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Membrane Transporters and Channels: Role of the Transportome in Cancer Chemosensitivity and Chemoresistance

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ABSTRACT

Membrane transporters and channels (collectively the transportome) govern cellular influx and efflux of ions, nutrients, and drugs. We used oligonucleotide arrays to analyze gene expression of the transportome in 60 human cancer cell lines used by the National Cancer Institute for drug screening. Correlating gene expression with the potencies of 119 standard anticancer drugs identified known drug-transporter interactions and suggested novel ones. Folate, nucleoside, and amino acid transporters positively correlated with chemosensitivity to their respective drug substrates. We validated the positive correlation between *SLC29A1* (nucleoside transporter *ENT1*) expression and potency of nucleoside analogues, azacytidine and inosine-glycodialdehyde. Application of an inhibitor of *SLC29A1*, nitrobenzylmercaptopurine ribonucleoside, significantly reduced the potency of these two drugs, indicating that SLC29A1 plays a role in cellular uptake. Three ABC efflux transporters (*ABCB1*, *ABCC3*, and *ABCB5*) showed significant negative correlations with multiple drugs, suggesting a mechanism of drug resistance. *ABCB1* expression correlated negatively with potencies of 19 known ABCB1 substrates and with Baker's antifol and geldanamycin. Use of RNA interference reduced *ABCB1* mRNA levels and concomitantly increased sensitivity to these two drugs, as expected for ABCB1 substrates. Similarly, specific silencing of *ABCB5* by small interfering RNA increased sensitivity to several drugs in melanoma cells, implicating *ABCB5* as a novel chemoresistance factor. Ion exchangers, ion channels, and subunits of proton and sodium pumps variably correlated with drug potency. This study identifies numerous potential drug-transporter relationships and supports a prominent role for membrane transport in determining chemosensitivity. Measurement of transporter gene expression may prove useful in predicting anticancer drug response.

INTRODUCTION

Membrane transporters, ion exchangers, and ion channels are encoded by numerous gene families, comprising ~4% of genes in the human genome, with 406 genes encoding ion channels and 883 encoding a broad variety of transporters, of which, 350 are intracellular transporters (1). These genes and their encoded proteins—the transportome—perform important functions for the cell: they provide nutrients, remove unwanted materials, and establish electrochemical gradients across membranes (2). Numerous Mendelian disorders caused by mutations in transporter and channel genes underscore their physiological relevance (3). Membrane transporters also play key roles in pharmacology, affecting the entry of drugs into cells and extrusion of drugs from them. In particular, some ABC (ATP-binding cassette) transporters such as the multiple drug resistance transporter ABCB1 (multidrug resistance 1, P-glycoprotein) mediate energy-dependent efflux of drugs and thereby play major roles in the development of drug resistance (4, 5). Electrochemical gradients established across membranes by transporters and channels influence drug partitioning into and out of cells and cell organelles, *e.g.*, the mitochondria (6).

Given the large number of transport molecules and potential drug substrates for them, only a very small percentage of the possible pharmacological interactions among them have been studied. Those interactions may be particularly important in the chemotherapy of cancer because the balance between beneficial and toxic effects is so finely tuned. The effectiveness of cancer chemotherapy may often depend on the relative transport capacities of normal and cancer cells. Nonetheless, a systematic study of the transportome's roles in chemosensitivity and chemoresistance is lacking.

Robustly positive or negative gene-drug correlations can reflect a role in sensitivity or resistance. Gene-drug relationships have been studied productively in the panel of 60 human cancer cell lines (the NCI-60) used since 1990 by the United States National Cancer Institute (NCI) to screen for anticancer agents (7). For example, in a study using cDNA microarrays, (8, 9), the potency of L-asparaginase, used in the treatment of acute lymphoblastic leukemia, was shown to correlate negatively with asparagine synthetase expression in the leukemia cell subpanel. That relationship provided support for the mechanism by which L-asparaginase was thought to work. Because ovarian cell lines in the panel showed a similar negative correlation, it was hypothesized that a subset of ovarian cancer patients might benefit from L-asparaginase treatment. Analogously, a negative correlation between dihydropyrimidine dehydrogenase expression and 5-fluorouracil (5FU) activity was consistent with the mechanism of 5FU inactivation by the enzyme (9). Similar studies have been done using Affymetrix oligonucleotide arrays (10) to establish gene-drug relationships across the NCI-60. However, none of these microarray studies included more than a modest fraction of the transporter genes, and their results were not always concordant (11).

In light of the importance of the transportome, we designed and produced microarrays (70-mer oligonucleotides) that detect mRNA expression of genes encoding the transporters and channels. Here, we focus on solute carriers (SLCs), ABC transporters acting as extrusion pumps, ion transport ATPases, and select ion channel and pore families. In combination, these genes encode a majority of proteins involved in membrane transport, specifically of drugs. We then applied those arrays to a study of the NCI-60 cells and correlated the resulting expression patterns with potency data for a set of 119 anticancer agents with putatively known mechanisms of action (7). This analysis established numerous significant drug-transporter relationships. We then exploited RNA interference (12, 13) and transport inhibitors to validate three of those relationships. This study provides a basis for additional exploration of drug transport pathways and for optimizing cancer chemotherapy.

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Note: Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org). Primary gene expression data for this study are publicly available at http://pharmacogenomics.osu.edu/. Additional information about array fabrication, experiments, and data analyses are also publicly available at the above web site.

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MATERIALS AND METHODS

Oligonucleotide Microarrays. A spotted 70-mer microarray was developed to measure transporter and channel gene expression as described previously (14, 15). Each probe was printed four times/array, and the median was used to enhance precision of the measurements and minimize the effect of single outlier points.

