC-OAT1 and -OAT3, ciPTEC-eYFP cells were selected for YFP fluorescence using BD FACSAria SORP flow cytometer (BD biosciences, San Jose, USA). Proliferating cell culture was maintained up to 90% confluency using T75 culture flasks (Greiner Bio-One, Kremsmünster, Germany) at 33°C and 5% v/v CO_2. Cells were seeded 7 or 8 days prior to the experiment in either T75 culture flasks (Greiner Bio-One), 12-well plates (Greiner Bio-One) or 96-well plates (Corning Life Sciences, New York, USA) with a seeding density of 55,000 cells/cm^2 for ciPTEC-parent, 63,000 cells/cm^2 for ciPTEC-OAT1 and 82,000 cells/cm^2 for ciPTEC-OAT3. Cells were subsequently incubated for 24 h at 33°C and 5% v/v CO_2 to stimulate proliferation, followed by 6 or 7 days at 37°C and 5% v/v CO_2 to allow differentiation and monolayer formation, referred to as maturation. Cells were cultured using Dulbecco's modified eagle medium (DMEM HAM's F12, Life Technologies, Paisly, UK), supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 5 µg/ml selenium, 35 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 40 pg/ml tri-iodothyronine (Sigma, St. Louis, USA), and 10% fetal calf serum (FCS, Greiner Bio-One), and is referred to as complete medium (CM). Medium in which FCS was omitted, is referred to as serum free medium (SFM). The medium was refreshed every 2 to 3 days, supplemented with 1% penicillin/streptomycin (Invitrogen) only at 33°C.

Viability assay

To evaluate cisplatin-induced toxicity, viability of ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 was evaluated by an MTT assay (Mosmann, 1983). Briefly, ciPTEC were matured in 96-well plates, washed 3 times with SFM, and subsequently exposed to cisplatin (8.7-222 µM, Sigma) in SFM for 24 h. To evaluate the effect of competitive transport inhibitors on cisplatin-induced toxicity, cisplatin was co-exposed with probenecid (100 µM, Sigma), diclofenac (50 µM, Sigma), cimetidine (1.25 mM, Sigma) or pyrimethamine (10 and 100 nM, Sigma). For probenecid, a pre-exposure during ciPTEC maturation was additionally performed for 6 days in CM. Following cisplatin exposure, cells were washed 3 times with SFM and incubated with 0.5 mg/ml MTT (Sigma) in SFM for 3 h at 37°C. Formazan crystals formed in viable cells were dissolved in dimethyl sulfoxide (DMSO, Merck, New Jersey, USA) on a microplate shaker (VWR, Radnor, USA) for 1 h. Absorption was measured at 560 nm, subtracting the background at 670 nm, using the BioRad Benchmark Plus (BioRad, Hercules, USA). All values were normalized to unexposed control. Results were plotted with GraphPad Prism (version 5.03, GraphPad Software, San Diego, USA) using non-linear regression with four parameters (variable slope) on log-transformed x-values.

Cisplatin accumulation

To evaluate the effect of OCT2- and MATE-mediated transport on intracellular cisplatin accumulation, ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 were matured in T75 culture flasks, washed once in CM and exposed to cisplatin (250 µM, Sigma), with and without OCT2 and MATE substrates cimetidine (1.25 mM, Sigma) or pyrimethamine (100 nM, Sigma) in CM for 90 min. Cells were washed 3 times in HBSS (37°C), harvested using accutase (Invitrogen) and placed on ice. Cell suspensions were centrifuged at 1,500 rcf for 5 min (4°C) and cell pellets were resuspended in 200 µl nitric acid (100 mM, Merck). Lysates were obtained by applying 3 freeze-thaw cycles of liquid nitrogen and a 37°C waterbath, followed by centrifugation at 10,000 rcf for 10 min (4°C). Cisplatin content of cell samples was evaluated using an atomic absorption spectrophotometer AAnalyst800 (Perkin Elmer, Waltham, USA) equipped with a transversally heated graphite tube atomizer and a platinum hollow cathode lamp (Photron, Melbourne, Australia), loaded with 20 µl of each sample, as described in detail before (Durr et al., 2013; Eljack et al., 2014). All values were corrected for blank (unexposed), normalized to ciPTEC-parent (for uninhibited control) or uninhibited control of each respective cell line

(for competitive inhibitors) and results were plotted with GraphPad Prism (version 5.03, GraphPad Software).

Gene expression

To evaluate expression of drug transporter genes involved in cisplatin influx and efflux, ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 were matured in 12-well plates, washed 3 times and exposed to SFM for 24 h, before total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer´s instructions. When evaluating Nrf2 pathway induction, SFM was supplemented with 50 nM bardoxolone methyl (CDDO-Me, Sigma), cDNA was synthesized using M-MLV Reverse Transcriptase (Promega, Madison, USA), according to the manufacturer's instructions. mRNA expression levels of transporters were determined using gene-specific primer probe sets obtained from Life Technologies: OCT2 (*SLC22A2*, hs01010723_m1), MATE1 (*SLC47A1*, hs00217320_m1), MATE2-K (*SLC47A2*, hs00945650_m1), MRP2 (*ABCC2*, hs00166123_m1), *NQO1* (hs01045993_g1), *GCLC* (hs00155249_m1), *GAPDH* (hs99999905_m1) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, USA). Real-time qPCR reactions were performed using CFX96 Touch Real-Time PCR system (BioRad) according to the manufacturer's instructions, and analysed by CFX Manager software (version 1.6, BioRad). mRNA levels were calculated using *GAPDH* as reference gene and expressed as either $-\Delta\Delta C_t$ or fold change ($2^{-\Delta\Delta Ct}$) compared to ciPTEC-parent. Results were plotted with GraphPad Prism (version 5.03, GraphPad Software).

