Expression of Human Organic Cation Transporter 3 in Kidney Carcinoma Cell Lines Increases Chemosensitivity to Melphalan, Irinotecan, and Vincristine

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Abstract
Renal cell carcinoma (RCC) is usually chemoresistant. This chemoresistance could be overcome if specific cytostatics are applied for which the RCC expresses an uptake transporter. In the present study, we investigated the expression of solute carrier (SLC) transporters in different RCC lines and their ability to interact with chemotherapeutics. We tested five RCC lines for the expression of different SLCs by reverse transcription-PCR and TaqMan real-time PCR. In two of five RCC lines, A498 and 786O, we observed a highly significant expression of SLC22A3 (hOCT3). Uptake of the organic cation [3H]MPP4 (4-methyl-pyridinium iodide) into these cells and also into hOCT3 stably transfected Chinese hamster ovary (CHO) cells was inhibited by irinotecan, vincristine, and melphalan. The $K_i$ values [determined from Dixon plots] for irinotecan, vincristine, and melphalan were 1.72 ± 0.45 μmol/L, 17 ± 4.81 μmol/L, and 366 ± 51 μmol/L, respectively. Cytotoxic activities of the selected drugs were tested by [3H]thymidine incorporation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays on CHO-hOCT3, A498 (high expression of hOCT3), and ACHN cell lines (low expression of hOCT3). The growth of CHO-hOCT3 was inhibited by 20% more with irinotecan and by 50% more with vincristine compared with nontransfected CHO cells. Melphalan produced 20% to 30% more inhibition in hOCT3-expressing cells compared with nonexpressing control cells. Similar results were obtained for A498 and ACHN cells. Thus, our data support the hypothesis that the sensitivity of tumor cells to chemotherapeutic treatment depends on the expression of transporter proteins mediating specific drug accumulation into target cells. [Cancer Res 2009;69(4):1494–501]

Introduction
Kidneys are important for the excretion of water soluble xenobiotic compounds. Due to this function, tubule cells in the kidney are exposed to high concentrations of carcinogens, which can lead to tumor development (1). The incidence of renal cell carcinoma (RCC) is 3% of all tumor malignancies. Every year, 31,500 new cases are registered in the United States, whereas 12,000 patients die from metastatic disease annually (2). Surgery at the earliest stage has proven to be the most successful treatment for renal cancer. However, this therapy is no longer helpful when metastases have developed. From already diagnosed RCC, 50% of patients have developed metastases. For this group, chemotherapy would be a choice, but from the tested cytostatics only a few showed some effect on RCC (2). Solid tumors are usually treated by the antineoplastic drug cisplatin. The main dose-limiting side effect of cisplatin is nephrotoxicity. The accumulation of cisplatin in kidneys is higher than in other organs and is transporter mediated (3).

The response to chemotherapy by tumor cells depends on the concentration of cytostatics reached inside the cells. Cytostatic drugs may permeate cell membranes by simple diffusion and by influx and efflux transporter proteins. Efflux transporters are represented by ATP-binding cassette (ABC) family proteins and are mainly connected with chemoresistance of cancer cells. Known members of the ABC family are ABCB1 (MDR1), ABCC1 (MRP1), ABCB2 (MRP2), and ABCG2 (BCRP; ref. 4). Efflux transporters can interact with many cytostatics of different chemical structures. For example, MDR1 interacts with daunorubicin, vincristine, etoposide, and cisplatin; MRP2 was shown to interact with cisplatin and methotrexate (5). In the kidneys, MRP2 and BCRP are expressed at the luminal side of proximal tubule cells and play important roles in secretion of metabolites and xenobiotics (6). Influx transporters belong to the solute carrier transporters (SLC). At present, there are 47 SLC families, organized by alignment similarities and substrate specificities. Many members of these families have a similar tertiary structure consisting of 12 predicted transmembrane domains. The energy for substance translocation can be obtained from the electro-chemical gradient of Na+, H+, and organic ions such as dicarboxylate, and the membrane potential (7). The SLC transporter families are broadly expressed in the human body, with specific localization of some transporters to epithelial barriers of human body. Thereby, SLC transporters influence uptake, distribution and excretion of many drugs, including cytostatics.