Array Hybridization. Total RNA was extracted from cell cultures maintained at the NCI under conditions and with passage numbers as used in a previous cDNA microarray study (9, 16). Expression of each gene was assessed by the ratio of expression level in the sample against a pooled control sample from 12 diverse cell lines of the NCI-60 (9). A total of 12.5 μg of total RNA was used for cDNA synthesis and then labeled with Cy5 or Cy3 (control) by amino-allyl coupling. The protocol is available online.[4] In brief, samples from test cells were labeled with Cy5, and the pooled RNA control was labeled with Cy3. The samples were then mixed, and the labeled cDNA was resuspended in 20 μl of HEPES buffer (25 mM, pH 7.0) containing 1 μl of tRNA, 1.5 μl of polyA$^+$, and 0.45 μl of 10% SDS. The mixture was hybridized to the slides for 16 h at 65°C. Slides were washed, dried, and scanned in an Affymetrix 428 scanner to detect Cy3 and Cy5 fluorescence. For the current study, each of the 60 cell line samples was studied by array experiment once, with each probe spotted on the same array four times, using RNA samples obtained from the NCI, to ensure that the cells were under the same conditions and passage numbers as used in a previous cDNA microarray study (9). This facilitates the validation by comparison to already published gene expression data using genes commonly represented on different array types. The amount of RNA/sample was sufficient for only one array hybridization. In Supplementary Tables 1–3, the genes with concordant expression patterns were highlighted, indicating that these results are reproducible. Recently, we have performed array experiments using RNA samples from the NCI-60 cells cultured in our laboratory, with a new batch of 70-mer arrays. A majority of the gene-drug relationships were confirmed by this replicate experiment as well. However, because the mRNAs were collected under different conditions, we decided against using the mean of the two experiments. We make these results available on a public web site for those who wish to look into specific genes in more detail.[5]

Spot Filtering. Background subtraction and calculation of medians of pixel measurements/spot was carried out using GenePix Software 3.0 (Foster City, CA). Spots were filtered out if they had both red and green intensity < 250 units after subtraction of the background or if they were flagged for any visual reason (odd shapes, background noise, and so forth).

Normalization. Most statistical analyses were carried out using the statistical software package R.[6] The plot of $M = \log_2 R/G$ *versus* $A = \log_2 \sqrt{R \times G}$ shows dependence of the log ratio M on overall spot intensity A (data not shown), where R and G are red and green intensity, respectively. Therefore, an intensity-dependent normalization method was preferred over a global method. To correct intensity and dye bias, we used location and scale normalization methods, which are based on robust, locally linear fits, implemented in the *SMA* R package (17). This method is based on transformations:

$$R/G \rightarrow \log_2 \mathrm{R/G} - \mathrm{cj\ (A)}$$

$$= \log_2 \mathrm{R/kj\ (A)} \times \mathrm{G} \rightarrow (1/\mathrm{aj}) \times \log_2 \mathrm{R/kj\ (A)} \times \mathrm{G},$$

where $c_j(A)$ is the Lowess fit of the M *versus* A plot for spots on the j^{th} grid of each slide, and a_j is the scale factor for the j^{th} grid (to obtain equal variances along individual slides). After performing these transformations, the gene expression level of each probe was set to be the median of the four copies of that probe. The box plots of the log ratios for each of the 60 slides are centered close to 0 with similar spreads, and on average, 10 outliers/slide were observed (data not shown). In this situation, we decided not to adjust for scale normalization between slides because the noise introduced by scale normalization of different slides may be more detrimental than a small difference in scale.

Correlation Analysis between Gene Expression and Drug Activity. Growth inhibition data (GI_{50} values for 60 human tumor cell lines) were those obtained by the Developmental Therapeutics Program.[7] Values were expressed as potencies by using the negative log of the molar concentration calculated in the NCI screen. We focused on 119 drugs for which the mechanism of action is largely understood (7). The drug data can be found online.[8] Pearson correlation coefficients were calculated for assessment of gene-drug relationships. Confidence intervals and unadjusted P values were obtained using Efron's bootstrap resampling method (18), with 10,000 bootstrap samples for each gene-drug comparison. To reduce the number of false-positive correlations among 87,000 comparisons, we controlled for false discovery rate as described previously (19). However, because of computational limitations introduced by the bootstrapping technique, using 10,000 samplings yielded only bootstrap estimators with a resolution of 0.0001. To control false discovery rate at the level 0.05, criteria would have to be too stringent, *i.e.*, if only $P = 0$ was regarded as significant. Therefore, an arbitrary cutoff of 0.001 was used for the unadjusted bootstrap P values. This cutoff is expected to detect more true gene-drug associations at the expense of increasing the number of false-positive ones to be validated by other means.

Small Interfering RNA (siRNA)-Mediated Down-Regulation of Gene Expression. siRNA duplexes for *ABCB1* were chemically synthesized by Qiagen, Inc. (Valencia, CA). The target sequence was 5′-AAGCGAAGCAGTGGTTCAGGT-3′, beginning from nt 2113 of the *ABCB1* mRNA sequence NM_000927, as recommended.[9] Chemically synthesized mock siRNA (fluorescein-labeled, nonsilencing) was also purchased from Qiagen, Inc. siRNA duplexes for *ABCB5* were synthesized using the Silencer siRNA construction kit (Ambion, Austin, TX). The three target sequences were as follows: 5′-AAAGGAGCTCAAATGAGTGGA-3′ (ABCB5_745); 5′-AAGTGGAGAATCGCTGACCTT-3′ (ABCB5_930); and 5′-AACAGTTTTCTCGATGGCCTG-3′ (ABCB5_1114), which are located at nt 745, 930, and 1114 of the *ABCB5* mRNA sequence BC044248, respectively.

Cell lines, obtained from Division of Cancer Treatment and Diagnosis at NCI were cultured in RPMI 1640 containing 10% heat-inactivated FCS in a 5% CO_2 incubator at 37°C. Transfection was performed with TransMessenger Transfection Reagent (Qiagen, Inc.). To down-regulate *ABCB1* or *ABCB5*, cancer cells were transfected with 0.3 or 0.6 μM siRNA. For RNA extraction, cells were harvested 48 h after transfection. To measure cytotoxic drug potency, cells grown in 6-well plates were subcultured into 96-well plates 24 h after transfection.

Cytotoxicity Assay. 5FU, azacytidine, camptothecin, and mitoxantrone were obtained from Sigma. The other compounds were from the Developmental Therapeutics Program at NCI. Drug potency was tested using a proliferation assay with sulforhodamine B, a protein-binding reagent (20), except for cell lines HL-60 and K-562. In each experiment, 3000–5000 cells/well were seeded in 96-well plates and incubated for 24 h. Anticancer drugs were added in a dilution series in 6 replicated wells. After 4 days, incubation was terminated by replacing the medium with 100 μl of 10% trichloroacetic acid (Sigma, St. Louis, MI) in 1 × PBS, followed by incubation at 4°C for at least 1 h. Subsequently, the plates were washed with water and air-dried. The plates were stained with 100 μl of 0.4% sulforhodamine B (Sigma) in 1% acetic acid for 30 min at room temperature. Unbound dye was washed off with 1% acetic acid. After air-drying and resolubilization of the protein-bound dye in 10 mM Tris-HCl (pH 8.0), absorbance was read in a microplate reader at 570 nm. To determine IC_{50} values, the absorbance of control cells without drug was set at 1. Dose-response curves were plotted using SigmaPlot software (RockWare, Golden, CO). Each experiment was performed independently at least three times. Student's t test was used to determine the degree of significance. To study the effect of an ABCB1 inhibitor, 10 μM verapamil (Sigma) were added to the cells 20 min before and during exposure to drugs, and the plates were incubated for 3 days.

