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Profiling SLCO and SLC22 genes in the NCI-60 cancer cell lines to identify drug uptake transporters

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Abstract

Molecular and pharmacological profiling of the NCI-60 cell panel offers the possibility of identifying pathways involved in drug resistance or sensitivity. Of these, decreased uptake of anticancer drugs mediated by efflux transporters represents one of the best studied mechanisms. Previous studies have also shown that uptake transporters can influence cytotoxicity by altering the cellular uptake of anticancer drugs. Using quantitative real-time PCR, we measured the mRNA expression of two solute carrier (SLC) families, the organic cation/zwitterion transporters (SLC22 family) and the organic anion transporters (SLCO family), totaling 23 genes in normal tissues and the NCI-60 cell panel by quantitative real-time PCR. By correlating the mRNA expression pattern of the SLCO and SLC22 family member gene products with the growth inhibitory profiles of 1,429 anticancer drugs and drug candidate compounds tested on the NCI-60 cell lines, we identified SLC proteins that are likely to play a dominant role in drug sensitivity. To substantiate some of the SLC–drug pairs for which the SLC member was predicted to be sensitizing, follow-up experiments were performed using engineered and characterized cell lines over-expressing SLC22A4 (OCTN1, organic cation/carnitine transporter 1). As predicted by the statistical correlations, expression of SLC22A4 resulted in increased cellular uptake and heightened sensitivity to mitoxantrone and doxorubicin. Our results indicate that the gene expression database can be used to identify SLCO and SLC22 family members that confer sensitivity to cancer cells.

Keywords

uptake transporters; gene profiling; solute carrier; multidrug resistance; drug discovery

Introduction

One of the major mechanisms of adaptive cellular anticancer drug resistance (multidrug resistance (MDR)) is diminished cellular accumulation conferred by a combination of decreased transporter-mediated drug uptake and increased energy-dependent efflux of drugs (1). This occurs in concert with a variety of metabolic changes in cells that affect the ability of cytotoxic drugs to kill cells, including alterations in the cell cycle, increased repair of DNA damage, reduced apoptosis, and altered metabolism of drugs. Of these mechanisms, the one most commonly encountered in the laboratory is the increased efflux of a broad range of cytotoxic drugs mediated by ATP-Binding Cassette (ABC) transporters (2). P-glycoprotein (P-gp), encoded by *ABCB1* (also known as *MDR1*), stands out among ABC transporters by conferring the strongest resistance to a wide variety of drugs and has been demonstrated to be implicated in clinical drug resistance (3). In addition to P-gp, 11 of 48 known human ABC transporters have been shown to play some role in the drug resistance of cancer cells *in vivo* and/or *in vitro* (4).

Recently, we have used a bioinformatic approach to identify ABC transporter substrates. We profiled mRNA expression of all 48 ABC transporters in 60 diverse cancer cell lines (the NCI-60) used by the National Cancer Institute (NCI) to screen for anticancer activity of >100,000 compounds submitted for testing. In our previous study, by correlating the expression profiles with the growth inhibitory profiles of a subset of 1,429 compounds (incorporating anticancer drugs and drug candidates) tested against the cells, we successfully identified several cytotoxic substrates recognized by different ABC transporters (5).

As mentioned above, resistance can also result from reduced transporter-mediated drug uptake, and the net accumulation of an anticancer drug in a cell is probably influenced by the concurrent actions of uptake and efflux transporters. The solute carrier (SLC) gene series encodes a large family of passive transporters, ion-coupled transporters, and exchangers that rely on a concentration gradient across the membrane or co/counter-transport to facilitate substrate transport. In the physiological setting, SLC transporters are responsible for the absorption and excretion of a wide variety of endogenous and exogenous compounds. The human organic anion transporting peptide (OATP or SLC21) family is comprised of 11 members that transport endogenous organic anions (e.g. bile salts, bilirubin) and xenobiotics. The organic cation (and carnitine) transporters (OCT, SLC22) family has members that are able to transport organic cations/zwitterions and anions (6–8). Within the SLC22 family there are six main cation transporters; SLC22A1 (OCT1), SLC22A2 (OCT2), SLC22A3 (OCT3), SLC22A4 (OCTN1), SLC22A5 (OCTN2) and SLC22A16 (OCT6), three of which are known to transport the zwitterion carnitine (SLC22A4, SLC22A5 and SLC22A16) (8).

Studies have proved that uptake transporters can indeed confer sensitivity to anticancer drugs (9–14). For example, methotrexate has been shown to be a substrate for organic anion-transporting polypeptide 1B3 (SLCO1B3, OATP1B3) (9). (Note: In this report, we refer to individual proteins of the SLCO and SLC22 family by using series numbers for SLC genes and also the general protein nomenclature. See <http://www.genenames.org/>). Similarly, studies have shown that the organic cation transporters SLC22A1 (OCT1), SLC22A2 (OCT2), and SLC22A3 (OCT3) mediate cell sensitivity to platinum drugs such as cisplatin, carboplatin, and oxaliplatin (12–15).

Huang et al. have exploited the NCI-60 database to correlate oligonucleotide array data with the potencies of 119 standard anticancer drugs and showed that SLC29A1 plays a role in the cellular uptake of the nucleoside analogues azacytidine and inosine-glycodialdehyde (16). In this study, we have measured the mRNA expression of two solute carrier (SLC) families in normal human tissues and the NCI-60 cell line panel. Since reproducible, quantitative

correlations between the expression and sensitivity were required, we chose to measure transcript expression by quantitative real-time PCR to gain a perspective on the potential role of SLCO and SLC22 transporters in drug response. We show that positively correlated drug-gene pairs reveal SLC transporters conferring chemosensitivity to their respective drug substrates. In particular, the pharmacogenomic approach based on the correlation of expression and sensitivity datasets derived from the NCI-60 cell panel identifies SLC22A4 (OCTN1, organic cation/carnitine transporter 1) as a candidate drug transporter. We generated a KB-3-1 cell line transfected with a plasmid expressing SLC22A4, and our *in vitro* experiments confirm that SLC22A4 mediates the cellular uptake of mitoxantrone and doxorubicin, thereby conferring cellular sensitivity to these agents.

