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New Clues for Nephrotoxicity Induced by Ifosfamide: Preferential Renal Uptake via the Human Organic Cation Transporter 2

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Abstract: Anticancer treatment with ifosfamide but not with its structural isomer cyclophosphamide is associated with development of renal Fanconi syndrome leading to diminished growth in children and bone problems in adults. Since both cytotoxics share the same principal metabolites, we investigated whether a specific renal uptake of ifosfamide is the basis for this differential effect. First we studied the interaction of these cytotoxics using cells transfected with organic anion or cation transporters and freshly isolated murine and human proximal tubules with appropriate tracers. Next we determined changes in membrane voltage in proximal tubular cells to understand their differentiated nephrotoxicity. Ifosfamide but not cyclophosphamide was significantly transported into cells expressing human organic cation transporter 2 (hOCT2) while both did not interact with organic anion transporters. This points toward a specific interaction of ifosfamide with hOCT2, which is the main OCT isoform in human kidney. In isolated human proximal tubules ifosfamide also interacted with organic cation transport. This interaction was also seen in isolated mouse proximal tubules; however, it was absent in tubules from OCT-deficient mice, illustrating the biological importance of this selective transport. Ifosfamide decreased the viability of cells expressing hOCT2, but not that of control cells. Coadministration of cimetidine, a known competitive substrate of hOCT2, completely prevented this ifosfamide-induced toxicity. Finally, ifosfamide but not cyclophosphamide depolarized proximal tubular cells. We propose that the nephrotoxicity of ifosfamide is due to its selective uptake by hOCT2 into renal proximal tubular cells, and that coadministration of cimetidine may be used to prevent ifosfamide-induced nephrotoxicity.

Keywords: Oxazaphosphorine; Fanconi syndrome; nephrotoxicity; isolated proximal tubule; human kidney

Introduction

The oxazaphosphorine prodrug cyclophosphamide (CPA) and its structural isomer ifosfamide (IFO) are widely used in cancer chemotherapy. IFO exhibits greater antitumor efficacy than CPA in some malignancies.[1]

CPA and IFO are metabolized mainly in the liver via two different cytochrome P450 mediated pathways, 4-hydroxylation and N-dechloroethylation. 4-Hydroxylation generates the active alkylating species (ifosfamide-mustard or phosphoramide mustard) and the urotoxic byproduct acrolein (ACR), while N-dechloroethylation gives origin to presumed

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inactive metabolites and the presumed toxic byproduct chloroacetaldehyde (CAA).[2] In clinical practice, side effects from ACR (hemorrhagic cystitis) are managed by coadministration of 2-mercaptoethanesulfonic acid (MESNA), which chemically inactivates ACR.[3]

The use of IFO is limited by serious side effects like renal Fanconi syndrome,[4,5] which has never been associated with administration of CPA.[6] Renal Fanconi syndrome is caused by a generalized dysfunction of renal proximal tubular (PT) cells, and is defined clinically by excessive urinary excretion of glucose, amino acids, phosphate, bicarbonate and small molecular weight proteins. Some authors have suggested that CAA is responsible for IFO nephrotoxicity.[7,8] However, CAA is a metabolite of both IFO and CPA. Therefore, to explain the selective nephrotoxicity of IFO, a higher formation of CAA from IFO than from CPA has been hypothesized. Indeed, a higher CAA formation after treatment with IFO than after treatment with CPA has been observed in patients.[1] However, since this difference in metabolism is merely a quantitative difference, at least some proximal tubular damage should be expected following high doses of CPA, which has not been observed.[9,10] Thus, an additional factor should be critical for the development of renal Fanconi syndrome following IFO treatment.

Both IFO and CPA are highly hydrophilic and do not diffuse readily through the lipid bilayer of cells, and consequently intracellular accumulation of these drugs must be mediated by specific transport systems. Several drug transporters, such as breast cancer resistance protein (BCRP), and multidrug-resistance associated proteins (MRP1, MRP2, and MRP4), are involved in the active efflux of parenteral oxazaphosphorines, their cytotoxic mustards and conjugates from hepatocytes and tumor cells.[11] The kidneys, especially the proximal tubule, possess a transporter battery to facilitate the vectorial movement of hydrophilic substances across cell membranes. These transporters are key determinants for the pharmacokinetics and pharmacodynamics of many drugs. No specific renal uptake system for IFO or CPA has yet been identified.

It has already been shown that both IFO and CPA inhibit the luminal transport of organic cations in the renal PT cell line LLC-PK.[12] IFO and CPA have been proposed to be substrates for the organic anion (OAT) and cation (OCT) transporters.[13] The isoforms 1 and 3 of OATs[14] and the isoform 2 of OCTs[15] are predominantly expressed in the human kidney, where they are localized in the basolateral membrane of PT cells. Thus, it can be speculated that the missing factor critical to the selective nephrotoxicity of IFO is a specific interaction with a renal transporter, mediating IFO accumulation in PT cells. Since the cytochrome P450 components CYP3A4 and CYP2B6 are also present in the kidney,[16] a cell-specific IFO accumulation could cause local production of metabolites and would explain selective toxicity in the PT.

We have investigated whether there is a selective interaction of IFO or CPA with OCTs and OATs, which could play

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a role in their specific uptake in PT cells. Indeed, we provide evidence not only for interaction of but also for transport of IFO but not of CPA via OCT. Moreover, we have investigated whether IFO has a preferential interaction with PT cells by comparing the acute effects of IFO, CPA, and their major metabolites and presumed toxic derivatives on membrane voltage and conductance. These cellular parameters are important, because they are coupled to the active reabsorption of glucose, amino acids, and phosphate, which are all impaired in renal Fanconi syndrome. Finally, we propose a scientific rational for clinical measures which might be able to prevent this unwanted and detrimental side effect of IFO treatment.