The chemosensitivity of HL-60 and K-562 cells to inosine-glycodialdehyde and azacytidine was assessed with the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt assay (Sigma). Cells were seeded (5,000 cells/well for K-562 and 10,000 cells/well for HL-60) in 96-well plates and incubated for 24 h before exposure to graded concentration of each

[4] Internet address: http://derisilab.ucsf.edu/pdfs/amino-allyl-protocol.pdf.
[5] Internet address: http://pharmacogenomics.osu.edu/.
[6] Internet address: http://www.r-project.org.
[7] Internet address: http://dtp.nci.nih.gov.
[8] Internet address: http://discover.nci.nih.gov.
[9] Internet address: http://www1.qiagen.com/products/genesilencing/cancersirnaset.aspx.

drug for 72–96 h. The effect of a SLC29A1 transport inhibitor was assessed by adding 100 nM nitrobenzylmercaptopurine ribonucleoside (Sigma) for 20 min before and during drug exposure. Each experiment was performed independently at least twice.

Real-Time Quantitative Reverse Transcriptase-PCR (RT-PCR). Total RNA was prepared by using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis (visual presence of sharp 28S and 18S bands). The RNA was quantitated by spectrophotometry. One μg of total RNA was incubated with DNase I and reverse transcribed with oligo(dT) with Superscript II RT-PCR (Life Technologies, Inc.). One μl of reverse transcriptase product was amplified by primer pairs specific for selected genes. Primers were designed with Primer Express software (Applied Biosystems, Foster City, CA), and *ACTB* (β-actin) was used as a normalizing control. Relative gene expression was measured with the GeneAmp 7000 Sequence Detection system (Applied Biosystems). Conditions and primer sequences are available on request.

RESULTS

Correlating Transportome Gene Expression with Drug Activity. The 70-mer microarray comprises 632 probes targeting 461 transporter genes and 151 channel genes, as well as 100 probes for unrelated genes (14, 15). The array was designed before the human genome sequence became available. It includes probes for 215 SLC genes of ~300 SLC genes that have been cloned to date (SLC, transporter of SLC families, including the structurally similar SLC ion exchangers), 40 of the 48 ABC transporters (ATP-driven extrusion pumps), plus a few contigs assembled from expressed sequence tags of putative SLC transporters not represented in the current annotation of the human genome. Furthermore, we include probes for a majority of ATPases (active ATPase ion transporters other than the ABC transporters) and channel genes encoding Na^+, K^+, Ca^{2+}, and Cl^- channels. Therefore, our array covers a majority of genes relevant to drug transport. Twenty genes were represented by probes for two different domains of the target molecule to permit comparison of the results from different sites of hybridization.

After the experimental data on transporter expression patterns had been analyzed, previously published cDNA and Affymetrix expression data for limited numbers of the genes (9, 10) were used as controls against which to compare our results. Hierarchical clustering of cell lines based on gene expression and corroboration of gene expression data by comparing multiple expression data sets are reported as supplementary information online.

To determine the relationship between gene expression for the 732 probes and growth inhibitory potencies of the 119 drugs, we calculated the Pearson correlation coefficient for each gene-drug pair. Of the resulting 87,108 gene-drug correlations, 2.5% were distributed either >0.262 or < -0.267 (histogram not shown). Therefore, on the assumption that the large majority of relationships would, in fact, be uncorrelated, we used $r > 0.3$ as a heuristic criterion for potentially significant correlations to cut down on the number of gene-drug pairs, the statistical significance of which we then assessed by computing unadjusted bootstrap *P* values (based on 10,000 bootstrap samples for each pair; Ref. 18). We next used the *P* values to prioritize gene-drug pairs for additional attention. For genes not previously known to be related to chemosensitivity, we used $P = 0.001$ as a heuristic cutoff. For transporter genes previously implicated in chemosensitivity to any of the 119 drugs, we used $P = 0.05$ as the cutoff in searching for additional substrates.

Using these criteria, we identified 177 gene-drug pairs (~0.2%) that showed positive correlations and 210 pairs (~0.2%) that showed negative correlations. A total of 145 genes was potentially linked in this way to at least one drug. Once a candidate gene had been identified using the stringent criterion ($P < 0.001$), we again used the more relaxed value, $P < 0.05$, to identify additional potential substrates in the 119 drug set. Table 1 shows representative genes and substrates. For each gene listed, the number of correlated drugs is shown for both cutoff points, $P < 0.001$ and $P < 0.05$ (Table 1; a full listing is found in Supplementary Tables 1–3). The criterion for assessing the validity of expression values for a candidate gene included concordance in gene expression (Pearson correlation coefficient, $r > 0.3$) in at least one comparison between the 70-mer array and other expression data sets for the NCI-60 when available (Supplementary Tables 1–3).

SLCs and Chemosensitivity. SLCs encode the transportome for amino acids, peptides, sugars, monocarboxylic acid, organic cations, phosphates, nucleosides, and water-soluble vitamins. Table 1 and Fig. 1 summarize the results for SLC genes that showed significant Pearson correlation for at least one drug (see Supplementary Information for a complete list). The fact that several of these transporters have previously been implicated in drug transport provided a validation of the correlation analysis. In other cases, correlated drugs were similar in structure to natural substrates of the respective transporters, strengthening the hypothesis that these drugs are indeed substrates. Nucleobase transporters (*SLC23A2*) and nucleoside transporters of both ENT (equilibrative) and CNT (concentrative) families showed positive correlations with a number of antimetabolite drug analogues (Fig. 1*A*), as expected for transporters that facilitate drug entry into cells. This observation is consistent with the hypothesis that these transporters are essential for nucleoside drug uptake (21).