ASP accumulation

To evaluate the accumulation of hOCT2 model substrate ASP^+ (Pietruck and Ullrich, 1995; Cetinkaya et al., 2003); ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 were matured in 96-well plates (Schophuizen et al., 2013). Prior to the experiment, cells were washed 3 times in HEPES-Tris buffer, consisting of 132 mM NaCl, 4.2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 10 mM Hepes, buffered to pH=7.4 using Tris pH=8.8. Next, cells were exposed to ASP^+ (4-di-1-ASP^+, 0.8-50 µM, Thermo Scientific, Waltham, USA) with and without cimetidine (1.25 mM, Sigma), pyrimethamine (10 and 100 nM, Sigma) or probenecid (100 µM, Sigma) for 1 h at 37°C. For probenecid, a pre-exposure during ciPTEC maturation was additionally performed for 6 days in CM to study the effect of long-term OAT activity inhibition on ASP^+ transporting proteins. After accumulation for 1 h, the fluorescent signal

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was measured (excitation 485 nm; emission 590 nm) using a Victor X3 plate reader (Perkin Elmer, Waltham, USA). Values were corrected for extracellular signal (empty wells with corresponding ASP^+ concentration) and either presented as arbitrary units (for kinetic curves) or normalized to control (for competitive inhibitors). Results were plotted with GraphPad Prism (version 5.03, GraphPad Software) using Michaelis-Menten regression analysis for concentration-dependent transport rate curves.

Glutathione level

To evaluate total cellular glutathione level, ciPTEC were matured in 6-well plates and glutathione level was evaluated using the Glutathione Assay Kit (Sigma), according to the manufacturer's instructions. Briefly, cells were harvested using accutase (Invitrogen), washed with HBSS and deproteinized with 5% sulfosalicylic acid (Sigma). Lysates were obtained by applying 2 freeze-thaw cycles of liquid nitrogen and a 37°C water bath, followed by 5 min incubation on ice and centrifugation at 10,000 rcf for 10 min (4°C). Samples were measured undiluted and absorption was evaluated at 412 nm every min for 5 min, using the Benchmark Plus (BioRad). All values were corrected for blank, normalized to ciPTEC-parent and results were plotted with GraphPad Prism (version 5.03, GraphPad Software).

Data analysis and statistic

All data analysis and statistics were performed using GraphPad Prism (version 5.03, GraphPad Software) and presented as mean±S.E.M. of three independent experiments (n=3) performed in experimental triplicate, unless stated otherwise. For calculation of EC_{50} values, log cisplatin concentration versus viability was plotted after background subtraction. Statistics were performed by Student's t-test (two-tailed, $\alpha=0.05$) or by two-way ANOVA (two-tailed, $\alpha=0.05$) using the Bonferroni post test ($p<0.05$), as indicated in the figures.

Results

The toxic potential of cisplatin was evaluated in parent ciPTEC by exposure for up to 72 h, which resulted in a concentration- and time-dependent loss in cell viability as measured via the MTT assay, presented in Figure 1A. To elucidate the role of influx transporter activity on cisplatin-induced toxicity, ciPTEC-parent was co-exposed to cisplatin in presence of the OCT2 substrate cimetidine or CTR1 substrate $CuSO_4$ for 24 h. Both substrates significantly attenuated toxicity of cisplatin, demonstrating their involvement in uptake and related toxicity of the drug (Figure 1B). Next, cisplatin exposure to ciPTEC either expressing OAT1 or OAT3 demonstrated reduced sensitivity compared to ciPTEC-parent, reflected by increased TC_{50} values, for which the largest shift was observed for ciPTEC-OAT3 (Figure 1C, Table 1). The involvement of OAT-mediated transport in cisplatin-induced toxicity was investigated further by cisplatin exposure in the presence of OAT substrates probenecid and diclofenac. These did not affect the cytotoxicity after 24 h nor after 7 days of pre-exposure, suggesting that OAT-mediated influx is not directly involved in reducing cisplatin sensitivity, nor that OAT transport results in the induction of protective cellular changes on a long term (Table 1). It should be noted that 7-day exposure to competitive inhibitor solvent (DMSO, 0.1% v/v) slightly reduced cisplatin-induced toxicity, which may be explained by inactivation of the drug by prolonged exposure to DMSO (Table 1) (Hall et al., 2014). In order to avoid possible adaptive responses that may affect drug sensitivity, cisplatin preconditioning at shorter incubation times was not performed. Moreover, longer term exposures usually take place *in vivo* when patients are treated with cisplatin, making 24 or 48 h exposures clinically more relevant. To investigate the intracellular capacity to handle oxidative stress induced by cisplatin, total glutathione content was analysed in the three cell lines. No significant changes were observed in glutathione content between ciPTEC-parent, ciPTEC-OAT1 ($p=0.44$) and ciPTEC-OAT3 ($p=0.56$) (Supplementary Figure 1). As decreased sensitivity to cisplatin might be due to the transduction process used to over-express OAT1 or OAT3, we evaluated cisplatin sensitivity in ciPTEC expressing enhanced yellow fluorescent protein (ciPTEC-eYFP) generated by the same transduction methodology. No difference in toxicity ($p=0.53$) was observed between ciPTEC-eYFP (TC_{50} 39±7 µM) and ciPTEC-parent (TC_{50} 34±1 µM, Table 1), supporting our hypothesis that OAT protein expression is responsible for diminished cisplatin-sensitivity in OAT1 or OAT3 expressing cells and excluding nonspecific aberrations induced by the transduction methods.