Experimental Section

Cell Culture and Proximal Tubule Isolation. hOAT1, hOAT3[17] and hOCT2[18] were each stably expressed in HEK293 cells (ATCC, Rockville, MD, USA). Cells were grown at 37 °C in 50 mL cell culture flasks (Greiner, Frickenhausen, Germany) in DMEM (Biochrom, Berlin, Germany) containing 3.7 g/L $NaHCO_3$, 1.0 g/L D-glucose and 2 mM L-glutamine (Gibco BRL/Life Technologies, Eggenstein, Germany), gassed with 8% CO_2. To this medium were added 100,000 U/L penicillin, 100 mg/L streptomycin (Biochrom), 10% fetal calf serum and 0.8 mg/mL Geneticin (Gibco). Experiments were performed after 4−8 days with cells grown on glass coverslips of passages 11−42. Culture and functional analysis of cells was approved by the Landesumweltamt Nordrhein-Westfalen, Essen, Germany. LLC-PK_1 cells (kind gift of Dr. M. Mohrmann, University Freiburg, Germany) were grown (passages 182−191) as already published.[19]

S3 segments of PT of mouse or human kidney samples were isolated mechanically with fine forceps in DMEM culture medium containing 5 mM albumin at 4 °C as described before.[20,21] Tubule segments were transferred to the perfusion chamber and fixed between two glass holding pipettes for transport measurements.

Male FVB (Harlan-Winkelmann, Borchen, Germany) and OCT1/2 knockout (KO) mice (FVB 6622-M, Taconic, Ry, Denmark) 7−9 weeks old and weighing 25−30 g were used. Experiments were approved by a governmental committee on animal welfare and were performed in accordance with national animal protection guidelines.

Human kidney samples were obtained from a 60 and a 54 year old female patient undergoing tumor nephrectomy. A piece of normal kidney tissue surrounding the tumor was transferred into chilled HCO_3^--free phosphate buffer immediately after nephrectomy. The procedure was approved by the ethics commission of the Universitätsklinikum Münster, and written consent was obtained from the patients.

IFO and CPA Interaction with hOAT1 and hOAT3. For uptake measurements stably transfected HEK293 cells[17] and mock transfected control cells were cultivated in Petri dishes. After 2−3 days (confluence growing) these cells were plated into 24-well plastic dishes (Sarstedt, Nümbrecht, Germany) at a density of 2×10^5 cells per well. Transport assays were performed after 3 days in Ringer solution (in mM: NaCl 130, KCl 4, $CaCl_2$ 1, $MgSO_4$ 1, NaH_2PO_4 1, HEPES 20, D-glucose 18, pH adjusted to 7.4). The cells were washed twice with 500 μL of Ringer solution and for uptake measurements incubated for 5 min at room temperature in Ringer solution containing 10 μM *p*-[^{3}H]aminohippurate (PAH) (hOAT1-transfected cells), or 10 nM [^{3}H]estrone-sulfate (ES) (hOAT3-transfected cells). For inhibition studies, the test solutions included additionally 100 μM IFO or CPA. The incubation with radioactively labeled compounds was stopped after 5 min, and the extracellular tracer was removed by washing three times with 750 μL of ice-cold Ringer solution. The cells were dissolved in 0.5 mL of 1 N NaOH, and the ^{3}H-content was determined after neutralization of the probes with 0.5 mL of 1 N HCl by liquid scintillation counting (Canberra-Packard, Dreieich, Germany). Uptake of the tritiated substrates into nontransfected cells allowed the determination of maximal inhibition of transport.

Fluorescence Measurements with 4-(4-(Dimethylamino)-styryl)-*N*-methylpyridinium (ASP^+). As substrate for OCT in hOCT2 expressing HEK293 cells or in freshly isolated mouse or human PT, the fluorescent organic cation ASP^+ was used as already published.[22,23] In short, dynamic fluorescence microscopy with cells grown on glass or isolated mouse or human tubules was performed in the dark with an inverted microscope (Axiovert 135, Zeiss, Oberkochen, Germany) equipped with a 100× 1.45 oil immersion objective. Data acquisition and analysis were performed with Metafluor Software (Visitron Systems, Puchheim, Germany).

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Excitation light (488 nm) from a polychromator system (VisiChrome, Visitron Systems, Puchheim, Germany) was reflected to the tubules by a dichroic mirror (560 nm), and emission was detected after passing an emission filter (575−640 nm) by a Photometrics CoolSNAP$_{EZ}$ digital camera (Roper Scientific, Tucson, USA). Signal intensity of a defined and fixed region of interest across the cell monolayer or the tubule was analyzed. In all fluorescent measurements, the initial linear slope of cellular fluorescence increase was used as transport parameter.[24] As superfusion solution a HCO_3^--free, Ringer-like solution was used which contained (in mM) NaCl 145, K_2HPO_4 1.6, KH_2PO_4 0.4, D-glucose 5, calcium-gluconate 1.3, $MgCl_2$ 1, pH adjusted to 7.4.