As a concrete example, consider *SLC29A1* (*equilibrative nucleoside transporter 1*; Ref. 22), which correlates positively with azacytidine (Figs. 1*A* and 2*A*). Fig. 2*A* shows a sorted list of the correlations of *SLC29A1* with each of the 119 drugs. This list identifies possible substrates of the transporter. The highly correlated nucleoside analogue drugs are indicated. To validate positive correlations between *SLC29A1* expression and the potency of inosine-glycodialdehyde and azacytidine, we exposed leukemia cells HL-60 and K-562, which express a high level of *SLC29A1*, to graded concentrations of these compounds in the presence and absence of nitrobenzylmercaptopurine ribonucleoside, a tight-binding inhibitor of *SLC29A1* (23). The presence of nitrobenzylmercaptopurine ribonucleoside reduced the cytotoxic effects of inosine-glycodialdehyde > 10-fold and that of azacytidine 2~3-fold in both cell types (Table 2), indicating that SLC29A1 (equilibrative nucleoside transporter 1) plays an important roles in cellular uptake of these compounds.

Impaired transport of folate drugs is a potential mechanism of drug resistance, whereas high transport capacity would be expected to increase chemosensitivity. As shown in Fig. 1*B*, *SLC19A1*, a member of the reduced folate carrier protein family, correlated positively with folate analogues such as aminopterin derivative and methotrexate. These results are consistent with previous findings (24, 25) and extend the spectrum of putative substrates, although the known *SLC19A1* substrate aminopterin did not reach statistical significance ($r = 0.20$; $P = 0.07$), possibly because other sensitivity/resistance mechanisms may predominate. Although not known to transport folates, *SLC19A2* and *A3* also showed positive correlations with antifolate drugs, a finding that requires additional verification.

Amino acid transporters have received less attention as drug carriers, although the activity of polar amino acid analogues is likely to depend on transporter function. Fig. 1*C* shows several amino acid transporters that correlated with the potency of amino acid analogues, a finding not previously noted. For example, *SLC38A2* (or *ATA2*), a member of the amino acid transport system A, correlated positively with acivicin and L-alanosine, the amino acid analogue drugs. *SLC25A12*, which encodes a calcium-stimulated aspartate/glutamate

Table 1 *Select transporter and channel genes showing significant correlations with chemosensitivity*[a]

	No. of drugs					
	$P < 0.001$		$P < 0.05$			
Gene	$r > 0$	$r < 0$	$r > 0$	$r < 0$	Substrate	Representative drug
SLC[b] transporters						
SLC23A2	1	0	8	0	Nucleobase	5FU
SLC28A1	0	0	7	0	Nucleoside	Aminopterin, 6MP
SLC28A3	2	0	38	0	Nucleoside	Thioguanine, cytarabine (araC), gemcitabine
SLC29A1	2	0	21	0	Nucleoside	CCNU, azacytidine, thioguanine
SLC29A2	0	0	2	1	Nucleoside	alpha-2-Deoxythioguanosine, inosine-glycodialdehyde
SLC19A1	0	0	19	0	Folate	6MP, gemcitabine
SLC19A2	2	0	24	1	Folate	Tetraplatin, iproplatin, an-antifol, trimetrexate
SLC19A3	0	0	4	0	Folate	an-antifol
SLC1A1	1	0	24	1	Amino acid	L-Asparaginase, L-alanosine
SLC1A4	3	1	11	12	Amino acid	Asaley, Taxol analogue, Acivicin, L-alanosine
SLC3A1	0	0	4	0	Amino acid	L-Asparaginase
SLC7A2	0	0	13	0	Amino acid	L-Alanosine
SLC7A3	0	0	12	0	Amino acid	L-Asparaginase
SLC7A8	0	0	0	14	Amino acid	*N*-phosphonoacetyl-L-aspartic acid
SLC7A9	0	0	3	1	Amino acid	Acivicin
SLC7A11	2	0	12	5	Amino acid	Anthrapyrazole, colchicine, L-alanosine
SLC15A1	0	0	5	0	Peptide	Fluorodopan, teroxirone, etoposide, L-asparaginase
SLC25A12	4	0	29	0	Aspartate glutamate	Thioguanine, *N*-phosphonoacetyl-L-aspartic acid
SLC25A13	0	0	0	41	Aspartate glutamate	L-Asparaginase, CPT, Hepsulfam
SLC38A2	1	0	14	0	Amino acid	Maytansine, acivicin, L-alanosine
SLC38A5	2	0	25	0	Amino acid	Clomesone, Colchicine, L-asparaginase
SLC2A5	0	2	0	12	Glucose	Aminopterin, aminopterin
SLC2A11	2	0	38	0	Glucose	Anthrapyrazole, oxanthrazole
SLC9A3R2	2	0	21	0	Sodium/hydrogen	CPT,9-MeO, L-asparaginase
LOC133308	7	0	51	0	Sodium/hydrogen	CCNU, 6MP, doxorubicin, Taxol analogue
SLC4A7	18	0	56	0	Sodium bicarbonate	Mitomycin, spiromustine, CPT,10-OH, mitoxantrone
ABC transporters						
ABCB1	0	3	2	32	Multiple drugs	Bisantrene, Taxol analogue
ABCB5	0	1	1	25	Unknown	CPT,7-CI
ABCC3	0	2	0	18	Organic anions	Vincristine, methotrexate-derivative
Ion pump						
ATP1A1	0	5	6	43	Sodium/potassium	Uracil mustard, CPT, 11-formyl (RS)
ATP1A3	0	3	0	24	Sodium/potassium	CCNU, daunorubicin, 5-6-dihydro-5-azacytidine
ATP1B1	0	10	0	34	Sodium/potassium	CCNU, tetraplatin, inosine-glycodialdehyde
ATP1B3	0	0	2	20	Sodium/potassium	Daunorubicin, 5FU
ATP1G1	0	0	4	14	Sodium/potassium	Tetraplatin, Taxol analogue
ATP2A1	1	0	12	0	Calcium	Morpholino-adriamycin, doxorubicin, 5FU
ATP2A3	0	0	37	0	Calcium	BCNU, gemcitabine
ATP2B3	0	0	0	8	Calcium	Colchicine-derivative, Taxol analogue
ATP2B4	0	11	0	44	Calcium	Tetraplatin, methotrexate, 5FU, Taxol analogue
ATP2C1A	0	3	0	31	Calcium	Iproplatin, mechlorethamine, deoxydoxorubicin
ATP6V1D	0	0	0	25	Proton	Daunorubicin, methotrexate, Taxol analogue
Ion channel						
AQP1	0	4	0	20	Water	Aminopterin, an-antifol, methotrexate
AQP4	0	1	0	10	Water	L-Alanosine
AQP9	1	0	11	1	Water, urea, arsenite	Taxol analogue
MIP	2	0	46	0	Water	Pipobroman, Halichondrin B
CACNA1D	0	4	0	37	Calcium	Mitozolamide, cyclodisone, deoxydoxorubicin

[a] Underlined drugs negatively correlate with expression of the corresponding genes while all other drugs have positive correlations. For each gene, the number of drugs with positive or negative correlation (r) is shown with two cutoff points, $P < 0.05$ and $P < 0.001$. $r > 0$, positive correlation; $r < 0$, negative correlation. For a complete list, see Supplementary Tables 1–3.