Materials and Methods

Chemicals

Tetraethylammonium chloride (TEA), mitoxantrone dihydrochloride, and doxorubicin hydrochloride were obtained from Sigma (St. Louis, MO). NSC59729 and NSC251819 were obtained from the DTP, NCI. Solutions of TEA (10 mM), mitoxantrone (20 mM), doxorubicin (20 mM), NSC59729 (20 mM), and NSC251819 (20 mM) were prepared using the same buffer as employed in uptake assays (described below). Stock solutions were aliquoted and stored at -80°C . [ethyl 1- ^{14}C]TEA bromide (55.0 mCi/mmol) and [^3H (G)]mitoxantrone (1.5 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO), and [^{14}C]doxorubicin hydrochloride (55.0 mCi/mmol) was from Amersham (Buckinghamshire, UK). All other chemicals and reagents were of analytical grade.

Preparation of total RNA

Total RNA from human normal tissues was obtained from Clontech (Palo Alto, CA). Total RNA from the 60 cancer cell lines was prepared and provided by DTP (For detail, see <http://www.dtp.nci.nih.gov/branches/btb/ivclsp.html>). For consistency in RNA quality, DTP has adopted standard operating procedures that include the use of matched serum batches and harvesting the cells at a particular confluence (17). Nevertheless, the quality (purity and integrity) of the RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). The RNA was quantitated using a spectrophotometer (Ultrospec 3100 *pro*, Amersham).

Real-time quantitative RT-PCR

Expression levels of the SLCO and SLC22 family genes were measured by real-time quantitative RT-PCR using ABI PRISM 7900HT (Applied Biosystems, Foster City, CA). For information on specific oligonucleotide primers and TaqMan probes for each of the SLC members, see Supporting Information Table S4 and Supporting Information Table S5. Synthesis of cDNA from total RNA samples was carried out using TaqMan Reverse Transcription Reagents (Applied Biosystems) with 1 μg total RNA/50 μL reaction volume. The cDNA (0.5 μL RT sample) was amplified using TaqMan Universal PCR Master Mix Reagents (Applied Biosystems) in a total volume of 10 μL . The PCR mixture was pre-incubated at 50°C for 2 min, incubated at 95°C for 10 min, and amplified by 40 cycles at 95°C for 15 s and 60°C for 1 min. No-template (water) reaction mixtures were prepared as negative controls.

Data processing

During the PCR amplification, fluorescence emission was measured and recorded in real time. Crossing point values were calculated using the ABI PRISM 7900HT software package. The raw results were expressed as number of cycles to reach the crossing point. If the desired product was not detected, the corresponding value was adjusted to crossing points indicating

no expression. To assess the contribution of experimental error, cell lines were assessed in triplicate. The average pair-wise correlation of triplicate expression profiles was 0.90. One of the most important steps in the design of quantitative PCR experiments is the choice of adequate internal controls. A reliable standard gene is expected to show unchanged expression under all experimental conditions. We found that the expression levels of five housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, and zeta polypeptide, Ubiquitin C, hypoxanthine phosphoribosyltransferase 1, and beta actin) are highly variable across the 60 cell lines and 29 human tissues (data not shown; however, see (18)). Hence, as in our previous study (5), they were not used as controls. Since the majority of the studied genes are not expected to be consistently changed across the cells, we chose to internally normalize the samples (genes in a cell) with respect to the mean expression of the characterized genes. Finally, the values were multiplied by -1 so that positive values indicate higher expression.

Drug database

More than 100,000 chemical compounds have been tested in the NCI-60 screen by the DTP. For this study, we focused on a subset consisting of 1,429 compounds that have been tested at least four times on all or most cell lines in the NCI-60 and whose screening data met quality control criteria described elsewhere (19). This subset includes most of the drugs currently used clinically for cancer treatment, along with many candidates that have reached clinical trials. Details of all drugs are available at <http://discover.nci.nih.gov/> and <http://spheroid.ncifcrf.gov/spheroid/>.

Statistical analysis of real-time quantitative RT-PCR

Basic descriptive statistics were performed using the CIMminer tool (<http://discover.nci.nih.gov>) and the R statistical programming language (www.r-project.org). A two-dimensional agglomerative hierarchical cluster analysis, with average linkage algorithm and distance metric $1-r$, where r is the Pearson's correlation coefficient, was performed using CIMminer to group the 60 cell lines as well as SLCO and SLC22 family members based on their expression profiles. The resulting matrix of numbers was displayed in clustered image map form (20). To select drug candidates for detailed follow-up, we used both a simple Bonferroni procedure and the Benjamini-Hochberg False Discovery Rate procedure (21) to adjust for multiple testing of all 28 genes and all 1,429 compounds simultaneously.

Establishment of stable transfectants

A plasmid containing the full-length cDNA of human *SLC22A4* (reference sequence: NM_003059) was obtained from OriGene (Rockville, MD). The cDNA was amplified by PCR using specific primers (Lofstrand, Gaithersburg, MD). The amplified PCR product was subcloned into expression vector pcDNA 3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA). The inserted *SLC22A4* was sequenced by the NCI DNA Sequencing Facility. Using Lipofectamine (Invitrogen), the expression construct was transfected into KB-3-1 cells. KB-3-1 cells transfected with mock vector (pcDNA3.1/V5-His-TOPO/*lacZ*) served as a control. Stable clones were selected with 0.8 mg/mL G418 sulfate. Among the G418-resistant clones, the stable transfectants expressing *SLC22A4* (OCTN1) were characterized by real-time quantitative RT-PCR, Western blot analysis, immunocytochemical analysis, and an assay for uptake of [14 C]TEA, a known substrate of *SLC22A4* (OCTN1). The selected clones (*SLC22A4* (OCTN1)/KB-3-1 and Mock/KB-3-1) were used for drug sensitivity and uptake assays.