Uptake of IFO and CPA by hOCT2-HEK293 and HEK293 Cells. To determine whether the presence of hOCT2 is important for the uptake of IFO and CPA, hOCT2-HEK293 (hOCT2) and HEK293 WT cells (kind gift of Prof. H. Koepsell, University Würzburg) were incubated for 10 min at 37 or 4 °C with 500 μM oxazaphosphorines. After incubation, cells were washed three times with ice-cold Ringer-like solution and hypoosmotic lysis was induced by addition of water. Lysates were sonicated for 10 min and centrifuged for 10 min at 13,000 rpm. For the precipitation of proteins two volumes of acetonitrile were added to one volume of cell lysate, mixed and centrifugated. Ten microliters of the supernatant was injected into a HPLC system (HP 1100 series liquid chromatograph connected to a HP 1100 MSD model G1946A mass spectrometer (Hewlett-Packard, Camas, WA, USA)) with a reversed phase column (PUROSPHER RP 18ec, 55 × 2, 3μ, Merck, Darmstadt, Germany). CPA and IFO were eluted under gradient conditions with a mobile phase consisting of eluent A (water with 0.1% formic acid) and eluent B (methanol), a flow rate of 0.4 mL/min and a column temperature of 35 °C. After injection, the column was washed for 3 min with eluent A. Within the next two min, the gradient was increased up to 60% of eluent B, was kept at that concentration for one min, and was then decreased again within one min to the initial conditions (total run time 11 min). For detection, the mass spectrometer with electrospray interface was used in the positive ion mode. As drying gas ultrapure nitrogen with a flow of 10 L/min was used at a temperature of 350 °C. The nebulizer pressure was set at 20 psi, and the electrospray needle was maintained at −4000 V. Sodium adducts of IFO and CPA were measured at *m*/*z* 261.1. The measurements were linear over an oxazaphosphorine concentration range of 0.025−2.5 μM. As both substances were measured individually, no chromatographic separation was necessary. The presence of IFO or CPA metabolites (N-dechloroethylation, 4-hydroxylation or activation products) was determined by measuring at *m*/*z* 198.6, 221.0, 275.1, 277.1 and 293.1.

Assessment of Toxicity. Cell viability was assessed by a colorimetric assay, which is based on the conversion of dissolved yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenases of living cells.[25] Thus, this MTT assay offers precise quantification of cell viability in mammalian cell cultures. WT or hOCT2 cells grown in 96-well plates were incubated 24 h at 37 °C with or without 500 μM IFO or CPA. In additional experiments, cells were incubated with 500 μM IFO + 100 μM cimetidine. At the end of the incubation period, 10 μL of MTT reagent (5 mg/mL MTT in PBS) was added to each well and incubated for further 3 h. To stop the reaction, plates were frozen for 1 h and SDS (100 μL of 0.01 M HCl in SDS 10%, 12 h, 37 °C) was used to extract the resulting formazan dye. Absorbance was measured spectrophotometrically with a sensitive microplate reader (Infinite M200, Tecan, Zürich, Switzerland) at wavelengths of 550 and 590 nm. Wells with drugs, but without cells, served for background corrections.

Patch-Clamp Experiments. Membrane voltages (V_m) and membrane conductances (g_m) were measured with the slow-whole-cell patch-clamp technique as already published.[19] This technology permits electrical access to epithelial cells without disturbing their cellular content. V_m was measured in the current clamp mode of a patch-clamp amplifier in a running bath (exchange 20/min) at 37 °C. Whole cell currents were determined before, after, and during every experimental procedure by application of voltage steps in 10 mV intervals up to ±30 mV with duration of 0.5 s (Figure 2). By means of a sinus wave of 1 kHz and 100 mV amplitude (Function Generator Voltcraft, Conrad, Hirschau, Germany), which was fed intermittently by the stimulus input of the amplifier, pipet capacitance and access conductance could be measured. g_m was calculated following Ohm's law assuming a network between these conductances. For experiments, aliquots of cytotoxics or metabolites were prepared freshly in control solution (see above).

Chemicals. IFO and CPA were obtained from Baxter Oncology (Frankfurt, Germany), CAA and ACR from Merck-Schuchardt (Hohenbrunn, Germany), ASP^+ from Molecular Probes (Leiden, Netherlands). [^{3}H]PAH (3.25 Ci/mmol) was provided by PerkinElmer Life Sciences (Boston, MA, USA), [^{3}H]ES (ES, ammonium salt, [6,7-^{3}H(N)], 43.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). All other substances and standard chemicals were from Sigma or Merck (Darmstadt, Germany).

Statistical Analysis. Data are presented as mean values ± SEM, with *n* referring to the number of experiments or monolayers. In electrophysiological experiments, mean val-

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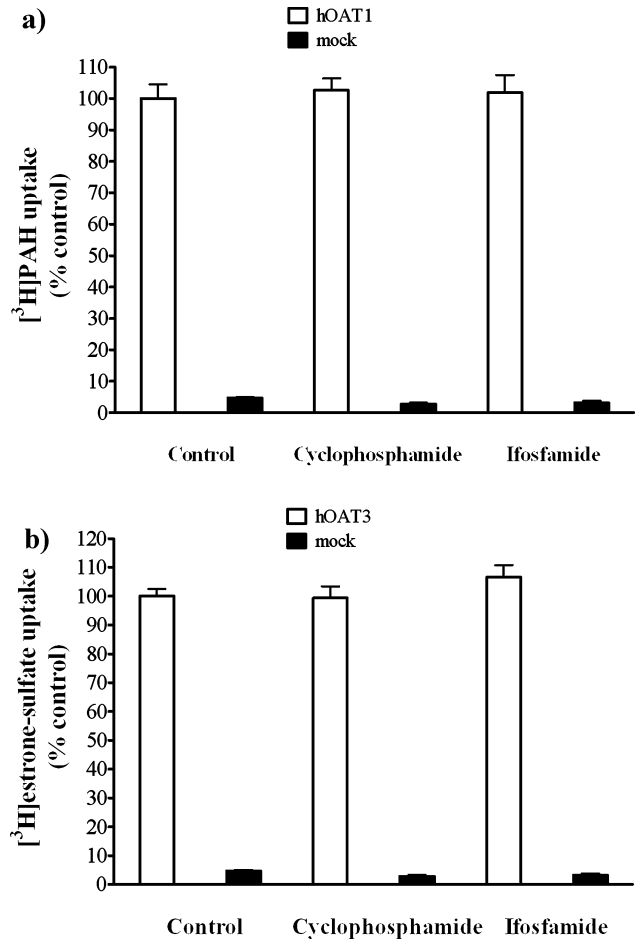

Figure 1. Summary of [^{3}H]PAH (panel a) and [^{3}H]estrone-sulfate (panel b) uptake, which is not inhibited by 100 μM IFO or 100 μM CPA in hOAT1 (panel a) and hOAT3 (panel b) cells, respectively. Data points are means ± SEM of 3 experiments with quadruple determinations each. Black columns represent results obtained with mock cells. Note no effect of IFO or CPA on organic anion transport.

ues were compared with the mean of pre- and postcontrol values within one experimental series; for irreversible effects comparison was made to the precontrol. IC_{50} values were obtained by sigmoidal curve fitting using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA). Unpaired/paired two-sided Student's *t*-test or ANOVA with Tukey post-test were used where appropriate to prove statistical significance of the effects. A P value <0.05 was considered statistically significant.