[b] SLC, solute carrier.

carrier protein (Aralar1) located in the mitochondrial inner membrane, showed positive correlation with *N*-phosphonoacetyl-L-aspartic-acid. In contrast, *SLC25A13*, which encodes Citrin, another calcium-stimulated aspartate/glutamate transporter in mitochondria homologous to Aralar 1, showed negative correlation with L-asparaginase (−0.55), possibly by providing aspartate precursor to the cells. Moreover, the correlation coefficient was −0.96 (confidence interval −1.00 to −0.87) for the six leukemic lines and −0.98 (confidence interval −1.00 to −0.92) for the six ovarian lines. This result parallels previous studies with the NCI-60 that implicated *asparagine synthetase* in drug resistance, particularly in leukemic cells (9). *SLC25A13* and *asparagine synthetase* play roles in urea and arginine synthesis (26), and both are located in chromosome 7q21.3, separated by <100 kb (*SLC25A13* is centromeric of *asparagine synthetase*). Possible coordinate expression or chromosomal amplification involving these two genes should be considered for future study.

Several SLC genes correlated with multiple drugs of different structures (Supplementary Table 1). This correlation pattern may reflect functions of the transporter other than a transporter-substrate relationship. For example, some nutrient transporters (glucose, amino acids, organic anions, and peptides) may be up-regulated because of the increased energy needs of cancer cells. Although amino acid transporter *SLC7A11* positively correlated with L-alanosine (Fig. 1), it also showed significant negative correlations (Table 1). SLC7A11 forms a heteromultimeric complex with SLC3A2 (the amino acid transport system X_C^-), and both showed negative correlation with multiple drugs, including cisplatin (Supplementary Table 1). The amino acid transport system X_C^- mediates cystine entry coupled with the exodus of glutamate and thereby regulates intracellular glutathione levels. This has been shown to contribute to cisplatin resistance in cancer cell lines (27). Similarly, negative correlations between drugs and glucose transporters may be related to the impact of glucose metabolism and apoptosis (28, 29). Thus, glucose transporters could affect drug potency either by serving as drug carriers or by modulating

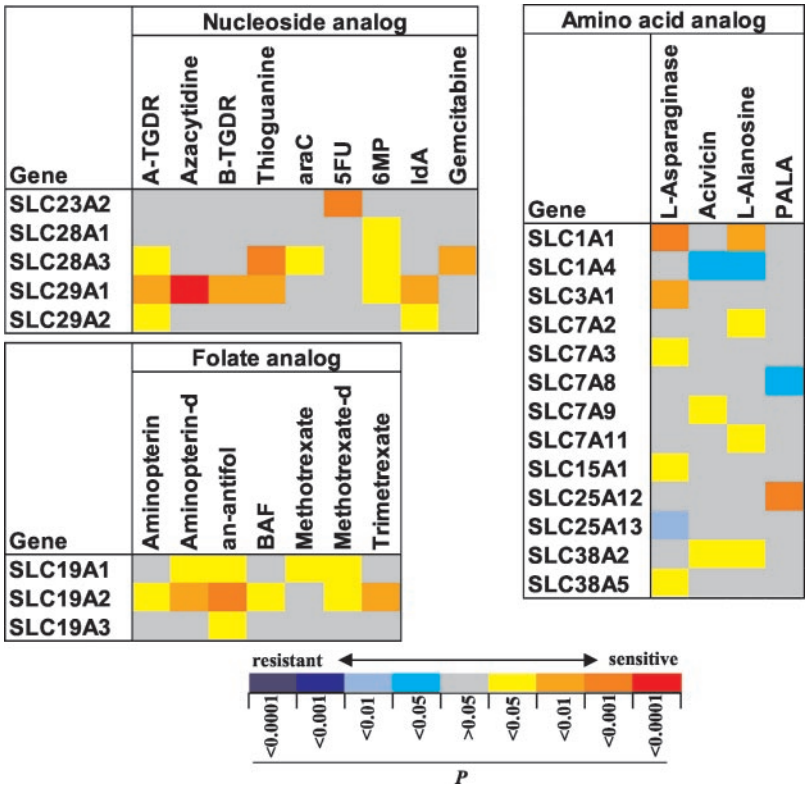

Fig. 1. Correlation between potencies of anticancer drugs and expression of nucleoside, folate, and amino acid transporters. The color code represents the bootstrap *P* and reflects the sign of the correlation coefficient. *A*, nucleoside transporters and nucleoside analogues: A-TGDR, α-2′-deoxythioguanosine (NSC 71851); azacytidine (NSC 102816); B-TGDR, β-2′-deoxythioguanosine (NSC 71261); thioguanine (NSC 752); araC, cytarabine (NSC 63878), 5FU, fluorouracil (NSC 19893); 6MP, 6-mercaptopurine (NSC 755); IdA, inosine-glycodialdehyde (NSC 118994); gemcitabine (NSC 613327). *B*, folate transporters and folate analogues: aminopterin (NSC 132483); aminopterin-d, aminopterin derivative (NSC 134033); an-antifol (NSC 623017 and NSC 633713); BAF: Baker's-antifolate (NSC 139105); methotrexate (NSC 740); methotrexate-d, methotrexate derivative (NSC 174121); trimetrexate (NSC 352122). *C*, amino acid transporters and amino acid analogues: L-asparaginase (NSC 109229); acivicin (NSC 163501); L-alanosine (NSC 153353); PALA, *N*-phosphonoacetyl-L-aspartic acid (NSC 224131).

cellular drug toxicity. As shown in Table 1, expression levels of several glucose transporters (*e.g.*, *SLC2A5*) are positively or negatively correlated with numerous drugs. These results provide the rationale for additional analysis of underlying mechanisms, including drug transport and other biological functions of the SLC transporters.