Cell culture

The culture medium for stable transfectants was DMEM supplemented with 10% FBS (GIBCO (Invitrogen)), 100 U/mL penicillin G, 100 μ g/mL streptomycin, 2 mM L-glutamine, and 0.4

mg/mL G418. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ and 95% air.

Western blot analysis

Crude membrane fractions from the cells stably transfected with SLC22A4 (OCTN1) and mock-vector were collected using the Qproteome Cell Compartment Kit (QIAGEN, Valencia, CA). The proteins (10 µg/lane) were separated on 4 – 12% gradient SDS-polyacrylamide gels, then blotted onto a PVDF membrane. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 hr at room temperature (RT). After washing with TBS-T, the membrane was incubated with anti-V5-HRP antibody (Invitrogen) diluted 1:5000 in TBS-T overnight at RT. The membrane was then washed with TBS-T, and proteins were visualized using the SuperSignal West chemiluminescent substrate (PIERCE, Rockford, IL).

Immunocytochemical analysis

Cells were grown on chamber slides. Twenty-four hrs after seeding, the cells were fixed (100% methanol for 5 min at RT), permeabilized (0.1% Triton X for 3 min at RT), blocked (PBS/10% FBS for 30 min at RT), and incubated with anti-V5-FITC antibody (Invitrogen) diluted 1:500 in PBS/10% FBS for 2 hrs at RT. After a wash with PBS, VECTASHIELD mounting medium (Vector, Burlingame, CA) was added. Fluorescent cells were examined under a confocal laser scanning fluorescence microscope (LSM510, Carl Zeiss, Jena, Germany).

Drug sensitivity assay

Cells were seeded in 100 µL culture medium without G418 at a density of 3,000/well in 96-well plates and incubated for 24 hrs. Medium containing serially diluted mitoxantrone, doxorubicin, NSC59729, or NSC251819 was added to give the indicated final concentrations in three replicated wells. Cells were then incubated for 72 hrs. The antiproliferative activities of drugs were evaluated using CCK-8 (Cell Counting Kit-8) following the manufacturer's instructions (Dojindo, Gaithersburg, MD). Maximal cell survival (defined as 100%) represented wells without drug. Dose-response curves were plotted using GraphPad PRISM software (San Diego, CA).

Uptake assay

Cells were seeded in the culture medium without G418 at a density of 2.0×10^5 /well in 24-well plates and were incubated for 24 hrs. Before initiation of the assay, cells were washed with an uptake buffer containing 125 mM NaCl, 20 mM NaHCO₃, 3 mM KCl, 1.8 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM D-glucose, and 10 mM HEPES (pH 7.4) and preincubated in the same buffer for 5 min. The assay was initiated by replacing the uptake buffer with 0.1 ml of the same buffer containing radiolabeled drugs. For a time course of the uptake, cells were incubated with 3 µM [³H]mitoxantrone or [¹⁴C]doxorubicin for the designated time period in an incubator (5% CO₂, 95% air). For the competition studies, cells were incubated with 3 µM [³H]mitoxantrone or [¹⁴C]doxorubicin in the absence or presence of 30 µM of non-radiolabeled TEA, mitoxantrone, or doxorubicin for 10 min. Uptake was terminated by adding excess ice-cold uptake buffer. Cells were washed thoroughly three times with 1 mL ice-cold uptake buffer and then lysed by alkalization. The cell lysates were transferred to scintillation vials containing scintillation fluid, and the radioactivities were measured in a liquid scintillation counter (LS 6000SE; Beckman Coulter, Fullerton, CA). Cells washed with the uptake buffer immediately after addition of the assay mix were used as the zero-time point, representing nonspecific binding of the drug to the cells. A TEA uptake assay to select the clones expressing SLC22A4 (OCTN1) was performed by incubating the cells with 60 µM [¹⁴C]TEA for 10 min.

Statistical analysis for drug sensitivity assay and uptake assay

Differences between the mean values were analyzed by a two-sided Student's *t* test and results were considered statistically significant at $P < 0.05$.

Results

mRNA expression of SLCO and SLC22 family members in human tissues

Fig. 1A is a clustered image map ("heat map") (20) that displays the patterns of mRNA expression of the SLCO (11 genes) and SLC22 (17 genes) families across 29 human tissues (including fetal liver, brain, and kidney). Red and blue indicate high and low expression relative to the mean expression of a given transporter across all tissues, respectively (see also Table S1). Subsets of the SLC genes expressed abundantly in the liver, brain and kidney clustered on the heat map. For example, *SLCO1B1*, *SLCO1B3*, *SLC22A1*, *SLC22A7*, and *SLC22A9*, which are expressed at high levels in the liver, form a cluster. *SLC22A15*, *SLC22A17*, *SLCO1A2*, and *SLCO1C1*, expressed at high levels in tissues of the nervous system such as those of the brain and spinal cord, form another cluster. Whereas 9 out of 17 SLC22 family genes were expressed at high levels in the kidney, of the SLCO family, only *SLCO4C1* was expressed at high levels in that tissue. Other than the liver, brain, and kidney, high levels of gene expression were observed in the testis, lung, mammary gland, and retina. These results are consistent with those reported in human subjects (6–8).