Renal drug excretion in proximal tubules is mediated by a group of broad specificity transporters belonging to the SLC22 family (8). The organic anion transporters 1 and 3 (OAT1; SLC22A6; OAT3; SLC22A8) take up a multitude of negatively charged pharmaceuticals from the blood into proximal tubule cells (7). The organic anion transporter 2 and 3 (OCT2; SLC22A2; OCT3; SLC22A3) take up positively charged compounds (9). Due to their broad specificity, OATs and OCTs could transport cytotoxic drugs. Indeed, transport of methotrexate has been shown for OAT1 and OCT3 (10). OCT2, hMATE1, and hMATE2-K-mediated transport of cisplatin (11–13) and transport of oxaloplatin by OCT2, OCT3, hMATE1, and hMATE2-K was recently shown by Yonezawa and colleagues (13).

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The expression of uptake transporters of the SLC family by tumor cells would enable the accumulation of specific cytostatics in these cells, rendering them chemosensitive. Because the majority of RCCs originate from proximal tubule cells, we hypothesized that carcinoma cells may retain some OAT and OCT expression. This would allow for a tailored therapy with anionic or cationic cytostatics.

The aim of this study was as follows: first, to determine the expression of transporters belonging to SLC family in renal carcinoma cells, second, to verify the functional characteristics of the transporters in the tumor cells, third, to select the appropriate cytostatics for the transporters, and, fourth, to show transporter-mediated cytostatic effects on renal carcinoma cells.

Materials and Methods

Reagents. Cell culture materials including FCS, PBS, RPMI 1640, and DMEM-LG were purchased from Invitrogen (Groningen). Quantum 263 complete medium for tumor cells was obtained from PAA Laboratories GmbH. RNA Preparation kit SV Total RNA Isolation System and Mouse Leukocyte Virus (MuLV) reverse transcriptase was obtained from Promega GmbH. Chemical reagents (analytic grade) were purchased from Sigma-Aldrich. Hygromycin was bought from AppliChem. [3H]thymidine was provided by Sigma. Hygromycine was bought from AppliChem. [3H]MPP (1-Methyl-4-phenylpyridinium iodide) was from Biotrend. [3H]thymidine was provided by Amersham Bioscience. All renal carcinoma cell lines were kindly provided by the Department of Nephrology and Rheumatology (Georg-August-University Göttingen).

Semiqualitative reverse transcription-PCR. Renal carcinoma cells were cultivated till 90% confluence and total RNA was obtained using the SV Total RNA Isolation System kit. The concentration and quality of RNA were determined using a Gene Quant II spectrophotometer (Amersham Bioscience). All renal carcinoma cell lines were kindly provided by the Department of Nephrology and Rheumatology (Georg-August-University Göttingen).

Results

Expression evaluation of SLC family transporters in renal carcinoma cell lines. To study the expression of SLC transporters, we chose five renal carcinoma cell lines, A498, ACHN, 7860, RCCNG1, and LN78. For testing transporter expression, we selected the probably most important ones for drug uptake transporters, such as hOAT1, hOCT3, hLAT1, hLAT2. Within the OAT family, only OAT1 was expressed at a low mRNA level in all renal cell lines. Neither OCT1 nor OCT2 showed a significant expression in renal cancer cells (Fig. 1A), but OCT3 was present at high expressing levels in A498, and 7860, and at a lower level in LN78. In ACHN and RCCNG1, hOCT3 was not detectable by RT-PCR.

Quantitative real-time PCR. For the real-time PCR, the reaction mixture contained 12.5 µL of 2× reaction buffer, 1.25 µL 25× primer mixture, 5 µL cDNA solution, and 6.25 µL nuclease-free water in a total volume of 25 µL. The mixture was transferred into a 96-well plate, and covered with an optical adhesive film. The plate was then transferred in a BiPrism 7000 real-time PCR cycler (Applied Biosystems). The PCR program covering the following steps: 2 min at 52°C uridine glycosylase step, 10 min at 96°C TaqMan 5′-Prime activation step, 40 cycles: 15 s at 96°C, 1 min at 60°C. Fluorescence reading was performed on the last part of each cycle. Obtained data were analyzed by the BiPrism 7000 software.