Intracellular pH has been shown to affect cellular response to anticancer drugs. Two SLC ion exchangers, a bicarbonate transporter and a sodium-proton exchanger (30) function as pH regulators in tumor cells (30). Among members of the Na^+/H^+ exchanger family, *SLC9A3R2* showed positive association with multiple drugs (Table 1). Moreover, an expressed sequence tag encoding a hypothetical protein, LOC133308, that contains a Na^+/H^+ exchanger motif correlated positively with several drugs. Among the bicarbonate transporters, *SLC4A7* correlated positively with 56 drugs. Therefore, genes that affect pH may influence many different ionizable drugs by changing pH gradients that determine drug partitioning into cells and cell organelles.

ABC Transporters and Chemoresistance. Negative correlations indicative of a possible role in chemoresistance occurred with 11 ABC genes (Supplementary Table 2), of which, 9 had previously been implicated in drug resistance (4, 31). However, only 4 ABC transporter genes showed highly significant negative correlations with drugs ($P < 0.001$). Expression data obtained by other methods validated results for three of these genes, *ABCB1*, *ABCC3*, and *ABCB5*. (Table 1). The fourth gene, a known chemoresistance transporter, *ABCC1* (*multidrug-resistant protein 1*), was not additionally considered because various array types gave discrepant results.

Expression levels of *ABCB1* (or *multidrug resistance 1*, *P-glycoprotein*) significantly correlated with potency of many drugs (Table 1), consistent with numerous previous studies (8, 32, 33). Previously *ABCB1* mRNA and protein levels (32, 34) and function in terms of rhodamine efflux (33) have been correlated with drug activity data against NCI-60. These studies produced a list of drugs that have been predicted as P-glycoprotein substrates and experimentally verified. We plotted the ordered *ABCB1* correlation coefficients for all 119 drugs and obtained a clear separation between known ABCB1 substrates and nonsubstrates (Fig. 2*B*). Using the dual criteria of $P < 0.05$ and $r < -0.3$, we identified all known substrates of ABCB1 plus a geldanamycin analogue (GA) (NSC 330500) and Baker's antifol (BAF) (NSC 139105; Table 3). Negative ABCB1-GA and ABCB1-BAF correlations suggested that these drugs are substrates of the transporter. To validate this new finding, we used a chemically synthesized siRNA duplex to target *ABCB1* in NCI/ADR-RES and HCT-15 cells, which express high levels of *ABCB1*. Real-time RT-PCR demonstrated that 40 h after treatment, siRNA substantially reduced *ABCB1* mRNA levels by 74 and 68% in NCI/ADR-RES and HCT-15, respectively. When we compared growth inhibitory IC_{50} values for siRNA-treated and mock-treated control cells using a sulforhodamine B cell proliferation assay, the sensitivity of NCI/ADR-RES to paclitaxel, bisantrene, GA, and BAF was 2.0–7.6-fold greater in the siRNA-treated cells (Table 2 and Supplementary Fig. 3*A*). The sensitivity to 5FU, a non-P-glycoprotein substrate, was unaffected by siRNA silencing. The sensitivity of HCT-15 to GA and BAF was 1.3- and 1.5-fold greater in the siRNA-treated cells (Table 2). Because these changes were relatively small, we used chemical inhibitors to determine whether GA and BAF are indeed substrates of ABCB1 in HCT-15. We treated the HCT-15 cells with increasing concentrations of GA or BAF, with and without the presence of ABCB1 antagonist verapamil. For GA and BAF, respectively, increase in potency was 3.5- and 43.8-fold after verapamil treatment. Therefore, application of RNA interference gene silencing and ABCB1 antagonist supports the hypothesis that GA and BAF are ABCB1 substrates.

ABCC3, which encodes multidrug resistance-associated protein 3, showed significant negative correlation with a methotrexate derivative. That finding is consistent with a study that overexpression of *ABCC3* results in high-level resistance to methotrexate (35).

ABCB5, a putative ABC transporter of unknown physiological

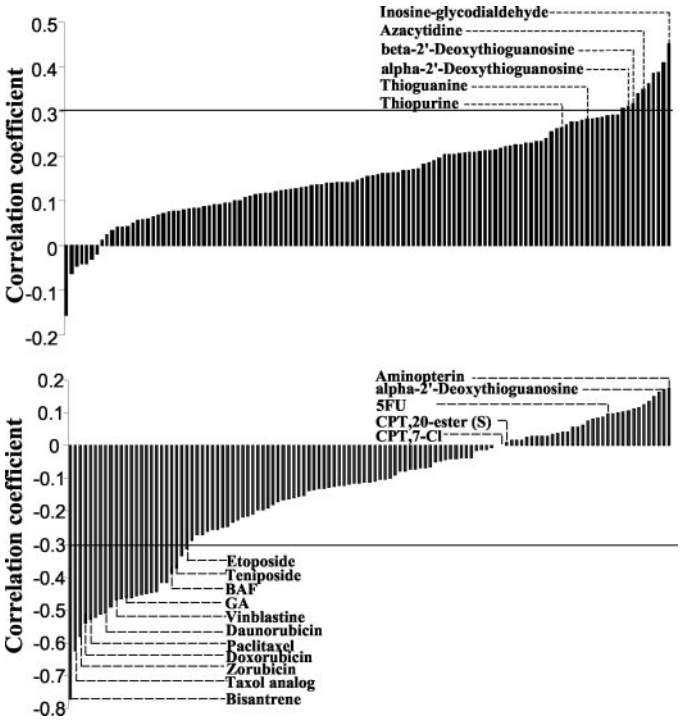

Fig. 2. Sorted correlation coefficients between *ABCB1* and *SLC29A1* expression and cytotoxic potencies of 119 drugs. *A*, plot of correlation coefficients of the nucleoside transporter SLC29A1. Positive values of $r > 0.3$ suggest possible transporter substrate relationships needed for drug entry into the cells. The highest positive correlation is seen for inosine-glycodialdehyde. *B*, sorted correlation coefficients for *ABCB1*. ABCB1-MDR1 substrates such as bisantrene and doxorubicin (*bold*) show strong negative correlations with *ABCB1* expression. A correlation coefficient of -0.3 is the approximate cutoff for statistical significance (see also Table 3).