mRNA expression in the NCI-60 cancer cell line panel

Fig. 1B shows the mRNA expression of SLCs across the NCI-60 cancer cell lines. The data indicate that SLC transporters are expressed at similar levels in normal tissues and across the cancer panel (Table S2). In comparison to published expression patterns of gene expression (5,19) the SLCO and SLC22 family members show less coherence. The distribution of SLC transporters on the gene dendrogram appears to be independent of sequence-homology categories. In our previous study on 48 ABC transporters, the pattern of expression correlated to some extent with tissue of origin. In particular, melanoma cells were found to express a characteristic set of ABC transporters, but renal, central nervous system (CNS), and ovarian cancer cell lines also tended to cluster (5). The present study suggests that the relation of SLC transporter expression to tissues is less explicit, as only melanoma and renal cancer cell lines tended to form clusters. However, consistently with earlier molecular profiles, the two luminal, hormone-dependent breast cancer lines, MCF7 and T47D, cluster together; and MDA-N, an ERBB2 transfectant of MDA-MB435 clusters with its parental line. Interestingly, *SLC22A6*, *SLC22A7*, *SLC22A8*, *SLC22A9*, and *SLC22A12* mRNAs were not detected in any of the 60 cancer cell lines, though they were found to be expressed in the human mammary (*SLC22A6*, A7, A8, A12), kidney (*SLC22A6*, A7, A8, A9, A12), liver (*SLC22A7*, A9) and retina (*SLC22A6*, A8) mRNA samples. The expression matrix of the 23 extant SLC transporters in the 60 cells is presented in Table S2.

Identification of candidate uptake transporters for anticancer drugs

We hypothesize that a strong correlation of an SLC transporter with the activity of a given compound is due to increased transporter-mediated cellular accumulation of the drug, and that we can thus predict substrates of individual transporters through bioinformatic analysis. To verify this hypothesis, we correlated the expression patterns of SLC transporters with growth inhibition data for 1,429 compounds from the NCI Developmental Therapeutics Program (DTP). In particular, we were looking for positively correlated drug-gene pairs, which may indicate that a given compound can inhibit growth of the cancer cells more strongly if an SLC transporter is overexpressed. Using the expression data presented in Table S2, we calculated the Pearson's correlation coefficient for each gene-drug pair.

Of the resulting 32,867 relationships (23 genes $\times$ 1,429 compounds), the correlation values showed a distribution of r values (Fig. 2) comparable to that of previous correlations for ABC transporters with the same compound subset (5). Our assumption was that the large majority of relationships would, in fact, be uncorrelated and this is indeed the case; 98.4 % of drug-gene correlations are $-0.4 < r < 0.4$. To narrow the list of correlated drug-gene pairs, we used both a simple Bonferroni procedure and the Benjamini-Hochberg False Discovery Rate procedure (21) to adjust for multiple testing of all 28 genes and all 1,429 compounds simultaneously. The analysis yielded several significantly positively correlated gene-drug pairs (at a false discovery rate less than 0.1, see Table S3). To verify that statistical correlations are based on a functional relationship (i.e., where an increase in the function of the gene product (uptake transporter) results in increased drug sensitivity), we performed follow-up experiments in the laboratory.

For our initial *in vitro* validation work we chose to focus on SLC22A4, which was expressed at high levels in kidney, bone marrow, lung, prostate, spinal cord and trachea human tissue samples. The number of drugs from the set of 1429 that showed correlations of at least 0.4 with SLC22A4 was 18, where less than one would be expected in a set of 1429 random correlations. Permutations of the cell lines showed that the mean number of correlations exceeding 0.4 among 1429 correlations computed with permuted cell lines was 1.71. If we take 0.4 as a reasonable threshold for significance, then the Benjamini-Hochberg estimate of the proportion of false positives (FDR) would be $1.71/18 < 0.1$. An initial analysis had indicated that mitoxantrone (NSC301739) and doxorubicin (NSC123127), two anticancer drugs used widely in the anticancer regimens, show a highly significant Pearson correlation with the expression of the SLC22A4 (OCTN1) gene product (Fig. 3 and Table S3). The correlations of the GI_{50} 's of mitoxantrone and doxorubicin with the log2 expression of SLC22A4 (OCTN1) were 0.45 ($p = 6.2 \times 10^{-5}$) and 0.43 ($p = 1.4 \times 10^{-4}$), respectively. Among the SLC genes examined in this study, these drugs revealed only one other (weaker) strong correlation with a second transporter; mitoxantrone with SLCO2A1 ($r = 0.35$), and doxorubicin with SLC22A5 ($r = 0.33$) (Table S3).

Analysis of the SLC22A4 transporter drug-gene pairs revealed that eighteen compounds gave a Pearson correlation coefficient ≥ 0.4 . The NSC compounds along with their correlation coefficients, structure, common name(s) and correlations with other SLC transporters are given in Table S6. 11 of the 18 compounds are organic cations or formulated as acid salts (forming cations in solution), including 8 of the top 10 compounds, which strongly supports the notion that SLC22A4 is an organic cation transporter. We used statistical tools to prioritize gene-drug pairs for additional attention. In addition to the clinically used anticancer agents, we included two compounds that were predicted to be SLC22A4 substrates based on significant drug-gene correlation values. NSC59729 (Sparsomycin, $r = 0.35$) and a structural analog, NSC251819 ($r = 0.34$), were also validated in cytotoxicity assays. These two compounds both possess alkyl sulfoxy moieties similar to those of busulfan (NSC750) described above. Their structures are presented in Fig. S1. (Drugs are designated by their NSC (National Service Center) numbers in the DTP drug database. Information on NSC compounds is available at <http://spheroid.ncicrf.gov/spheroid/>.)