Transport measurements. Chinese hamster ovary (CHO) cells stably expressing human OCT3, CHO-hOCT3, and RCC lines were harvested and plated into 24-well plates (2 × 105 cells per well). After 72 h of incubation, the cells were rinsed thrice with mammalian Ringer solution containing: 130 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L CaCl2, 1 mmol/L MgSO4, 1 mmol/L NaH2PO4, 20 mmol/L HEPES, and 20 mmol/L Na-glucose, the pH adjusted to 1 mol/L NaOH to 7.4. After washing, cells were incubated at RT with 200 µL of transport solution containing 1 µmol/L [3H]MPP in Mammalian Ringer. After 5 min incubation at RT, the cells were washed with ice-cold PBS and lysed with 500 µL of 1 mol/L NaOH solution for 20 min. The lysed mixture was neutralized with 500 µL of 1 mol/L HCl and transferred into scintillation vials. Incorporated radioactivity was counted by a scintillation counter (TriCarb 1500 Packard).

Cell viability assay. CHO-hOCT3 and RCC lines were harvested and seeded in 96-well plates at a concentration of 5 × 103 cells per well. After 24 h, the cells were incubated with different concentrations of melphalan and washed thrice with the medium. The cells were incubated in a medium containing 4 µmol/L of [3H]thymidine for 15 min. Thereafter, the cells were fixed with 5% trichloracetic acid for 30 min on ice. The cell pellets were washed twice with ice-cold PBS and once with 96% ethanol. Then, the pellets were dissolved in 1 mol/L NaOH for 20 min and neutralized with 1 mol/L HCl. Incorporated radioactivity was counted by a scintillation counter (TriCarb 1500 Packard).

Statistical analysis. Statistical analysis and graphical layout were performed with Microsoft Excel (Microsoft) and SigmaPlot 2001 (SPSS Science).
As also shown in Fig. 1A, hLAT1 is present at high levels in all five kidney carcinoma cell lines. The mRNA of hLAT2 is well-expressed in the cancer cells 7860 and RCCNG1, but in ACHN and A498 cells, the expression of hLAT2 was very low.

Using TaqMan real-time PCR, we found that A498 and 7860 RCC lines have the highest OCT3 expression levels (12.5 and 6 cycles difference compared with ACHN and RCCNG1 cells; Fig. 1B). An intermediate expression level of hOCT3 was observed in LN78 (4–5 cycles differences with ACHN and RCCNG1 cells). Therefore, for the next functional experiments, we selected A498 as highly hOCT3-expressing cells and ACHN as a cell line with very low levels of OCT3.

Expression of ABC family transporters in renal carcinoma cell lines. The accumulation of cytostatics in the tumor cells mediated by SLC transporters should depend on the activity and expression of efflux transporters. Therefore, we studied the expression of transporters for release of cytostatics. MDR1, MRP2, and MRP4 transporters are mainly present at the apical side in normal kidney tubule cells. All five tested cell lines showed high expression of MRP2 and 4 (Fig. 1C). Expression of MDR1 varied as follows: in A498, 7860 and ACHN cells expression was found at a high level, and in LN78 at a low level. RCCNG1 did not show any detectable MDR1 expression (as shown in Fig. 1C).

Uptake experiments. To characterize the functional expression of the transporters in the renal carcinoma cells, we performed uptake experiments with well-documented substrates of OATs (p-aminophenylphosphonic acid, PAH) or OCTs (N-methyl-4-phenylpyridinium iodide, MPP), respectively. The functional expression of OAT1 could not be confirmed by the specific uptake of [3H]PAH. Neither of the kidney tumor cells showed a probenecid inhabitable transporter-mediated PAH uptake (data not shown). Functional expression of OCT3 in the RCC and CHO cells was examined and quantified by uptake of [3H]MPP. As ACHN and A498 cell lines did not show any expression of other OCT members, MPP uptake should be mainly mediated by hOCT3. The MPP uptake results with A498 and ACHN cells were compared with those of OCT3-stably transfected CHO cells and mock-CHO cells as a model. As shown in Fig. 2A, the uptake of [3H]MPP in OCT3-CHO cells was 3.5 ± 0.01 pmol/5 min/10^6 cells, which was 11-fold higher than in nonexpressing mock-CHO cells (0.2 ± 0.01 pmol/5 min/10^6 cells; P < 0.00001). OCT3-mediated [3H]MPP uptake was significantly inhibited by 100 μmol/L quinine (0.55 ± 0.02 pmol/5 min/10^6 cells; P < 0.00001) and 500 μmol/L MPP (0.47 ± 0.05 pmol/5 min/10^6 cells; P < 0.00001). Tetraethylammonium (TEA) at a concentration of 1 mmol/L inhibited [3H]MPP uptake partially (1.32 ± 0.04 pmol/5 min/10^6 cells; P < 0.00001). Nontransfected CHO cells did not show a quinine- or MPP-inhibited uptake of [3H]MPP. As shown in Fig. 2B, the hOCT3-expressing A498 cells showed a 10-fold higher [3H]MPP uptake (2.11 ± 0.1 pmol/5 min/10^6 cells; P < 0.00001) compared with ACHN cells (0.29 ± 0.003 pmol/5 min/10^6 cells), which do not express OCT3. The [3H]MPP uptake in A498 cells was abolished by 100 μmol/L quinine (0.3 ± 0.003 pmol/5 min/10^6 cells; P < 0.00001) and by 500 μmol/L nonlabeled MPP (0.3 ± 0.01 pmol/5 min/10^6 cells; P < 0.00001). 1 mmol/L TEA caused a 65% decrease in [3H]MPP uptake compared with untreated cells (Fig. 2B).