function, showed strong negative correlation with camptothecin, 7-Cl ($P < 0.001$). Our array data indicated that *ABCB5* is selectively expressed in melanoma cells, suggesting a tissue-specific role in chemoresistance. To identify suitable *ABCB5* domains for siRNA-mediated gene silencing, we synthesized siRNA duplexes against three target domains and transfected them into SK-MEL-28 cells. Real-time RT-PCR demonstrated that siRNA-ABCB5_930 was most effective in down-regulating *ABCB5* (resulting in >80% reduction in *ABCB5* mRNA level). SK-MEL-28 cells transfected with siRNA-ABCB5_930 were 2–3-fold more sensitive to several drugs, including camptothecin, 10-OH camptothecin, and 5FU, than were control cells transfected with mock siRNA (Table 2, Supplementary Fig. 3*B*). In contrast, no change in potency was observed for mitoxatrone. These results support the hypothesis that *ABCB5* represents a novel chemoresistance gene. It remains to be determined whether the chemoresistance conferred by *ABCB5* expression is caused by increased drug efflux or to some other mechanism. *ABCB5* is selectively expressed in the 6 of 8 melanotic melanoma cells. It was also selectively expressed in MDA-MB435 and MDA-N, the Erb/B2 transfectant of MDA-MB435, supposedly of breast origin but with a pharmacological, genomic, and proteomic signature of melanotic melanoma (16). Because *ABCB1* (multidrug resistance 1) is only weakly expressed in melanomas (only 2 of 8 lines expressing low level of *ABCB1* mRNA), we postulate that ABCB5 plays an important drug resistance role in those cells. Recently, a cDNA derived from the ABCB5 locus was cloned and shown to be expressed in melanocytes (36). The cloned sequence indicated an unusual structure, with an extracellular ABC module, followed by five transmembrane domains and an intracellular ABC module (36). Function in cell fusion and rhodamine efflux was implied. However, the probe representing *ABCB5* on our arrays was derived from overlapping human expressed sequence tags assembled into a contig (14, 15), which is partially homologous with the cloned cDNA (36). Subsequent analysis of the mRNA expression of the *ABCB5* expressed sequence tag and the cloned *ABCB5* gene by real-time RT-PCR in 20 of the NCI-60 cell lines revealed that both mRNA isoforms are expressed at a constant ratio. Moreover, correlating the RT-PCR to drug activity data confirmed the notion that *ABCB5* may serve as a broadly selective drug resistance transporter.[10]

The known chemoresistance genes *ABCA2*, *ABCB2*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCC4*, and *ABCC5* were negatively associated with several drugs ($P < 0.05$; Supplementary Table 2). However, the suggested drug substrates differed from those previously reported and measured expression of these genes did not correlate well with results obtained from other array studies (Supplementary information online). This discrepancy may have been related to insufficient sensitivity of the 70-mer arrays, to cross-hybridization, or to other technical problems involving hybridization for these genes. As with all microarray gene expression studies, not all probes function optimally. Additional validation studies would be needed before chemoresistance could be inferred or excluded.

Several ABC transporters (*e.g.*, *ABCA1*, *ABCB3*, and *ABCG1*) showed positive drug correlations (Table 1). *ABCG1*, a half-transporter involved in cholesterol and phospholipid transport (37), correlated positively with doxorubicin and other ABCB1 substrates. The mechanism underlying these observations remains to be determined.

Role of Ion Pumps (ATPases) and Channels in Chemosensitivity/Resistance. To identify ion pumps associated with drug activity, we investigated ATPases that maintain cellular electrical gradients (Table 1 and Supplementary Table 3). Genes that encode *ATP1A1*, *1A3*, *1B1*, *1B3*, and *1G1* isoforms of the α, β, and γ subunits of Na^+/K^+-ATPase showed negative correlations with a number of drugs. The Na^+/K^+-ATPase is responsible for maintaining electrochemical gradients. It plays a role in cell proliferation (38) and appears to serve as resistance factor.

ATP2A1, *A3*, *B3*, *B4*, and *C1A* genes that encode subunits of the calcium pumps showed either positive or negative correlation with drugs. The opposite effects may be because of the mechanism of action and charge of the chemotherapeutic agent. Because calcium content, release, and transfer from the endoplasmic reticulum to mitochondria appears to play a key role in apoptosis (39), calcium fluxes may play important roles in drug toxicity. *ATP6V1D* encodes a subunit of vacuolar H^+-ATPase, which mediates acidification of intracellular organelles. It correlated negatively with 25 drugs. This finding is consistent with previous observations that pH regulation by the vacuolar ATPase is a factor in resistance to anticancer drugs (40). In sum, the findings reported here indicate that sodium/potassium, calcium, and proton ATPases all affect chemosensitivity.

Table 1 also lists genes that encode channels. *AQP1* and *AQP4*, which encode water channel proteins, correlated negatively with folate and amino acid drug analogues. Both *AQP1* and *AQP4* are highly expressed in brain tumors and carcinomas but are undetectable in normal epithelial cells (41, 42). *AQP9* and *MIP*, aquaporins involved in transport of water, urea, and glycerol, correlated positively with several drugs. We do not know at this point whether these gene products mediate drug transport directly or affect sensitivity and resistance by indirect mechanisms.

Ion channels modulate electrochemical gradients generated by ion pumps and ion exchangers. Maintenance of a strong electrochemical gradient is vital to the cell and a potentially strong influence on drug activity. K^+ and Cl^- leakage currents tend to polarize cells, whereas

[10] Y. Huang and W. Sadée, unpublished data.

Table 2 *Shift of the dose-response curves of cytotoxic drugs by down-regulation or inhibition of three transporters*[a]

Gene	Cell line	Drug	IC_{50} (μM) Control	IC_{50} (μM) Treatment[b]	Fold reversal[c]
ABCB1	NCI/ADR-RES	Paclitaxel	8.0	1.1	7.6
		Bisantrene	>100	23	>4.2
		Geldanamycin	6.8 ± 1.1	3.6 ± 0.6[d]	2.0 ± 0.5
		Baker's antifol	420 ± 120	131 ± 36[d]	3.2 ± 0.5
		5FU[e]	7.8 ± 1.7	8.1 ± 0.8	0.97 ± 0.28
	HCT-15	Paclitaxel	0.31	0.18	1.7
		Bisantrene	7.6	5.5	1.4
		Geldanamycin	8.6 ± 3.7	6.8 ± 3.3[d]	1.3 ± 0.1
		Baker's antifol	48 ± 5.2	34 ± 6.2[d]	1.5 ± 0.3
ABCB5	SK-MEL-28	CPT, 10-OH	0.48 ± 0.12	0.14 ± 0.02[d]	3.62 ± 1.35
		5FU	12 ± 4.6	6.4 ± 2.7[d]	1.96 ± 0.16
		Camptothecin	0.77 ± 0.07	0.38 ± 0.04[d]	2.01 ± 0.03
		Mitoxathone	1.08 ± 0.05	0.80 ± 0.13	1.38 ± 0.25
SLC29A1	HL-60	Azacytidine	0.55 ± 0.13	1.68 ± 0.30[d]	0.34 ± 0.14
		IdA	100 ± 45	>1000*	<0.10
	K-562	Azacytidine	3.4 ± 0.7	13.5 ± 3.8[d]	0.26 ± 0.04
		IdA	78	>1000	<0.08