The next step was to determine cisplatin accumulation in the three cell lines, which showed a reduced trend in ciPTEC-OAT1 (p=0.29) and ciPTEC-OAT3 (p=0.12) as compared to ciPTEC-parent after exposure to excess cisplatin concentrations for 90 min (Figure 2A). Hence, cisplatin accumulation in the cell lines was directly associated with their sensitivity to cisplatin as assessed by cell viability. This could be the result of decreased OCT2 expression levels or increased MATE1 and MATE2-k expression, which facilitate cisplatin influx and efflux, respectively. ciPTEC expressing OAT3 showed slightly but significantly decreased OCT2 (*SLC22A2*) gene expression of 0.4±0.1 fold, while the 0.5±0.1 fold reduction in ciPTEC-OAT1was not significant. On the other hand, MATE1 (*SCL47A1*) was 16.2±0.3 fold increased in ciPTEC-OAT1 and 6.1±0.7 fold in ciPTEC-OAT3 (Figure 2B, C_t values are shown in Supplementary Table 1). In addition, MATE2-k gene expression was increased in ciPTEC-OAT1, but decreased in ciPTEC-OAT3. Although MRP2 (*ABCC2*) expression has also been associated with reduced cisplatin sensitivity (Kawabe et al., 1999), we did not find differential regulation of this drug transporter between the three cell lines studied (Supplemental Table 2). To evaluate the functional contribution of influx and efflux transporters further, cisplatin accumulation was analyzed in the presence of the competitive OCT2 inhibitor cimetidine and resulted in a reduction of intracellular cisplatin levels, that was significant for ciPTEC-OAT1, but not for ciPTEC-parent (p=0.32) or -OAT3 (p=0.33). Pyrimethamine did not affect cisplatin accumulation at concentrations for which it is a selective MATE1 substrate (Ito et al., 2010), suggesting a limited role of MATE-mediated efflux in the current experimental conditions (Figure 2C). Competitive MATE inhibition by pyrimethamine did not affect cisplatin-induced toxicity following co-exposure for 24 h, again supporting the limited role of MATE in the current conditions (Table 2).

To study the influence of OAT expression on OCT2 protein activity in more detail, we investigated uptake of ASP^+ as specific substrate for the transporter in all ciPTEC cell lines in presence and absence of OCT2, MATE and OAT substrates. V_{max} of ASP^+ uptake was significantly reduced in ciPTEC-OAT1 (13.1±0.3 min^{-1}) and ciPTEC-OAT3 (21.6±0.6 min^{-1}) compared to ciPTEC-parent (23.5±0.1 min^{-1}), suggesting that OCT2-mediated uptake capacity of ASP^+ is reduced in ciPTEC expressing OAT1 or OAT3 (Figure 3A, Table 3). As expected, competitive inhibition of OCT2 by cimetidine reduced ASP^+ accumulation (Figure 3B), while the competitive MATE inhibitor

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pyrimethamine did not affect ASP^+ accumulation, suggesting that ASP^+ is transported by OCT2 and not by MATE, which is in line with cisplatin accumulation assays (Figure 3C). Competitive OAT inhibition by probenecid did not affect ASP^+ accumulation after 24 h (Figure 3D), neither after 7 days (Figure 3E) of treatment, suggesting that OAT-mediated uptake did not regulate OCT2 transport capacity.

The Nrf2 pathway is known to attenuate cisplatin-mediated toxicity by regulation of cisplatin transporters (Atilano-Roque et al., 2016). Therefore, the involvement of this pathway in the regulation of OCT2 and MATE transporters was evaluated in ciPTEC. Bardoxolone stimulated the Nrf2 pathway in ciPTEC parent, as assessed by increasing expression of both target genes *NQO1* and *GCLC* upon 24 h exposure by a factor of 6.1±0.1 and 4.1±0.2, respectively (Figure 4A). This was accompanied by a 1.7±0.2 fold increased MATE1, a 8.5±0.6 fold increased MATE2-k and a 0.3±0.1 fold decreased OCT2 expression, supporting the nephroprotective effect of Nrf2. A similar decrease in OCT2 expression was observed for ciPTEC-OAT1 and ciPTEC-OAT3, as described above. The involvement of Nrf2 in differential regulation of cisplatin transporters in ciPTEC-OAT1 and ciPTEC-OAT3 compared to ciPTEC-parent could however not be confirmed, as only the Nrf2 target *NQO1* was increased 2.7±0.2 fold in ciPTEC-OAT1, while *GCLC* was neither upregulated in ciPTEC-OAT1 nor ciPTEC-OAT3 (Figure 4B). This illustrates that stimulation of Nrf2 via bardoxolone is distinct from the gene expression pattern observed in ciPTEC-OAT1 and ciPTEC-OAT3, indicating that the differences in OCT2 and MATE expression observed are not solely caused by Nrf2 pathway induction.