Inhibition of [3H]MPP uptake by cytostatic substances. To elucidate the interaction of hOCT3 with cytostatic drugs, we examined the inhibition of hOCT3-mediated [3H]MPP uptake by a range of widely used cytostatic agents. The results are summarized in Fig. 3. [3H]MPP uptake was significantly inhibited by 100 μmol/L of irinotecan to 19.6% ± 0.7% (P < 0.0001), vincristine to 29.3% ± 1.7% (P < 0.0001), melphalan to 40.5% ± 0.7% (P < 0.0001), and prednisone to 64.1% ± 4.1% (P < 0.01), of the uptake into untreated cells. Doxorubicin, methotrexate, fluorodeoxuridine, and cyclophosphamide showed weak, but significant inhibition (P < 0.05) of [3H]MPP uptake, to 82.2% ± 3.5%, 90.1% ± 3.1%, 77.9% ± 3.1%, and 84.5% ± 1.4%, respectively, of untreated cells. Cisplatin, cytarabine, and busulfan did not inhibit an interaction with hOCT3 (Fig. 3).

The type of interaction and affinity of cytostatics with a high inhibitory effect on [3H]MPP uptake were analyzed by Dixon plots. Three experiments were performed for each drug, and a representative experiment is shown in Fig. 4A to C. All tested compounds interacted with hOCT3 in a competitive manner. The calculated K_i values for irinotecan, vincristine, and melphalan are 1.75 ± 0.45 μmol/L, 17 ± 4.5 μmol/L, and 366 ± 51 μmol/L, respectively.

**Figure 1.** Expression of human OAT1, OCT1-3, LAT1, and LAT2 in kidney carcinoma cell lines determined by RT-PCR. Total RNA was isolated, reverse transcribed, and used as a template for PCR using OAT1, OCT1, OCT2, OCT3, LAT1, LAT2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers. A, result of a representative experiment. Similar results were obtained from mRNA isolated from three independent passages of RCC cells. B, expression of human OCT3 in kidney carcinoma cell lines determined by TaqMan real-time PCR. The hOCT3 expression was normalized to expression level of GAPDH and hypoxanthine phosphoribosyltransferase. △Ct shows the difference between lowest expression (ACHN) and other tested RCC lines. Columns, mean calculated from three independent experiments with three repeats each; bars, SD. C, expression of human MDR1, MRP2, MRP4, and GAPDH in kidney carcinoma cell lines was determined by RT-PCR. The quantification was made against GAPDH as reference from the same cDNA to standardize and compare results. Neg. control, negative control.
Evaluation of the OCT3-mediated cytostatic activity of melphalan by [3H]thymidine incorporation. As melphalan is a DNA damaging agent, its cytotoxic activity was measured by the [3H]thymidine incorporation assay. We observed that, compared with CHO-mock cells, CHO-OCT3-expressing cells have 10% ± 3% (P < 0.05) less [3H]thymidine incorporation upon treatment with 10 μmol/L melphalan, 20% ± 4.5% (P < 0.01) less with 50 μmol/L melphalan, and 35% ± 6.4% (P < 0.01) less with 100 μmol/L melphalan, indicating that OCT3-expressing cells are more sensitive to melphalan (Fig. 5A). For A498 cells (OCT-3-expressing RCC), 35% ± 7.3% (P < 0.01) less [3H] thymidine incorporation was seen with 10 μmol/L melphalan, 30% ± 4.4% (P < 0.01) less with 50 μmol/L, and 2% (not significant) less with 100 μmol/L, compared with ACHN cells (Fig. 5B).