Results

IFO and CPA Interaction with hOAT1 and hOAT3. To test if IFO and CPA interact with hOAT1, the *cis*-inhibitory effect of 100 μM oxazaphosphorine on the uptake of *p*-[^{3}H]aminohippurate (PAH) by hOAT1 expressing cells was studied. hOAT1 cells showed a significantly higher PAH uptake than mock cells (Figure 1a). PAH uptake by hOAT1 in the presence of 100 μM IFO (102 ± 6%, n = 3) or of 100 μM CPA (103 ± 4%, n = 3) was not different from controls (100 ± 5%, n = 3) (Figure 1a). Similar experiments were performed with hOAT3, which showed a significantly higher [^{3}H]estrone-sulfate (ES) uptake than mock cells (Figure 1b). ES uptake by hOAT3 in the presence of 100 μM IFO (107 ± 4%, n = 3) or of 100 μM CPA (99 ± 4%, n = 3) was not different from controls (100 ± 3%, n = 3) (Figure 1b). These data show that there is no significant interaction of oxazaphosphorines with either hOAT1 or hOAT3.

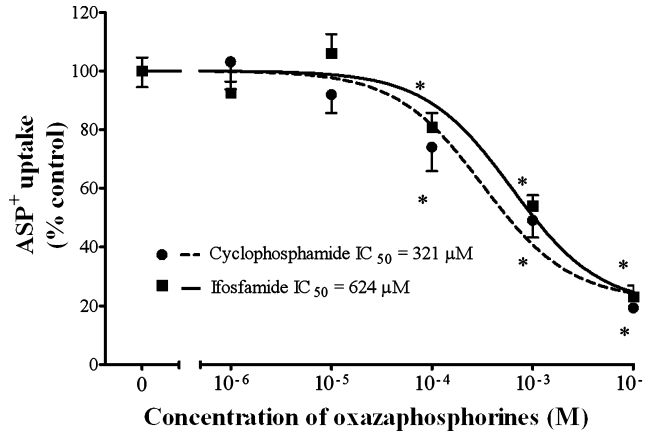

Figure 2. Concentration−response curves for the inhibition of initial ASP^+ uptake by IFO (N = 10−27) and CPA (N = 7−22) in hOCT2-HEK293 cells. Values are mean ± SEM expressed as % change of ASP^+ uptake in the absence of oxazaphosphorines. IC_{50} values determined from these curves were not significantly different. Asterisks indicate significant effects. Note an interaction of IFO as well as of CPA with hOCT2.

IFO and CPA Interaction with hOCT2. In contrast to what has been observed with OATs, both IFO and CPA at a concentration of 100 μM significantly inhibited the uptake of the fluorescent organic cation ASP^+ in hOCT2 expressing cells by 19 ± 5%, n = 10 and 26 ± 8%, n = 10 (Figure 2), respectively. For this reason, the concentration dependence of these oxazaphosphorines on ASP^+ uptake was further studied. As shown in Figure 2, both IFO and CPA inhibited ASP^+ uptake by hOCT2 with comparable IC_{50} values (624 and 321 μM, respectively). Even though these data clearly demonstrate that both oxazaphosphorines interact with ASP^+ transport, they do not show whether these substances are also substrates for hOCT2.

Uptake of IFO and CPA by hOCT2 and Wild Type (WT) Cells. The uptake of IFO by hOCT2 expressing cells (0.94 ± 0.25 fmol/cell, n = 5) was significantly higher than by WT cells (0.29 ± 0.03 fmol/cell, n = 5) (Figure 3a). Lowering the temperature to 4 °C resulted in a significant reduction of IFO uptake (0.31 ± 0.03 fmol/cell, n = 5) by hOCT2 to values similar to those in WT cells (Figure 3a). The lower temperature showed no effect on the IFO accumulation in WT cells (0.29 ± 0.07 fmol/cell, n = 4), confirming the unspecific nature of its uptake in the absence of hOCT2. The uptake of CPA by hOCT2 (0.54 ± 0.06 fmol/cell, n = 9) was not different from that in WT (0.53 ± 0.07