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Discussion

Cisplatin is a well-described nephrotoxicant, affecting the proximal tubule in particular. Here, we demonstrate that the human-derived proximal tubule epithelial cell model, ciPTEC, is sensitive to OCT2- and CTR1-mediated cisplatin-induced toxicity. Expression of OAT1 and OAT3 reduced ciPTEC sensitivity to cisplatin, which is independent of OAT transport activity, and is associated with a trend towards reduced intracellular cisplatin accumulation, explained by reduced OCT2-mediated uptake capacity.

The mechanism responsible for reduced cisplatin sensitivity in ciPTEC-OAT1 and ciPTEC-OAT3 involves reduced OCT2-dependent influx of the drug. ASP^+ is a model substrate for OCT2 and was used to evaluate OCT2 transport capacity, which was decreased in ciPTEC expressing OAT. We can only speculate on how this is regulated, but this could involve the Nrf2 pathway, as Nrf2-null mice developed more extensive nephrotoxicity after cisplatin treatment compared to wild types (Aleksunes et al., 2010). Nrf2-mediated signalling reduces expression of OCT2 in rat cortical tissue and MDCK cells, and increases expression of MATE1 in primary human proximal tubule cells, reducing renal cisplatin accumulation and toxicity (Shu et al., 2001; Atilano-Roque et al., 2016; Huang et al., 2017). In ciPTEC-parent, the involvement of Nrf2 in down-regulation of OCT2 and upregulation of MATE1 could be confirmed supporting previous *in vivo* and *in vitro* studies (Aleksunes et al., 2010; Atilano-Roque et al., 2016). Remarkably, we found a profound increase of MATE2-k gene expression, while the effect for MATE1 was less pronounced. Regulation of MATE2-k via Nrf2 was recently published when proximal tubule cells were exposed to flow (Fukuda et al., 2017). With respect to cisplatin-induced toxicity, OAT expression affected OCT2 and MATE1/2-k, for which the direct involvement of Nrf2 remains elusive as established target genes of this pathway were not consistently regulated. Therefore, the protective effect on cisplatin toxicity via bardoxolone-mediated Nrf-2 stimulation was not tested in the current study focusing on the differential effect of OAT expression. Alternatively, tyrosine kinase inhibitors reduced OCT2 activity and oxaliplatin-induced acute neuropathy *in vivo*, suggesting a role for tyrosine phosphorylation in regulation of OCT2. (Sprowl et al., 2016). It is important to note that cisplatin sensitivity and accumulation were reduced most pronounced for ciPTEC-OAT3, while ASP^+ accumulation was reduced mostly in ciPTEC-OAT1. This suggests that additional, non-elucidated cell

protecting mechanisms in parallel of the Nrf2 pathway or tyrosine kinase activity might play a role in the protection against cisplatin toxicity.

In addition to drug transport function, OAT1 and OAT3 are associated with several metabolite pathways, including fatty acid, amino acid, nucleic acid, glucose, pyruvate and glutathione metabolism (Ahn et al., 2011; Wu et al., 2013). As a result, a hypothesis was postulated on the role of drug transporters in detecting and maintaining levels of endogenous metabolites, antioxidants, signalling molecules, hormones and nutrients (Nigam, 2015; Bush et al., 2017). OAT-mediated transport is bidirectional and therefore its activity influences the intracellular concentration of counter-transported compounds, including metabolites of the citric acid cycle, predominantly alpha-ketoglutarate (Burckhardt and Burckhardt, 2003; Sweet et al., 2003; Kaufhold et al., 2011). Reduced or competitively inhibited OAT function might therefore reduce energy metabolism, illustrated by reduced active transport of para-aminohippuric acid upon inhibition of mitochondrial ATP production (Nagai et al., 1998). This, in turn, may affect drug-induced toxicity response in proximal tubules by pre-disposing redox state and supplementing or depleting the energy pool. Since reactive oxygen species (ROS) are key players in cisplatin-induced nephrotoxicity, the proximal tubule redox state and cellular content of the ROS scavenger glutathione are highly important for sensitivity to drug-induced toxicity (Wang and Lippard, 2005; Manohar and Leung, 2017). No significant differences in total glutathione content were observed among the ciPTEC cell lines, suggesting that redox metabolism is not directly affected by (lack of) OAT function in ciPTEC. The expression of OAT could have influenced the intracellular proton gradient thereby decreasing the membrane potential which drives transport by OCTs (Okuda et al., 1999; Budiman et al., 2000). This might explain the increased K_m values for ASP^+ accumulation in ciPTEC-OAT1 and ciPTEC-OAT3 as observed in the present study. Metabolomic analysis of compounds involved in energy production, especially of the citric acid cycle, may generate a more profound understanding of the role of OAT transporters in cellular homeostasis and drug sensitivity.