Establishment of stable transfectants of SLC22A4 (OCTN1)

We established stable transfectants of V5-tagged SLC22A4 (OCTN1) using KB-3-1 cell lines (derived from a single clone of human KB epidermoid carcinoma cells). KB-3-1 cells transfected with vector only plasmid were also established as a control (Mock/KB-3-1). The expression and function of SLC22A4 (OCTN1) in the stably transfected cells (SLC22A4 (OCTN1)/KB-3-1) were confirmed by a combination of real-time quantitative RT-PCR, Western blot analysis, immunocytochemical analysis, and accumulation assays. In the Western blot analysis, the protein was detected at approximately 62 kDa in two clones of SLC22A4

(OCTN1)/KB-3-1 (Fig. 4A). Immunocytochemical analysis using anti-V5-FITC antibody confirmed that the expressed protein was localized predominately in the plasma membrane (Fig. 4B). RT-PCR revealed the crossing point (CP) value (where a lower crossing point indicates greater expression) of SLC22A4 in the engineered SLC22A4/KB-3-1 cell line as 24.7. This expression is 5-fold higher than the highest expressing NCI60 cell line, NCI-H226 (CP = 27.17), and 9-fold the highest tissue expression; kidney (CP = 27.9). In the uptake assay, the accumulation level of a known SLC22A4 (OCTN1) substrate, ¹⁴C-tetraethylammonium (TEA), was 7.2-fold higher in SLC22A4 (OCTN1)/KB-3-1 cells than in Mock/KB-3-1 cells (Fig. 4C), indicating that the established cell lines express high levels of functionally competent SLC22A4 protein.

Sensitivity of SLC22A4 (OCTN1) KB-3-1 cells to mitoxantrone and doxorubicin

To test whether SLC22A4 (OCTN1) sensitizes the cells to mitoxantrone and doxorubicin treatment as predicted by the bioinformatic analysis, we compared SLC22A4 (OCTN1)/KB-3-1 with Mock/KB-3-1 in drug sensitivity assays (Fig. 5). The IC₅₀ values of mitoxantrone and doxorubicin in SLC22A4 (OCTN1)/KB-3-1 after 72-hr drug exposure were 4.6- and 3.6-fold lower (indicating greater sensitivity) than those in Mock/KB-3-1, respectively (Table 1). In experiments not documented here we also tested NSC59729 and NSC251819 and found that SLC22A4 (OCTN1)/KB-3-1 was 2.1- and 2.6-fold more sensitive to the two compounds, respectively, than was Mock/KB-3-1 ($P < 0.05$). Cisplatin, a compound not predicted to be toxic to SLC22A4-expressing cells, demonstrated equivalent cytotoxicity to both mock and transporter-expressing cell lines (data not shown).

Uptake of mitoxantrone and doxorubicin by SLC22A4 (OCTN1) KB-3-1 cells

Results obtained with the cytotoxicity assays indicated that the presence of SLC22A4 sensitizes cells. To test if the increased sensitivity of the cells could be explained by an increased uptake of the compounds, we performed accumulation experiments with radiolabeled mitoxantrone and doxorubicin. Cells were incubated with 3 μ M [³H]mitoxantrone or [¹⁴C]doxorubicin at 37°C and reactions were stopped at designated time points by adding excess ice-cold uptake buffer. As shown in Fig. 6, A and B, uptake of both drugs was significantly increased in SLC22A4 (OCTN1)/KB-3-1 cells compared with Mock/KB-3-1 cells. Addition of non-radiolabeled 30 μ M TEA (a known substrate of SLC22A4) to the reaction buffer resulted in a decrease in the accumulation level of [³H]mitoxantrone and [¹⁴C]doxorubicin in SLC22A4 (OCTN1)/KB-3-1 cells, with little effect in Mock/KB-3-1 cells (Fig. 6, C and D), consistent with the notion that TEA and mitoxantrone/doxorubicin are competing for a common transport mechanism.

Discussion

Compared with efflux transporters (i.e., ABC transporters that are considered targets to overcome MDR (4)), relatively little attention has been paid to uptake transporters in cancer research. However, considering that most anticancer drugs need to first enter cancer cells and accumulate in order to be effective, uptake transporters are expected to play a critical role in ensuring drug efficacy (22). There is already evidence that lowered expression of the copper influx transporter CTR1 (SLC31A1) is implicated in the development of cellular resistance to the cancer drug cisplatin (23), which is a substrate for the nutrient transporter (24).

The NCI-60 cancer cell lines have been more extensively profiled at the DNA, mRNA, protein, and functional level than any other set of cell lines in existence (See <http://dtp.nci.nih.gov/> and <http://discover.nci.nih.gov/cellminer>). In addition, patterns of drug activity across the cell lines and patterns of cell sensitivity across the set of tested drugs have been shown to contain detailed information on mechanisms of action and resistance (25,26). The NCI-60 database has been

successfully exploited to identify ABC transporters that confer MDR and compounds whose activity is decreased or potentiated by them (5,27,28). Using the same pharmacogenomic approach, identification of transporters that mediate the uptake of compounds should also be possible. Previous transcript expression profiling of the NCI-60 using cDNA arrays of <9000 elements (29), Affymetrix Hu6800 oligonucleotide chips (19) or a dedicated platform representing the 'transportome' (16) proved useful for identifying molecular biomarkers of chemoresistance. Our aim was to generate more reliable expression data for the SLC22 and SLCO genes to identify functionally relevant drug-gene pairs. Microarray studies are restricted by the limitations of the technology (such as low coverage or low sensitivity). Our earlier meta-analysis of the Staunton (29) and Scherf (19) arrays revealed that the datasets generated by the two platforms show very poor correlation (30). Combined, these two arrays contain only 8 of the 23 genes analyzed in our study (those with detectable expression); the Staunton cDNA array examined 7 SLC transporters that are also reported in our study (SLC22A3, SLC22A4, SLC22A15, SLC22A17, SLCO1A2, SLCO1B1 AND SLCO2B1), the strongest correlation being that of SLC22A3 ($r = 0.43$). The remaining Pearson correlation values range from $r = -0.5$ to $r = 0.29$, revealing little consensus between the common genes in these two datasets. The Scherf Affymetrix data reported only 2 genes common to our RT-PCR data (SLCO1A2, $r = 0.00$ and SLCO2A1, $r = 0.09$) with no correlation evident between the two sets of genes.