To prove hOCT3-mediated melphalan sensitivity, both CHO and RCC cells were incubated simultaneously with TEA to inhibit OCT3 (Fig. 5C and D). CHO-hOCT3 was more sensitive to melphalan at a concentration of 50 μmol/L compared with CHO-mock cells. In the presence of 50 μmol/L melphalan together with 2 mmol/L TEA, control cells and CHO-OCT3 showed no difference in [3H]thymidine incorporation (Fig. 5C). The TEA-dependent recovery of [3H]thymidine incorporation in CHO-hOCT3 was 12% ± 4.7% (P < 0.05) compared with cells treated only with melphalan. Renal carcinoma cells A498 with high hOCT3 expression are more sensitive to melphalan compared with the low hOCT3-expressing ACHN cells. The simultaneous treatment of A498 with 2 mmol/L TEA lead to a 5% recovery of the [3H]thymidine incorporation (Fig. 5D).

Evaluation of the hOCT-3–mediated cytostatic activity of irinotecan and vincristine. The sensitivity of CHO-hOCT3 cells to irinotecan was not significantly higher than that of the non-transfected CHO. MPP, TEA, and verapamil alone did not influence viability (Fig. 6A). The irinotecan effect on CHO-OCT3 cells was increased up to 25% ± 3.3% (P < 0.01) upon additional treatment with 1 μmol/L verapamil to block MDR1 (Fig. 6A). In the presence of 500 μmol/L MPP or 2 mmol/L TEA, the cell viability was reversed (P < 0.01). The viability of CHO-hOCT3 treated by vincristine was decreased by 40% ± 2.5% (P < 0.001), and after verapamil treatment it was decreased additionally by 19% ± 3% (Fig. 6B). The cytotoxic effect of vincristine was partially restored by 15% ± 1.7% (P < 0.01) upon adding 2 mmol/L TEA.

Vincristine and irinotecan sensitivities of RCC cells were also tested by the MTT assay (Fig. 6C and D). In contrast to OCT3-stably transfected CHO cells, irinotecan did not show significant differences in the cytotoxic effect between A498 and ACHN cells (Fig. 6C). Renal cancer cells tested by vincristine showed a behavior similar to CHO-OCT3 cells (Fig. 6D). Vincristine-treated A498 cells were 18% ± 2.5% more sensitive to the cytostatic compared with ACHN cells. The effect of vincristine was elevated to 25% ± 1.2% by additional treatment with 1 μmol/L verapamil. The cytotoxic effect of vincristine was slightly reversed in cells incubated with 500 μmol/L MPP and 2 mmol/L TEA.

Discussion

The cellular milieu is highly controlled by transporters and channels, which determine the influx and efflux of nutrients, ions,
metabolites, and drugs. Thereby, transporters play a pivotal role in cell function, differentiation, proliferation, and cell death. The importance of transporters in the strategy of tumor therapy and involvement in resistance of cancer cells to cytostatics is a widely accepted fact. The involvement of efflux pumps such as MDR and MRPs in cytostatics resistance and therapy has been intensively investigated in the last decade. In contrast, little is known about the contribution of influx or uptake transporters in tumor therapy and transporter-mediated sensitivity for cytostatics. Therefore, we initially examined the expression of several uptake transporters in renal carcinoma cell lines. Organic anion and cation transporters (OATs, OCTs) belong to the SLC22 gene family. The OAT and OCT transporters are highly expressed in the kidneys, but also in the liver, blood-brain barrier and several other tissues (15). The organic anion transporters have a very high affinity for negatively charged organic substrates, such as endogenous metabolites such as \( \alpha \)-ketoglutarate, prostaglandin E2, estrone sulfate, dehydroepiandrosterone sulfate, cyclic AMP, or cyclic guanosine 3',5'-monophosphate. Exogenous drugs known to be transported by OATs include \( \beta \)-lactam antibiotics, antiviral drugs, nonsteroidal anti-inflammatory drugs, diuretics, and cytostatics (16, 17). OATs are responsible for proximal tubule uptake and excretion of these substances. The organic cation transporters possess an affinity for positively charged endogenous (epinephrine, norepinephrine, etc.) and exogenous substances (MPP, TEA, quinine; ref. 9, 18). It was also shown that OCTs play an important role in the drug distribution in the organism (15), drug transport from blood to brain (19) and excretion from the body.