The present findings contradict previously reported publications, involving *in vivo* studies. Jacobs *et al.* suggested that cisplatin co-treatment with probenecid competitively inhibited OAT-mediated transport, thereby reducing the renal clearance of cisplatin and protecting patients from nephrotoxicity, even at dose-escalation (Jacobs et al., 1984; Jacobs et al., 1991). In line with this, reduced cisplatin excretion

and attenuated nephrotoxicity were observed in cisplatin-treated Oat1 knockout mice, and probenecid co-treated wild-type mice (Hu et al., 2017). The authors of the latter study suggested that cisplatin is converted into a highly reactive mercapturic acid metabolite, which is a substrate for OAT1 and OAT3, similar as described for cadmium and mercury (Zalups, 2000b; Zalups, 2000a; Cannon et al., 2001). However, the first conversion step involves extra-renal conjugation of cisplatin to glutathione, generating a water soluble metabolite filtered by the glomerulus. Proximal tubule cells are therefore mainly apically exposed to the conjugated metabolite, suggesting limited involvement of basolaterally located OAT1 and OAT3. This indicates that current *in vitro* models underestimate the complexity of drug-induced renal injury. To account for the contribution of metabolism, our *in vitro* model could be extended to include pre-exposure of cisplatin to cultured hepatocytes or precision-cut liver slices (Starokozhko et al., 2017). In contrast to studies in which OAT function contributes to nephrotoxicity, the beneficial effects of OAT-mediated transport on renal function have also been described. Ischemia reperfusion resulted in severe kidney injury in rats, which was accompanied by reduced expression of Oat1 and Oat3. Indomethacin prevented ischemia-induced downregulation of Oat1 and Oat3, while probenecid abolished indomethacin-mediated attenuation (Schneider et al., 2015). As cisplatin-induced proximal tubule toxicity can induce a similar decrease of Oat1 and Oat3 expression (Liu et al., 2012),. investigating indomethacin-mediated regulation of OAT1 and OAT3, might provide an interesting mechanism that can explain reduced renal adverse effects.

Most *in vitro* models applied in drug-induced toxicity prediction lack human- and tissue-relevant expression of transporters and metabolizing enzymes (Shaw et al., 2002; Chu et al., 2013; Van der Hauwaert et al., 2014; Wilmer et al., 2016). The proximal tubule model used here circumvents these issues as it demonstrates a tissue-specific expression profile in a human-derived stable cell line. To enable drug-induced toxicity evaluation of compounds that require influx by OAT transporters, OAT1 or OAT3 were re-introduced by lentiviral transfection of parent ciPTEC, generating ciPTEC-OAT1 and ciPTEC-OAT3 (Nieskens et al., 2016). As this method may have induced genetic aberrations, a similar transduction process was performed with eYFP, to show that the transfection procedure did not significantly affect cisplatin sensitivity.

Functional MATE-mediated transport was lacking in our experimental conditions, illustrated by the inability of pyrimethamine to modulate ASP^+ and cisplatin accumulation, and subsequent cisplatin-induced toxicity. This may be the result of limited polarization of ciPTEC under the current culture conditions, although activity of OCT2, OAT1 and OAT3 which are exclusively expressed at the basolateral membrane *in vivo*, was demonstrated. Although pyrimethamine is offered extracellularly, which may compromise inhibition of MATE-mediated cisplatin extrusion, , MATE protein levels were below the limit of detection with Western blotting in ciPTEC (data not shown). Therefore, MATE transport function was not studied further in ciPTEC. Additional experiments using a polarized culture system could be used to enhance the expression levels and possibly transport activity of MATE1 and MATE2-k.

ASP^+ and cisplatin have been described as MATE1-substrates *in vitro* and MATE1 has been shown to mediate efflux of cisplatin in rodents *in vivo* (Yonezawa et al., 2006; Ito et al., 2010; Nakamura et al., 2010; Wittwer et al., 2013). The driving force for MATE-mediated transport is a counter-directed gradient of H^+ (Tsuda et al., 2007). Transport of cisplatin was demonstrated upon intracellular acidification, resulting in a non-physiological outward-directed proton gradient and MATE-mediated influx (Nakamura et al., 2010). All experiments in ciPTEC were performed at a buffered pH of 7.4, thus lacking driving force for MATE. Important to consider is the limited cationic charge of cisplatin in buffers that contain a physiological chloride concentration, reflecting blood plasma, while it is twofold positively charged when exposed to low chloride, reflecting the cytoplasm (Andersson and Ehrsson, 1995; Jennerwein and Andrews, 1995). Apparent transport affinities might be different for the neutral or charged form, possibly affecting accumulation of the drug and further increasing the mechanistic complexity of cisplatin-induced toxicity. In short, the *in vivo* contribution of MATE-mediated efflux to cisplatin-induced nephrotoxicity in humans remains to be elucidated. Evaluating cisplatin-induced toxicity in bi-compartmental Transwell® or 3D culture models that allow for mimicking of the pH gradient as experienced in the physiological proximal tubule, may help to elucidate the involvement of MATE in this process.

In conclusion, we demonstrated that expression of OAT1 or OAT3 in a human-derived proximal tubule epithelial cell model resulted in reduced sensitivity to cisplatin. This was independent of OAT transport

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function and most likely explained by a reduced OCT2-mediated influx capacity. As organic anion transporters influence sensitivity to drugs that are typically handled by organic cation transporters, reliable *in vitro* drug-induced nephrotoxicity evaluation requires simultaneous expression of basolateral solute carrier transport proteins.