We chose to measure transcript expression by quantitative real-time RT-PCR. While PCR is a low throughput (and necessarily labor-intensive) technology, the number of genes of interest here did not warrant a more expensive high-throughput approach. A normalizing gene was not employed, as previous experience while measuring ABC transporter expression by RT-PCR showed high variability in the expression of 5 control genes, and mean expression normalization had reliably predicted drug-gene pairs of known ABC transporter substrates, and new substrates and selective compounds that were validated in vitro (5). We focused our attention on highly significant positive correlations between transporters and individual compounds, with an emphasis on clinically relevant drugs.

The number of positive correlations was similar in the case of SLC transporters (153 drug-gene pairs with $r \geq 0.50$) and ABC transporters (137 drug-gene pairs with $r \geq 0.50$). As there were fewer total SLC drug-gene pairs in this study, this indicates that 0.47 % of compounds were positively correlated with the expression of an SLC transporter, an increase over the 0.20 % discovery rate in the ABC transporter study (5). A positively correlated drug-gene pair indicates that increased expression of the gene is related to increased growth inhibitory activity. In the case of ABC efflux transporters, the mechanism by which the toxicity of positively correlated compounds such as NSC73306 is potentiated by P-glycoprotein is unclear (27,31). The higher ratio of positively correlated drug-gene pairs in the SLC-set is in accord with the function of these transporters as facilitators of influx (Fig. 2).

In contrast to the previous drug-gene correlations observed with ABC transporters (using the same drug activity dataset), the number of significant negative correlations was less pronounced. Of the 68,592 relationships ($48 \text{ genes} \times 1429 \text{ compounds}$) calculated for ABC transporters, 48 drug-gene pairs revealed significant inverse correlations ($r < -0.55$), suggestive of a transporter-substrate relationship in which the transporter protects the cells by keeping the recognized substrates below a cell-killing threshold. In contrast, only two drug-gene pairs ($r < -0.55$) with SLC transporters showed such a strong inverse correlation to any of the drugs analyzed – the naphthalendiones NSC623758 (SLCO2B1, $r = -0.65$) and NSC618315 (SLC22A18, $r = -0.6$). This low number of negative correlations (relative to drug-gene pairs observed among the ABC transporters) is entirely logical given that the SLC families are recognized as importers, and as such are not hypothesized to play a role in actively lowering cellular accumulation (Fig. 2).

It is not clear how coexpression of influx and efflux transporters that are diametrically opposed to one and other in function would affect cytotoxicity (which is tied in most cases to cellular accumulation), and therefore impinge upon correlations between drug activity and SLC/SLCO expression. Despite the considerable overlap in the substrate specificity of organic transporters and members of the ABC transporter family, the bioinformatics analysis has successfully identified mitoxantrone and doxorubicin as SLC22A4 substrates. Correlations between SLC22A4 expression for each of the three best-characterized multidrug resistance efflux transporters revealed that there is not a strong correlation between SLC22A4 and ABCB1 (P-gp, $r = -0.21$), ABCC1 (MRP1, $r = -0.10$) and ABCG2 ($r = -0.09$) expression. The three strongest ABCB1 expressing-cell lines do express lower-than average levels of SLC22A4: NCI-ADR-RES (ABCB1 = 12.28, SLC22A4 = -6.62), HCT-15 (ABCB1 = 10.08, SLC22A4 = -1.83) and UO-31 (ABCB1 = 4.68, -SLC22A4 = -1.33). Grube *et al.* have examined the expression of a SLC22A5/ABCB1 double-transfection cell line and showed that the cell line dramatically increased trans-cell transport, but not accumulation – suggesting that ABCB1 expression could negatively impact on the strength of correlations, yet the majority of NCI 60 cell lines do not express high levels of ABCB1, and removing NCI-ADR-RES, HCT-15 and UO-31 cell lines from the correlation analysis of SLC22A4 and ABCB1 reveals no correlation at all between their gene expression ($r = -0.01$).

There is strong structural consistency among the 18 compounds that correlated with SLC22A4 expression. All but one of the eighteen compounds contains at its core three or four fused rings with a high degree of aromaticity—the exception being NSC750, the alkylating agent busulfan (Table S6). Six of these compounds are highly structurally-related anthracycline derivatives; NSC123127 (doxorubicin, adriamycin), NSC354646, NSC357704, NSC275647 and NSC164011 (rubidazole, zorubicin), the structural variation being at either the terminus of the side chain or substitution on the primary amine of the daunosamine sugar (32). The anthracycline daunorubicin (NSC82115) also appeared in the 50 most correlated compounds with SLC22A4 expression ($r = 0.35$). Five additional compounds, mitoxantrone (NSC301739), piroxantrone (NSC349174), NSC355644, NSC693120 and NSC625530 are derived from anthracenediones; and two closely related compounds possess structural components of etoposide (NSC668380 and NSC644945)(33).

It is known that a given compound may be a low or high specificity substrate for multiple SLC transporters (see for example the SLC Tables at <http://www.bioparadigms.org/slc/intro.asp>). To ascertain whether there appeared to be functional overlap among the compounds that correlated with SLC22A4, we sought compounds with a Pearson correlation coefficient > 0.30 . Thirteen of eighteen compounds returned a positive correlation with one or more SLCO or SLC22 genes (six correlated with two or more alternate SLC genes) and remarkably the eicosanoid transporter SLCO2A1 (8 compounds) and the polyspecific cation transporter SLC22A5 (6 compounds) showed multiple common correlations (Table S6) suggestive of cross-recognition among SLC homologs. SLC22A5 (OCTN2) has been shown to be expressed in human heart endothelial cells (34), and while SLC22A4 expression in the human heart has not been reported previously, both transporters show similar mean-centered mRNA expression in this study (Fig. 1 and Table S1, SLC22A4: 0.52, SLC22A5: 0.82). It may be that SLC-facilitated uptake of the anthracyclines and anthracenediones described above is in part responsible for the clinically limiting cardiotoxicity of these agents (32). Furthermore, the degeneracy in substrate recognition among SLC transporters would result in a number of lower correlation coefficients for a compound (as is seen in our study), rather than the strong (negative) correlation coefficients for efflux substrates of the ABC transporters, as described above.