The ABC transporters are widely distributed. In the kidneys a few members of the ABC family such as MRP1, MRP2, MRP4, and MDR1 are highly expressed (20, 21). Physiologically these transporters pump out endogenous "waste," glutathione conjugates, and glucuronides, and exogenous hydrophobic xenobiotic such as benzapyrenes (22). The functions of ABC transporters include, of course, the excretion of cytostatics from cancer cells (23), which renders tumor cells chemoresistant. We performed in kidney cancer cell lines a screening of the ABC transporter family to compare the level of the expression with the expression of the SLC family members. Most of the tested ABC transporters were highly expressed. It will be very important in the planning of chemotherapy to consider the expression level of the ABC transporters because they counteract the uptake transporters.

As a target for cytostatic uptake in tumor cells, we examined the expression level of organic anion transporters in the kidney tumor cell lines 7860, RCCNG1, A498, LN78, and ACHN. Thus far, nothing was known of the expression of OATs in kidney tumor cells, and little about the interaction of cytostatics with organic anion transporters. Up to now, only the interaction of methotrexate with organic anion transporters was reported (23). On the other hand, the use of cytostatics in the cancer treatment may cause an accumulation of these drugs in the kidney proximal tubule cells and result in complications such as nephropathy (3). Our study revealed the expression of OAT1 in kidney cancer cell lines on the mRNA level. However, we observed a very low level of PAH uptake, and the uptake could not be inhibited by 500 \( \mu \)mol/L probenecid, a standard blocker of organic anion transporter systems. We conclude from our data that OAT1 was not functionally detectable in the kidney tumor cell lines. Therefore, organic anion transporters are not the appropriate target for chemotherapy of RCC with anionic cytostatics.

Figure 4. Kinetic analysis the inhibition of \([3H]MPP\) uptake by irinotecan, vincristine, and melphalan. Uptake experiment with (A) 1 \( \mu \)mol/L and (C) 10 \( \mu \)mol/L \([3H]MPP\) 20 nmol/L \([3H]MPP + 980 \text{ nmol/L or 9980 nmol/L MPP}\) in the presence of different concentrations of tested cytostatics compounds: (A) irinotecan, (B) vincristine, (C) melphalan. Data are presented as Dixon plots. Points, means of three repeats; bars, SE. The \( K_i \) was calculated from these plots.
Previous studies show that OCT2 is responsible for cisplatin accumulation inside the kidney and brain tissues. Nephropathies and neuropathies have been observed after chemotherapy with cisplatin due to accumulation of these cytostatics (3, 24). In HEK293, cells expressing organic cation transporter OCT1 and OCT2 as well as OCT2 and OCT3 cisplatin and oxaliplatin mediated cytotoxicity as reported by Yonezawa and colleagues (13).

Quantification of OCT3 expression within the five tested renal carcinoma cells lines by real-time PCR indicated a high variability of expression. A498 cells showed the highest mRNA expression of OCT3 compared with ACHN and RCCNG1 cells. To prove the functional expression of the transporter, we chose the nonexpressing ACHN cell line and highly OCT3-expressing cell line A498 to perform an OCT3-mediated uptake of the model substrate, [3H]MPP. As a positive control, we carried out the same experiments with hOCT3-stably transfected CHO cells and mock cells. The uptake experiment with A498 cells revealed a >10 fold higher [3H]MPP accumulation than in ACHN cells. Uptake was inhibited by unlabeled organic cations, MPP, quinine, and TEA. The MPP uptake data achieved with hOCT3 and mock CHO cells corresponded perfectly to the results of the renal carcinoma cells A498 and ACHN. These data show, for the first time, that OCT3 is functional in some renal cancer cells.