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Authorship contributions

Participated in research design: Nieskens, Peters, Van Asbeck, Russel, Masereeuw, Wilmer

Conducted experiments: Nieskens, Peters, Dabaghia, Korte, Jansen, Van Asbeck, Tavraz

Contributed new reagents or analytic tools: Peters, Friedrich, Tavraz

Performed data analysis: Nieskens, Dabaghia, Korte, Jansen, Van Asbeck

Wrote or contributed to the writing of the manuscript: Nieskens, Jansen, Dabaghie, Van Asbeck, Friedrich, Russel, Masereeuw, Wilmer

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Footnotes

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[b]Meeting abstracts where the work was previously presented:

The manuscript as presented here has not been previously submitted or presented

[c]Reprint requests can be directed to:

No reprints requested

Legends for Figures

Figure 1: Cisplatin sensitivity in ciPTEC is reduced by OAT1 and OAT3 expression, and is dependent on OCT2- and CTR1-mediated transport. (A) Time and dose- dependent decrease in viability of ciPTEC-parent following exposure to cisplatin (8.7-222 µM) for 24, 48 or 72 h as determined by MTT assay (mean±S.E.M., n=3). (B) Decreased viability of ciPTEC-parent upon cisplatin exposure could be partially restored by competitive inhibition of OCT2 (cimetidine;1.25 mM) or CTR1 ($CuSO_4$; 1.25 mM) for 24 h, determined by MTT assay (mean±S.E.M., n=3, *p<0.05, **p<0.01; compared to control by Student's t-test). (C) Reduced sensitivity of ciPTEC-OAT1 and ciPTEC-OAT3 following exposure to cisplatin (8.7-222 µM) for 24 h compared to ciPTEC-parent, as determined by MTT assay (mean±S.E.M., n=3). Results are normalized to unexposed ciPTEC.

Figure 2: Decreased intracellular cisplatin accumulation and OCT2 gene expression in ciPTEC-OAT1 and ciPTEC-OAT3. (A) Whole-cell cisplatin content is decreased in ciPTEC-OAT1 and ciPTEC-OAT3 compared to ciPTEC-parent, following exposure to cisplatin (250 µM) for 90 min. Results are normalized to ciPTEC-parent (mean±S.E.M., n=3, performed with 2 experimental replicates, no significant differences were found by one-way ANOVA). (B) Gene expression of OCT2 (*SLC22A2*) is decreased, while gene expression of MATE1 (*SLC47A1*) and MATE2-k (*SLC47A2*) is increased in ciPTEC-OAT1 and ciPTEC-OAT3 compared to ciPTEC-parent, using *GAPDH* as reference gene (mean±S.E.M., n=3, performed with 1 experimental replicate, *p<0.05, ***p<0.001; compared to ciPTEC-parent by one-way ANOVA). (C) Whole-cell cisplatin content is decreased by co-exposure to OCT2-substrate cimetidine (1.25 mM), but is not affected by co-exposure to MATE-substrate pyrimethamine (100 nM) during exposure to cisplatin for 90 min in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3. Results are normalized to control without substrate (mean±S.E.M., n=3, performed with 2 experimental replicates, **p<0.01; compared to control by Student's t-test).

Figure 3: Reduced ASP^+ accumulation reflects attenuated OCT2 transport capacity in ciPTEC-OAT1 and ciPTEC-OAT3. (A) ASP^+ (0.8-50 µM) accumulation for 60 min is decreased in ciPTEC-OAT1 and ciPTEC-OAT3 compared to ciPTEC-parent, evaluated by intracellular fluorescence intensity after 60 min (mean±S.E.M., n=3). (B, C, D) ASP^+ (3.1 µM) accumulation for 60 min in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 is reduced by (B) competitive inhibition of OCT (cimetidine,1.25 mM), but not affected by competitive inhibition of (C) MATE (pyrimethamine,10 and 100 nM) or (D) OAT (probenecid, 100 µM), evaluated by intracellular fluorescence intensity. Results are normalized to unexposed ciPTEC-parent (mean±S.E.M., n=3, *p<0.05, **p<0.01, ***p<0.001; compared to control by Student's t-test). (E) ASP^+ (3.1 µM) accumulation in for 60 min in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 is not affected by 7-day competitive OAT inhibition (probenecid,100 µM), evaluated by intracellular fluorescence intensity. Results are normalized to unexposed ciPTEC-parent (mean±S.E.M., n=2, no significant differences were found for treatment by Student's t-test).

Figure 4: Nrf2 pathway activation by bardoxolone in ciPTEC-parent is associated with cisplatin transporter regulation, but is not increased in ciPTEC-OAT1 and -OAT3, compared to parent ciPTEC. (A) Gene expression of Nrf2 target genes *NQO1* and *GCLC* is increased after 24 h pre-exposure to bardoxolone (50 nM), which is associated with decreased OCT2 (*SLC22A2*) gene expression, while gene expression of MATE1 (*SLC47A1*) and MATE2-k (*SLC47A2*) is increased in parent

ciPTEC, using *GAPDH* as reference gene (mean±S.E.M., n=3, performed with 1 experimental replicate, *p<0.05, **p<0.01, ***p<0.001; compared to control by Student's t-test). (B) Gene expression of Nrf2 target genes *NQO1* and *GCLC* is not consistently increased in ciPTEC-OAT1 and ciPTEC-OAT3, compared to ciPTEC-parent, using *GAPDH* as reference gene (mean±S.E.M., n=3, performed with 1 experimental replicate, ***p<0.001; compared to ciPTEC-parent by one-way ANOVA).