Among the top-scoring correlations, we were interested in the SLC22A4 (OCTN1)-mitoxantrone and -doxorubicin pairs. SLC22A4 (OCTN1) has been characterized as a low-

affinity carnitine transporter (35), and is best known for its association with Crohn's disease (36). Although some members of SLCO and SLC22 family such as *SLC22A15* have been implicated in carcinogenesis (37), *SLC22A4* (OCTN1) expression has not been linked to cancer. We found *SLC22A4* to be expressed abundantly in the 60 cancer cell lines that originate from a variety of different tissues, such as lung (NCI-H226, EKVX, NCI-H322M, NCI-H460), melanoma (LOX IMVI), CNS (SF-295), kidney (A498, UO-31), and breast (HS578T). Among the 60 cell lines, only OVCAR-8/ADR-RES (38), which overexpresses *MDR1* and is highly resistant to doxorubicin (adriamycin), did not express *SLC22A4*.

Currently, there is no data to suggest the involvement of *SLC22A4* in pharmacological response, yet it cannot be ruled out that it affects cell survival by modulating the electrochemical gradient, or by altering the uptake of physiological compounds and toxins. To test whether the observed correlations reflect an actual ability of the protein to confer sensitivity, we established stable transfectants of *SLC22A4* (OCTN1), and characterized their phenotype in drug sensitivity assays. Expression of *SLC22A4* (OCTN1) resulted in heightened sensitivity to both mitoxantrone and doxorubicin. Although the levels of increased sensitivity were relatively low, ample evidence indicates that even small (2- to 4-fold) changes in drug sensitivity can have a significant impact on the clinical efficacy of anticancer treatment (2). For many anticancer drugs, toxic-to-therapeutic ratios are low and, therefore, even a small change in drug sensitivity can hamper chemotherapy. Expression of *SLC22A4* (OCTN1) also resulted in increased cellular uptake of mitoxantrone and doxorubicin. Presumably, *SLC22A4* (OCTN1) mediates uptake of the weak base compounds, most of which bear positive charges within the physiological pH range and also in the acidic conditions characteristic of the tumor microenvironment (39).

Our bioinformatics results and *in vitro* experiments imply that uptake transporters can facilitate uptake of hydrophobic drugs that have charged forms, and increase total uptake of those drugs even in the presence of a background level of diffusion across the plasma membrane. This is in contrast to Grigat *et al.*, who used competition assays with ergothionein against verapamil and a number of non-cytotoxic histamine antagonists to conclude that *SLC22A4* is not a 'multispecific' cationic drug transporter (40). Since gene expression levels of *SLC22A4* (OCTN1) in our transfectants are comparable to those in some normal tissues (e.g., kidney) and cancers, physiological amounts of *SLC22A4* (OCTN1) may determine the sensitivity of the cell to drugs such as mitoxantrone and doxorubicin. Thus, both effectiveness of anticancer drugs to treat cancer and the toxicity of these drugs in normal tissues may be determined partially by levels of that transporter (and, by extension, other SLC and SLCO transporters).

Expression of *SLC22A4* (OCTN1) also made cells more sensitive to two other compounds, NSC59729 and NSC251819, which were predicted to be substrates for *SLC22A4* (OCTN1) from the correlation analysis (Supplementary Table S3 and Table 1). Overall, these results support the idea that correlation of the gene expression of the SLCO and SLC22 families with activity patterns in the 60 cell lines can be used to identify their potential substrates among the >100,000 compounds tested by the NCI. The correlation data could potentially provide information on significant relationships between drugs and uptake transporters in order to guide the development of transporter-targeted chemotherapy.

It should be noted that the correlative approach described here has statistical limitations: the sample size, the experimental uncertainty, and the fact that multiple tests of significance are being performed simultaneously (hence, the "multiple testing" corrections we describe in the text and Materials and Methods). There are also biological limitations: first, as is the case for almost all transcript profiling studies, there remains uncertainty about the relationship between mRNA and protein expression, and the relationship of both to function; second, correlation does not imply causality; and third, the potential for cross-recognition of substrates would be

expected to weaken strong drug-gene correlations. The correlated drug-gene pairs shown in Table S3 serve as a starting point for the generation of additional hypotheses. The results presented here indicate that (1) several compounds (drugs and drug candidates) inhibit the growth of cancer cells more strongly if an SLC transporter is overexpressed; and (2) SLC transporters can play a decisive role in the chemosensitivity of cancer cells. Additional follow-up experiments are underway to identify further SLC transporters that may influence chemosensitivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ABC, ATP-binding cassette; DTP, Developmental Therapeutics Program; NCI, National Cancer Institute; NSC, National Service Center; OCTN, organic cation/carnitine transporter; P-gp, P-glycoprotein; SLC, solute carrier; SLCO, solute carrier organic anion transporting polypeptide; TEA, tetraethylammonium.

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Figure 1. mRNA expression of SLCO and SLC22 family members in normal tissue samples (**A**) and the NCI-60 cancer cell line panel (**B**). These clustered image maps show patterns of gene expression assessed by real-time quantitative RT-PCR. Red and blue indicate high and low expression, respectively. The hierarchical clustering on each axis was done using the average-linkage algorithm with $1-r$ as the distance metric, where r is the Pearson's correlation coefficient, after subtracting column means. Included among the NCI-60 cancer cell lines are leukemias (LE), melanomas (ME), and cancers of breast (BR), central nervous system (CNS), colon (CO), lung (LC), ovarian (OV), prostate (PR), and renal (RE) origin. Three independent

real-time quantitative RT-PCR measurements were performed. For more details, see Supporting Information Table S1 and Supporting Information Table S2.