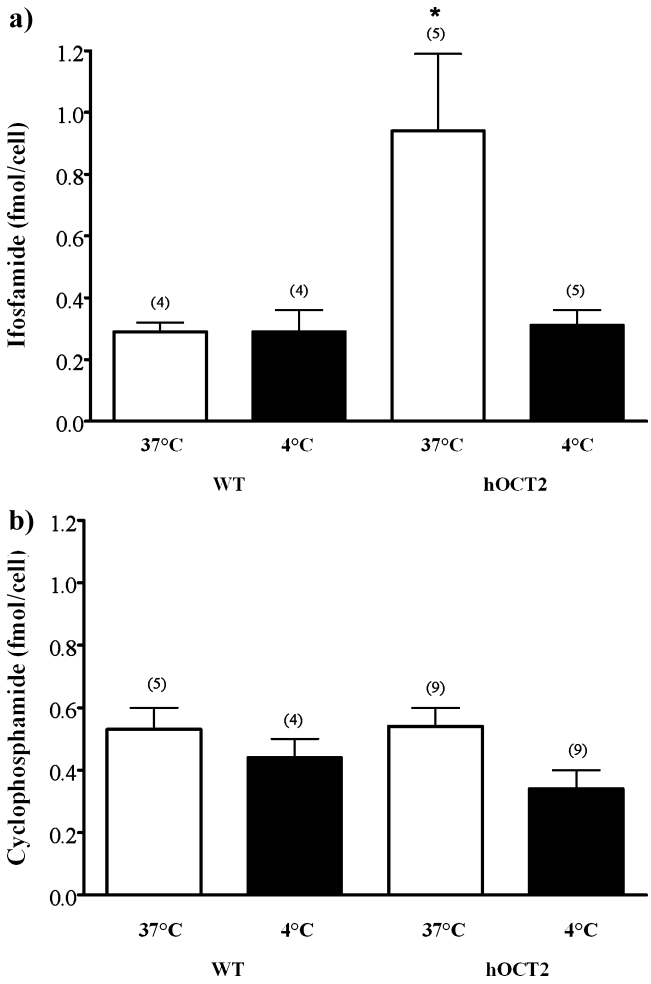

Figure 3. Summary of IFO (panel a), or CPA (panel b) accumulation in wild type (WT) and hOCT2 expressing cells after 10 min incubation with 500 μM oxazaphosphorine. Experiments performed at 4 °C are also shown (black columns). Values are mean ± SEM expressed as fmol of IFO or CPA per cell. * indicates a statistically significant effect. Above the columns is the number of observations. Note significant intracellular accumulation of IFO in hOCT2 transfected cells only.

fmol/cell, $n = 6$) cells or after lowering the temperature (4 °C) (0.34 ± 0.06 fmol/cell, $n = 9$ and 0.44 ± 0.06 fmol/cell $n = 6$ in hOCT2 and WT cells, respectively), confirming its unspecific nature (Figure 3b). No metabolites of IFO or CPA could be detected by mass spectrometry. These data prove a specific hOCT2 mediated uptake of IFO while CPA is not transported by hOCT2.

Toxicity of IFO and CPA. The dehydrogenase activity test was used to test the toxic effects of 500 μM oxazaphosphorines in WT and hOCT2 expressing cells. Figure 4 shows the results of these experiments as decrease of dehydrogenase activity after 24 h of incubation. Only incubation of hOCT2 cells with 500 μM IFO caused a significant decrease of dehydrogenase activity (−10.6 ± 1.5%, $n = 8$), which could totally be suppressed by contemporaneous incubation with 100 μM cimetidine (2.0 ± 2.2%, $n = 4$) as specific hOCT2

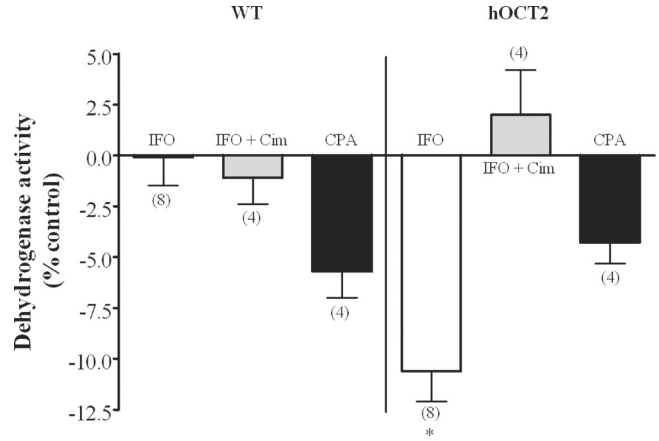

Figure 4. Summary of effects of 24 h incubation with 500 μM IFO or CPA on dehydrogenase activity in wild type (WT) and hOCT2 expressing cells. Experiments performed in the presence of 100 μM cimetidine (Cim) are also shown. Values are mean ± SEM expressed as percentage of control cells incubated for the same time without oxazaphosphorines. * indicates a statistically significant effect. Above the columns is the number of observations. Note significant toxicity of IFO in hOCT2 transfected cells only and abrogation of IFO cytotoxicity by coincubation with cimetidine.

substrate. These data demonstrate that IFO uptake into cells exerts a toxic component within 24 h. Moreover, these detrimental effects could be prevented by coincubation with a competitor for transport by hOCT2, i.e. cimetidine.

IFO Interaction with OCT Transport in Freshly Isolated Mouse and Human Proximal Tubules. IFO significantly reduced ASP^+ uptake across the basolateral membrane of freshly isolated S3 segments of mouse PT by OCT with a surprisingly low IC_{50} value of 2.5 μM (Figure 5). This effect was completely absent in PT of knockout mice deficient of OCT1 and 2. In freshly isolated human PT (S3 segments) 1 mM IFO led to a significant reduction of ASP^+ uptake by 41.4 ± 9.4%, $n = 17$ of two kidney samples, which compares well to the 55% inhibition of ASP^+ uptake by hOCT2 expressed in cells with 1 mM IFO. ASP^+ uptake in human PT was inhibited by 5 mM TEA^+ by 70.4 ± 9.4%, $n = 17$, indicating the specific contribution of OCT2 to ASP^+ uptake in these S3 segments.