Functional expression of OCT3 in renal cancer cells renders this transporter an appropriate candidate for kidney tumor therapy with cytostatics. However, little is known of the interaction of OCT3 with cytostatics. Koepsell and colleagues (9) reported the interaction of mitoxantrone with OCT3. Therefore, we examined the interaction of several cytostatics on the hOCT3-mediated [3H]MPP uptake in stably transfected CHO cells. One hundred moles of irinotecan, vincristine, and melphalan inhibited OCT3 facilitated MPP uptake by >60%, whereas methotrexate, doxorubicin, fluoro adenine, cyclophosphamide, and prednisone reduced the OCT3 activity less but still significantly. hOCT6 showed in contrast to hOCT3 very high affinity (K_m 5.2 μmol/L) for doxorubicin (25). OCT6 was not expressed in the kidney and renal carcinoma cells. Consequently, OCT6 cannot be considered in the kidney tumor therapy. Cisplatin, cytoarabinoside, and busulfan did not show any interaction with hOCT3. Zhang and colleagues (12) reported the interaction of oxaliplatin with OCT1 and OCT2, but not with OCT3. They showed the cytostatic effect of oxaliplatin, which was much higher than that of cisplatin in six colon cancer cell lines.

Dixon plot analysis revealed a competitive inhibition of OCT3 and the highest affinity for irinotecan followed by vincristine and melphalan. Because these cytostatics were not available in radioactive form, uptake by OCT3 could not be tested directly. However, the OCT3-mediated uptake of these three cytostatics could indirectly be shown by the transporter-dependent cytostatic effect evaluated by thymidine incorporation and cell viability. CHO-OCT3 cells and OCT3 expressing A498 tumor cells showed less thymidine incorporation after melphalan treatment compared with cells without OCT3 expression. Melphalan taken up into the cells binds to -NH2 and -SH groups of proteins or nucleic acids (26). The mustard-nucleoside derivates generated by melphalan (27) prevent DNA replication, resulting in reduced thymidine incorporation. Simultaneous incubation of melphalan and TEA resulted in a partial restoration of the thymidine incorporation in OCT3-expressing CHO cells. These experiments strongly favor OCT3-mediated translocation of melphalan and the increase of the cytotoxic effect on OCT3-expressing cells. The cytotoxic effect of melphalan could not be prevented by TEA in the A498 tumor cells, most probably because of high expression of L-type amino acid transporter (LAT1) in these cells, as shown.

![Figure 5.](image-url)
by RT-PCR in this study. The affinity of LAT1 for melphalan was recently reported (28).

Comparable evidence for the OCT3-mediated cytostatic effect in CHO and A498 cells was observed with irinotecan and vincristine by a cell viability test. Irinotecan is a strong inhibitor of topoisomerase I (29) that plays an important role in DNA duplication, RNA translation, and in cell cycle. Irinotecan is a positively charged substance that has a partial structural similarity to MPP. After uptake into the cell, irinotecan is converted to SN38 by hydrolysis. SN38 is inactivated by glucoronidation (30) the cytotoxic activity is dependent on the concentration of the active SN38 product inside the cells and on MDR1, which exports SN38 (31).

Vincristine is a potent cytostatic that is used for the treatment of blood cancers such as non–Hodgkin’s lymphoma and lymphoblastic leukemia. The main intracellular target for vincristine is the mitotic spindle. Vincristine binds to tubulin molecules and prevents their polymerization (32). After vincristine treatment, cells cannot divide and this leads to cell death by apoptosis (33).

A498 and CHO-OCT3 cells showed a greater sensitivity for vincristine than for irinotecan. The effect of vincristine and irinotecan could be increased by additional incubation with a potent MDR inhibitor verapamil (5). MDR1 expression was detected in CHO cells (34). It was found that OCT3-CHO treated with irinotecan and verapamil had a lower viability compared with nonexpressing CHO cells. Vincristine was more toxic than irinotecan. A possible reason could be the high affinity to tubulin, the resulting complexes having a low constant of dissociation. Additional treatment with verapamil amplified the cytotoxic effect of vincristine. This cytostatic effect was only slightly reversed by a substrate of OCT3. This finding suggests that even low amounts of vincristine disturb the microtubule net in an irreversible manner.

One pronounced side effect of vincristine is neuropathy (35). This side effect could be due to OCT3-mediated transport because OCT3 is expressed in the central neural system (9).

In the present study, we examined the dependence of cytostatic activities on influx transporters expressed in renal carcinoma cell lines. As a proof of principle, we showed the OCT3-mediated...
cytostatic sensitivity. This fact renders hOCT3 an appropriate candidate for individualized kidney tumor therapy. Along these lines, it is worthwhile considering to test for hOCT3 expression and to tailor the cytostatics therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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