Figure 2.

Distribution of r values for the SLC22 and SLCO drug-gene pairs (white bars). The distribution is overlaid with the r value distributions for the same compound drug-gene pairs set with ABC transporters (gray bars), showing the overall similarity in correlation distribution for the two transporter families. The Pearson correlation coefficient r values for ABC transporters are weighted slightly to the negative, as a negative r value is predictive for compounds that are substrates for the efflux transporters, whereas the SLC/SLCO r values are skewed slightly to the positive as substrates of SLC/SLCO transporters would have enhanced cellular accumulation and therefore increased cytotoxicity when a given SLC/SLCO transporter is over-expressed.

Figure 3.

Prediction of substrates for an SLC (SLC22A4 (OCTN1)) from correlation analysis. Scatter plot showing the correlation (r) of *SLC22A4* mRNA expression with sensitivity of the NCI-60 cancer cell lines to mitoxantrone (**A**) and doxorubicin (**B**). **C** and **D**, Chemical structures of mitoxantrone and doxorubicin. (NSC numbers of drugs are shown in parentheses.) Both the GI₅₀ and crossing point (CP) values across the NCI-60 panel were mean-centered and multiplied by -1 (dlogGI₅₀ and dCP respectively) to indicate activity and expression relative to the mean.

Figure 4.

Confirmation of protein expression and function of SLC22A4 (OCTN1) in established stable transfectants used in all follow-up experiments to evaluate whether correlations reveal functional interaction. **A**, Crude membrane fraction from cells stably transfected with mock vector (Mock/KB-3-1) or SLC22A4 (OCTN1) was subjected to Western blot analysis with anti-V5-HRP antibody. Two clones (Clone #1 and Clone #2) of SLC22A4 (OCTN1)/KB-3-1 in which protein expression was confirmed are shown. **B**, Cellular localization of SLC22A4 (OCTN1) in the SLC22A4 (OCTN1)/KB-3-1 and Mock/KB-3-1 cells was assessed by immunocytochemical analysis using anti-V5-FITC antibody. Result of SLC22A4 (OCTN1)/KB-3-1 Clone #1 is shown as a representative. **C**, Uptake of a known substrate ([¹⁴C]TEA, 60 μ M) for SLC22A4 (OCTN1) by SLC22A4 (OCTN1)/KB-3-1 and Mock/KB-3-1 cells. $\dagger P < 0.005$, Mock/KB-3-1 vs. SLC22A4 (OCTN1)/KB-3-1.

Figure 5.

Validation of the prediction by drug sensitivity assay. Growth inhibition of the SLC22A4 (OCTN1)/KB-3-1 (◆) and Mock/KB-3-1 (○) cells treated with either mitoxantrone (**A**) or doxorubicin (**B**) for 72 hrs was evaluated by CCK-8 assay. Result of SLC22A4 (OCTN1)/KB-3-1 Clone #1 is shown as a representative. Each experiment was performed independently at least three times. Note that error bars are mostly obscured by the data points, and that differences are statistically significant (as shown in Table 1).

Figure 6.

Uptake of mitoxantrone and doxorubicin by the SLC22A4 (OCTN1)/KB-3-1 and the Mock/KB-3-1 cells. **A** and **B**, Time course of uptake of [^3H]mitoxantrone (3 μM) and [^{14}C]doxorubicin (3 μM) by the SLC22A4 (OCTN1)/KB-3-1 (◆) and the Mock/KB-3-1 (○) cells. **C** and **D**, Uptake of [^3H]mitoxantrone (3 μM) and [^{14}C]doxorubicin (3 μM) by the SLC22A4 (OCTN1)/KB-3-1 (black bars) and the Mock/KB-3-1 cells (gray bars) in the absence (control) or presence of 30 μM of non-radiolabeled TEA, mitoxantrone, or doxorubicin. Uptake values are shown in fold change to that of the Mock/KB-3-1 cells incubated in the absence of non-radiolabeled drugs. Data are the means $\pm$ SD of values from three independent experiments, each done in duplicate. † $P < 0.005$, ‡ $P < 0.05$, SLC22A4 (OCTN1)/KB-3-1 vs. Mock/KB-3-1 without non-radiolabeled compounds. * $P < 0.005$, ** $P < 0.05$, SLC22A4 (OCTN1)/KB-3-1 without non-radiolabeled compounds vs. SLC22A4 (OCTN1)/KB-3-1 with non-radiolabeled compounds.

Table 1

The IC₅₀ values of mitoxantrone (NSC301739), doxorubicin (NSC123127), sparsomycin (NSC59729) and NSC251819 in the SLC22A4 (OCTN1)/KB-3-1 and Mock/KB-3-1 cells.

	IC ₅₀		Sensitivity factor [*]
	Mock/KB-3-1	SLC22A4/KB-3-1	
Mitoxantrone (NSC301739) (nM)	12.69 ± 1.71	2.75 ± 0.16	4.6
Doxorubicin (NSC123127) (nM)	33.36 ± 3.37	9.31 ± 0.11	3.6
Sparsomycin (NSC59729) (mM)	0.59 ± 0.13	0.28 ± 0.09	2.1
NSC251819 (mM)	15.75 ± 3.98	6.06 ± 0.62	2.6

* The sensitivity factor is defined as the ratio of the mean IC₅₀ value in the Mock/KB-3-1 cells to that in the SLC22A4 (OCTN1)/KB-3-1 cells. Data are expressed as mean ± SD of values from three to four independent experiments with each done in triplicate. *P* < 0.01, Mock/KB-3-1 vs. SLC22A4 (OCTN1)/KB-3-1.