IFO and CPA Effects on Membrane Voltage (V_m) and Membrane Conductances (g_m) in LLC-PK1 Cells. To our surprise, IFO caused a significant membrane depolarization of 4 ± 1 mV ($n = 21$), while CPA showed no significant effect on V_m ($n = 14$). Figure 6 shows an original patch-clamp trace documenting these effects of IFO or CPA. Figure 7a shows the summary of effects of IFO and CPA and of their main metabolites CAA and ACR, all at a concentration of 1 mM, on V_m. Plasma concentrations of 0.85 and 0.8 mM have been described in patients treated with CPA[26−29] and IFO,[30,31] respectively. The concentrations of CAA and ACR used are well above accepted physiological values and were chosen for direct comparison to IFO and CPA effects. In human, about 10% of a given dose will be

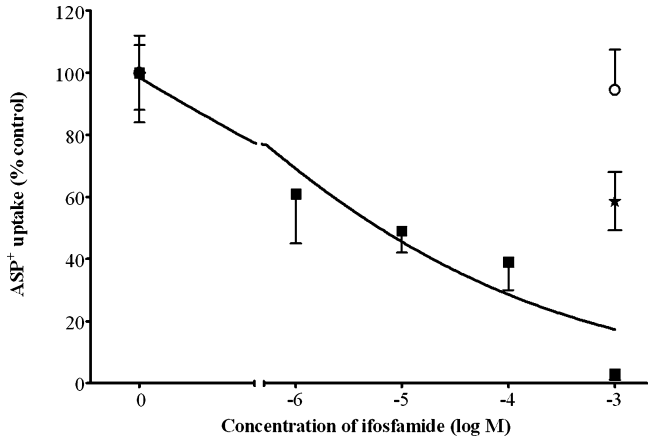

Figure 5. Concentration−response curves for the inhibition of initial ASP^+ uptake by IFO in freshly isolated mouse proximal tubules (S3 segments) of wild type mice (black squares, solid line, $N = 10-16$). In addition the effects of 1 mM ifosfamide on freshly isolated proximal tubules of OCT1/2 deficient mice (open circles, $N = 21$) and of human kidneys (black stars, $N = 17$) are included. Values are mean ± SEM expressed as % change of ASP^+ uptake in the absence of oxazaphosphorines. IC_{50} value determined from this curve for mouse proximal tubules was 2.5 μM.

metabolized to CAA and about 1% to cytotoxic active metabolites including ACR.[3]

CAA and ACR caused a partially reversible and an irreversible membrane depolarization of 5 ± 1 ($n = 16$) and 13 ± 3 ($n = 14$) mV, respectively. These depolarizations were immediate and slow. When the effect was reversible, the depolarization was also slow. Depolarization was always accompanied by a decrease in g_m. Figure 7b shows the effects of IFO, CPA, CAA and ACR, all at a concentration of 1 mM, on g_m. IFO caused a significant reversible decrease of g_m ($-9 \pm 4\%$, $n = 18$) while CPA showed no significant effect ($n = 13$). A similar effect was observed with CAA and ACR, which caused a reversible and an irreversible decrease of g_m ($-18 \pm 7\%$, $n = 11$ and $-22 \pm 4\%$, $n = 7$), respectively. These data point to a unique ability of IFO to depolarize LLC-PK1 cells by decreasing g_m. An example of IFO's ability to depolarize V_m of a proximal tubular cell and inhibit g_m can be seen in Figure 6. Matching the clinical observation of CPA treatment with no influence on PT function, CPA had no effect on V_m or g_m (Figure 6).

Discussion

Cyclophosphamide (CPA) is the most widely used alkylating agent in the treatment for hematological malignancies and a variety of solid tumors. Ifosfamide (IFO) is said to be more effective in a wide range of malignant diseases.[32] IFO and CPA have a wide variety of toxicities. However, in contrast to CPA treatment, IFO treatment is associated uniquely with nephrotoxicity and the development of a renal Fanconi syndrome.[10,33] A basic tenet in the literature is that both IFO and CPA are biologically inactive prodrugs that become active after metabolism in the liver and possibly also in the kidney.[11,34]

As only IFO but not CPA exerts nephrotoxicity in humans, predominantly interfering with PT function, we have tested the possible interaction of these oxazaphosphorines with hOAT1, hOAT3 and hOCT2. In this work, we identified hOCT2 as the specific transport system which is responsible for IFO uptake by the human PT. In line with the lack of nephrotoxicity of CPA in humans, CPA was not transported by hOCT2. Furthermore we could exclude OATs as additional uptake mechanisms for these oxazaphosphorines.

At a concentration of 100 μM, both IFO and CPA showed no interaction with hOAT1 and hOAT3. The same concentrations caused a significant inhibition of hOCT2. IFO and CPA inhibited the hOCT2 with a similar power, as evident from the comparison of their IC_{50} values. However, inhibition of ASP^+ uptake by hOCT2 does not necessarily imply translocation by the transporter. Our experiments measuring IFO and CPA in lysates of cells incubated with these oxazaphosphorines proved that IFO uptake only is mediated via hOCT2, in contrast to CPA. The uptake of IFO by WT cells was of unspecific nature, because it was not different from that in hOCT2 cells at 4 °C. At this low temperature, specific carrier-mediated transport processes are mostly inhibited. Since uptake of CPA was not temperature-