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Tables

Table 1: Cisplatin-induced toxicity is independent of OAT-mediated transport in ciPTEC-OAT1 and ciPTEC-OAT3. TC_{50} values (represented in µM) calculated from MTT viability assays following cisplatin (8.7-222 µM) exposure for 24 h are not affected by 24 h (n=3) and 7 day (n=6) competitive inhibition of OAT (probenecid, 100 µM; diclofenac, 50 µM) in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 (mean TC_{50}±S.E.M., *p<0.05, ***p<0.001; compared to ciPTEC-parent, no significant differences were found for treatments by two-way ANOVA, N.D. is not determined).

	co-exposure (24 h)		**co- and pre-exposure (7 days)**	
	-	+	-	+
ciPTEC-parent				
probenecid	33 ± 3	29 ± 4	47 ± 4	45 ± 3
diclofenac	34 ± 1	34 ± 1	N.D.	N.D.
ciPTEC-OAT1				
probenecid	53 ± 2*	49 ± 3	45 ± 6	45 ± 6
diclofenac	45 ± 6	45 ± 4	N.D.	N.D.
ciPTEC-OAT3				
probenecid	92 ± 8***	93 ± 7***	80 ± 2***	76 ± 6***
diclofenac	64 ± 11*	62 ± 13*	N.D.	N.D.

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Table 2: Cisplatin-induced toxicity is independent of MATE-mediated transport in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3. TC_{50} values (represented in µM) calculated from MTT viability assays following cisplatin (8.7-222 µM) exposure for 24 h are not affected by competitive MATE inhibition (pyrimethamine, 10 and 100 nM) in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 (mean TC_{50}, n=2).

	control	**pyrimethamine**	
		10 nM	**100 nM**
ciPTEC-parent	41	45	46
ciPTEC-OAT1	59	57	57
ciPTEC-OAT3	103	111	136

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Table 3: ASP^+ accumulation is decreased in ciPTEC-OAT1 and ciPTEC-OAT3. K_m (represented in µM) and V_{max} values (represented in min^{-1}) calculated from Michaelis-Menten fitted ASP^+ accumulation assays in ciPTEC-parent, ciPTEC-OAT1 and ciPTEC-OAT3 (mean±S.E.M., n=3, *p<0.05, **p<0.01, ***p<0.001; compared to ciPTEC-parent by one-way ANOVA).

	K_m	V_{max}
ciPTEC-parent	2.3 ± 0.3	23.5 ± 0.1
ciPTEC-OAT1	4.7 ± 0.4*	13.1 ± 0.3***
ciPTEC-OAT3	6.2 ± 0.3***	21.6 ± 0.6**