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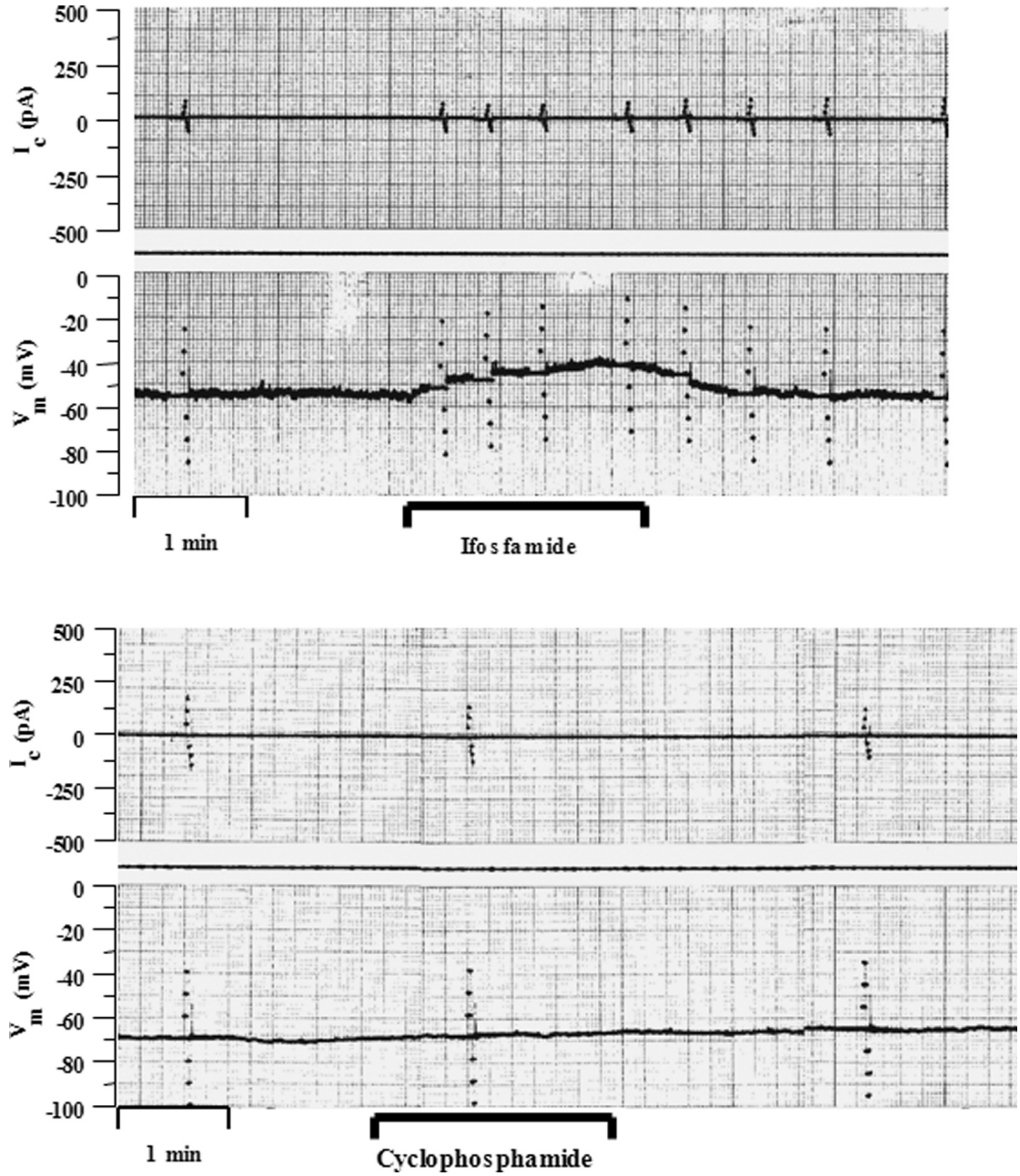

Figure 6. Original patch-clamp traces documenting the effects of IFO or CPA (1 mM each) on membrane voltage (V_m, measured in millivolts (mV), lower traces) and whole cell currents (I_c, measured in picoamperes (pA), upper traces) of individual LLC-PK1 cells. Note an almost immediate and slow but pronounced depolarization following exposure to IFO whereas CPA does not exert any effect.

dependent in hOCT2-expressing and in nonexpressing cells, we exclude a transporter-mediated uptake of this compound. Importantly, CPA and IFO, two structural isomers, can bind with approximately the same affinity to hOCT2. However, most importantly, only IFO is transported by hOCT2 into the cell.

After identification of hOCT2 as possible transport mechanism we verified this interaction in freshly isolated tubules of mice and also of human kidney. In mouse PT a surprisingly high IC_{50} value for the interaction of IFO with OCT could be measured and no inhibition was seen in tubules isolated from OCT1/2-deficient mice. Higher substrate affinities for rodent compared to human OCT is well-known and is probably responsible for increased renal excretion of many drugs by rodents.[35,36] In the human kidney hOCT2 is the main OCT,[15,20] and consistently IFO also

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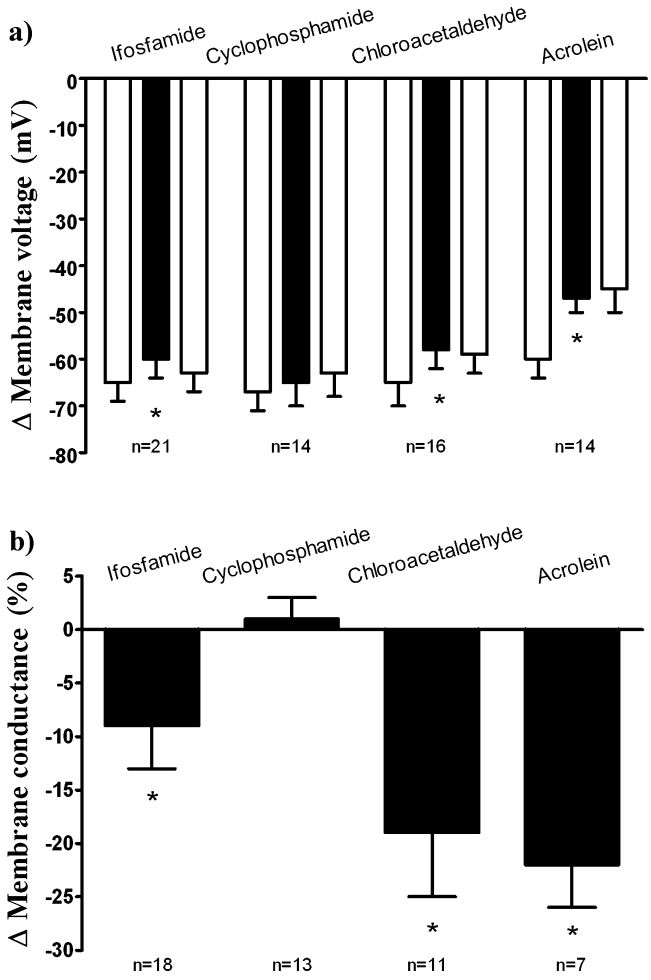

Figure 7. Summary of changes in membrane voltage (V_m) (panel a) and membrane conductance (g_m) (panel b) induced by 1 mM IFO, CPA, chloroacetaldehyde (CAA) and acrolein (ACR) in LLC-PK1 cells investigated with the slow-whole-cell patch-clamp technique. Values are means ± SEM of V_m (mV) or Δg_m (%) with (*N*) referring to the number of successful measurements. Asterisks indicate statistically significant effects. Empty bars represent pre- and postcontrols. Note the significant depolarization and decrease in membrane conductance caused by IFO but not by CPA.

interfered with ASP^+ uptake by isolated human PT. The degree of inhibition induced by 1 mM IFO corresponded well with the apparent affinity obtained in hOCT2 expressing cells.

hOCT2 plays also an important role for the development of IFO toxicity, as demonstrated by the experiments where the dehydrogenase activity was measured as an index of cell vitality. Only in cells expressing the transporter, a significant decrease of this enzymatic activity could be induced by incubation with IFO but not with CPA. Interestingly, incubation of hOCT2 cells with IFO together with cimetidine, a known substrate for hOCT2, completely suppressed IFO-induced toxicity. This suggests a possible therapeutic indication for selective kidney protection during chemotherapy with IFO, which typically is given exclusively by an intravenous route. However, the IFO uptake mechanisms in tumor cells remain unknown.

The existence of a specific renal transport pathway for IFO can explain its selective nephrotoxicity. Due to the high plasma concentrations achieved during the treatment with IFO, this oxazaphosphorine could be significantly accumulated by hOCT2 and then metabolized to toxic compounds directly in PT cells. It has already been demonstrated that human pediatric kidneys can metabolize oxazaphosphorines to CAA because of the presence of cytochromes P450-3A4 and P450-2B6 in tubular cells.[16]

Several single nucleotide polymorphisms with functional implications have been identified in hOCT2,[37] in this way furnishing a possible explanation to the different susceptibility of patients to drug side effects. Since hOCT2 is localized at the basolateral membrane, it can extract IFO directly from the blood. However, other transport systems in the luminal membrane could also contribute to the accumulation of IFO from urine filtrate into PT cells.

Furthermore we now provide circumstantial evidence that the dogma that IFO would be a biologically inactive prodrug is incorrect. By use of real time patch-clamp technology, we have shown an IFO-specific biological effect: immediate depolarization of the membrane voltage associated with a decrease in membrane conductance in LLC-PK1 cells. In these experiments using cells in culture, acute administration of drugs in Ringer-like solution, and electrophysiological analysis of effects within seconds excludes cellular formation of metabolites after drug uptake. IFO itself has been shown to be stable in aqueous solutions. Furthermore, we proved by mass spectrometry in yet another model (i.e., transfected HEK293 cells) that no metabolites could be appreciated. In humans, metabolites of oxazaphosphorine cytotoxics can analytically be found after several hours of administration. These results suggest an interaction of IFO with renal PT for which LLC-PK1 cells serve as a well-known and well-studied model. The mechanism of cellular interaction of IFO remains to be clarified; the decrease in membrane conductance, however, suggests an immediate effect on ion channels rather than on cell metabolism such as ATP production.

The known toxic metabolites chloroacetaldehyde (CAA) and acrolein (ACR) also caused a depolarization associated with a decrease in membrane conductance, suggesting that these changes are a common feature of their tubular cell toxicity. This hypothesis recently found an indirect confirmation in a work demonstrating similar effects of CAA and ACR in IHKE cells, a human PT cell line, where both caused a marked increase of intracellular Ca^{2+}. The observed oscillations of Ca^{2+} concentration could start a signal

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pathway leading to cell death.[38] Our electrophysiological patch-clamp setup allowed testing cytotoxic drugs or metabolites individually in a system where the PT cells retain their differentiated epithelial properties.

Our data also shed some light on the pathophysiology of renal Fanconi syndromes. So far, no genes for primary forms of renal Fanconi syndrome have been identified; also, secondary forms are not well understood.[39] Mutations in mitochondrial complexes 1−5 can show clinical signs and symptoms of renal Fanconi syndrome. Recently mutations in the Na^+-phosphate cotransporter have been associated with an autosomal recessive form of Fanconi syndrome and hypophosphatemia.[40] However, this gene defect did not show a complete renal Fanconi syndrome. Previous research into cellular mechanisms in a variety of models led to various hypotheses as to why this global renal PT insufficiency might occur.[41] Our electrophysiological data clearly document a decreased membrane conductance upon administration of cytotoxics or their presumed toxic metabolites. However, until specific genetic causes of primary renal Fanconi syndromes have been elucidated, some uncertainty will remain.

Understanding the cellular basis of other renal Fanconi syndromes will certainly help in creating the basis for developing rational treatment options for this serious renal tubular problem affecting many patients worldwide (most common cause in children is nephropathic cystinosis; most common cause in adults is HIV treatment).

In conclusion, this work demonstrates for the first time that IFO has a direct biological effect on renal PT cells in contrast to CPA. IFO, but not CPA, is a substrate for hOCT2, which in the human kidney is exclusively expressed in the PT. These findings provide a cell-specific explanation for IFO nephrotoxicity and offer a new potential target for selective protective interventions in cancer treatment with oxazaphosphorines. Ultimately, this will enable oncologists to improve the treatment of their pediatric and adult patients suffering from cancer by adjusting cytotoxic drugs toward maximal benefit and minimal side effects.

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