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Figures

Figure 1

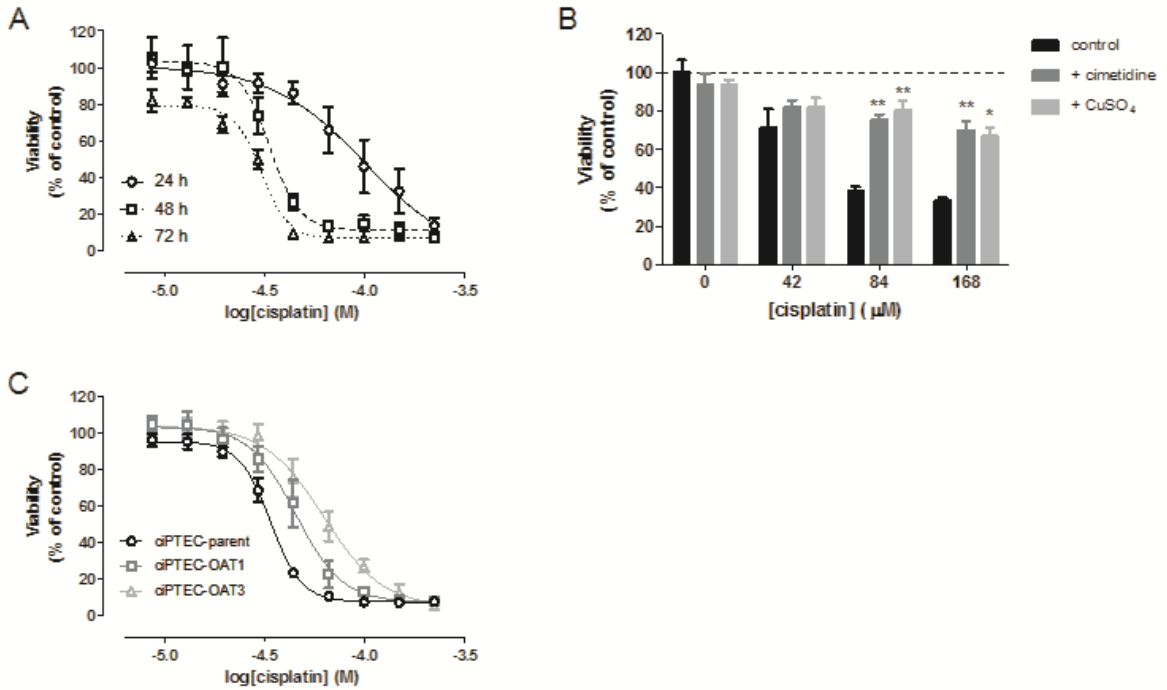

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Figure 2

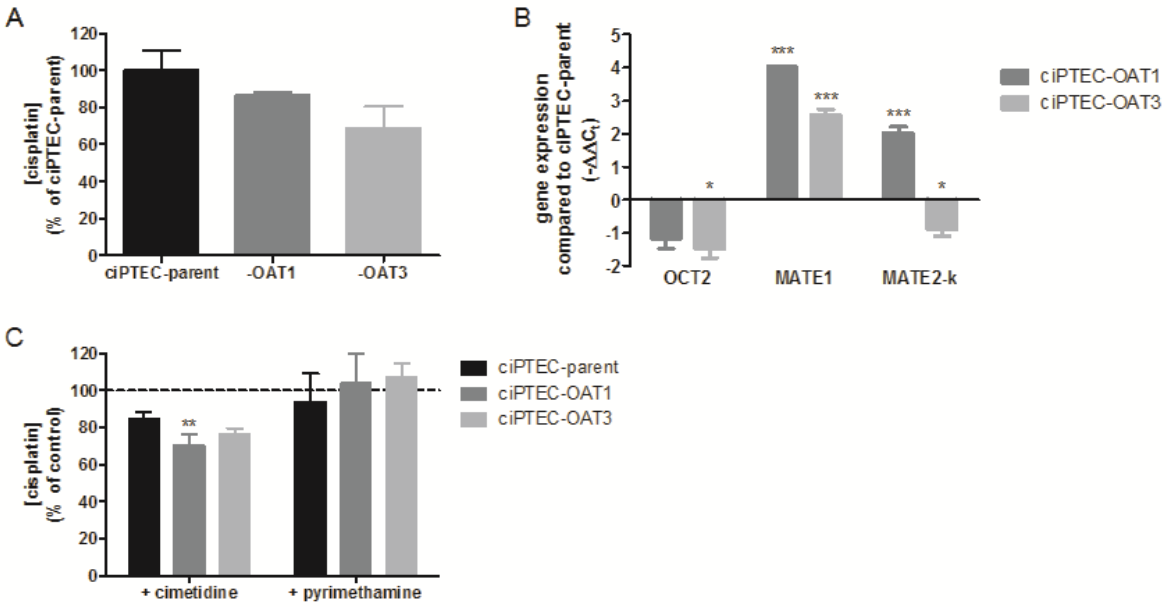

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Figure 3

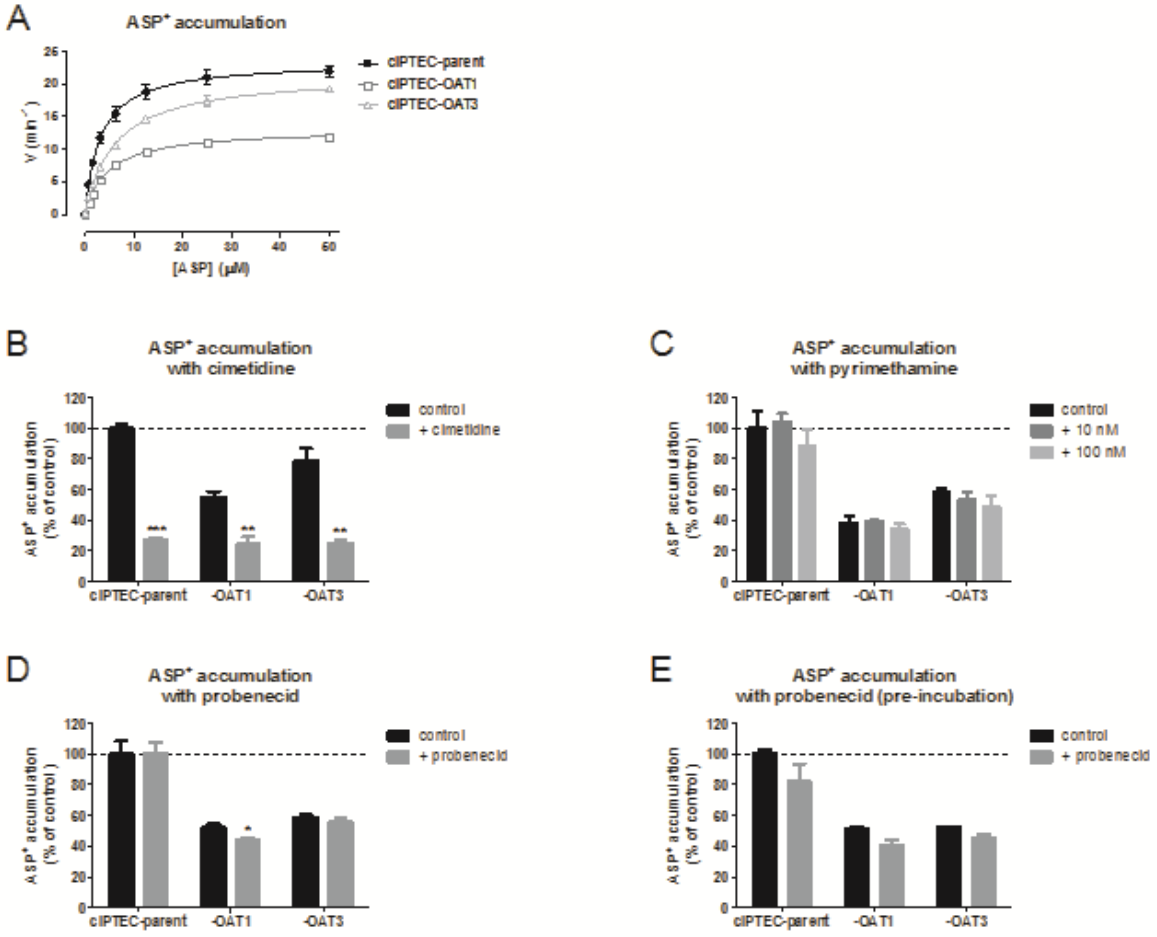

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Figure 4