[a] Small interfering RNA (siRNA) was used to target *ABCB1* and *ABCB5*. For SLC29A1, the known inhibitor nitrobenzylmercaptopurine ribonucleoside (NBMPR) (100 nM) was used to suppress transport activity. *ABCB1* expression levels were suppressed by 74 and 68% in NCI/ADR-RES and HCT-15, respectively. *ABCB5* expression levels were suppressed by >80% in SK-MEL-28 cells. IC_{50} is the concentration that produced 50% inhibition of cell growth compared to controls. Results represent the mean of two or mean ± SD of at least three independent experiments.

[b] Treatment with siRNA for *ABCB1* and *ABCB5* or with NBMPR for *SLC29A1*.

[c] For *ABCB1* and *ABCB5*, fold reversal is the IC_{50} for cytotoxic drug in mock-treated cells divided by the IC_{50} for drug in siRNA-treated cells. For SLC29A1, fold reversal is the IC_{50} in control cells divided by the IC_{50} in NBMPR-treated cells.

[d] $P < 0.05$ compared with the cells treated with mock siRNA.

[e] 5FU, 5-fluorouracil; CPT, camptothecin; IdA, inosine-glycodialdehyde.

Ca^{2+} and Na^{+} channels depolarize them. These two types of flux would be expected to have opposite effects on drug equilibration across cell membranes. However, Ca^{2+} flux is also important in apoptotic signaling, as noted above, so the net effect on drug potency is difficult to predict. In this study, *CACNA1D*, which encodes the α 1D subunit of the L-type calcium channel, showed negative correlation with several drugs, including deoxydoxorubicin (Table 1). Interestingly, L-type calcium channel antagonists block ABCB1 and thereby are thought to overcome drug resistance (43). It remains to be seen whether blocking CACNA1D could have contributed to this effect. Several genes that encode subunits of sodium, chloride, potassium, and other cation channels correlated with drug activity, confirming that ion channels can modulate drug response—possibly by affecting the cell's resting potential or by providing key metal ion cofactors. It will be important to understand the role of ion channels in the cell's response to toxic stimuli because perturbation of the ADP-ATP ratio during the course of a cytotoxic reaction directly alters electrochemical gradients.

Table 3 *Drugs showing significant negative correlation with ABCB1*[a]

Drug	NSC no.	70-mer array *P*	70-mer array *r*	cDNA array *P*	cDNA array *r*
Taxol analogue_7	666608	0.000	−0.62	0.004	−0.50
Taxol analogue_10	673187	0.000	−0.52	0.013	−0.42
Bisantrene	337766	0.001	−0.77	0.062	−0.49
Taxol (paclitaxel)	125973	0.001	−0.53	0.000	−0.54
Taxol analogue_3	658831	0.001	−0.45	0.004	−0.42
Taxol analogue_5	664402	0.001	−0.49	0.007	−0.40
Taxol analogue_6	664404	0.002	−0.51	0.024	−0.39
Baker's-antifol*	139105	0.002	−0.39	0.034	−0.30
Taxol analogue_2	656178	0.002	−0.45	0.003	−0.43
Vinblastine sulfate	49842	0.003	−0.47	0.050	−0.33
Geldanamycin*	330500	0.004	−0.46	0.002	−0.48
Taxol analogue_11	673188	0.011	−0.47	0.003	−0.48
Oxanthrazole	349174	0.012	−0.46	0.791	−0.07
Taxol analogue_8	671867	0.015	−0.42	0.004	−0.42
Taxol analogue_9	671870	0.016	−0.44	0.000	−0.49
Anthrapyrazole derivative	355644	0.016	−0.45	0.572	−0.12
Daunorubicin	82151	0.017	−0.51	0.300	−0.23
Etoposide	141540	0.020	−0.31	0.560	−0.09
Doxorubicin	123127	0.020	−0.54	0.287	−0.27
Zorubicin	164011	0.021	−0.58	0.207	−0.31
Taxol analogue_1	600222	0.041	−0.46	0.002	−0.53
5,6-Dihydro-5-azacytidine	264880	0.463	−0.16	0.034	−0.30

[a] The results from both 70-mer oligo arrays and cDNA arrays[8] are shown. Correlation coefficients (*r*) and bootstrap *P* values are shown for both 70-mer oligo and cDNA array data. The listed drugs fulfill two criteria: $P < 0.05$ and $r < -0.3$. We identified 19 putative ABCB1 substrates, all but two of which were known substrates. The two new suggested substrates (*) were validated by small interfering RNA. Note that the cDNA array failed to identify several substrates but yielded $r = -0.3$ for 5,6-dihydro-5-azacytidine, which is unlikely to be a substrate of ABCB1.

DISCUSSION

This study is the first to assess drug-transporter relationships comprehensively at the genomic level. Correlations between expression of the transportome and chemosensitivity in the NCI-60 panel reveal numerous significant gene-drug correlations. A number of those correlations correspond to known transporter-drug substrate relationships, thereby validating the approach. Moreover, the use of siRNA to down-regulate transporters provides additional validation for *ABCB1* and *ABCB5* as resistance genes and for *SLC29A1* as a sensitivity gene. In aggregate, the results here emphasize the important role that multiple types of membrane transport molecules can play in sensitivity and resistance to cytotoxic drugs. In addition to the direct effects on drug transport that have already been discussed, indirect mechanisms may also modulate sensitivity. Transporters and channels may, for example, affect chemosensitivity indirectly by providing nutrients to cancer cells, modifying the propensity to apoptosis or modulating the electrochemical gradient. For validated transporter-drug pairs, the correlations calculated here enable one to search for additional likely substrates from the database of >100,000 chemicals tested at the NCI. The additional correlations suggest possible roles and specificities for additional transport molecules. Overall, the databases presented here provide a wealth of information on structure-activity and pharmacogenomic relationships to guide the development and selective use of drugs for cancer. There is no possibility that we could mine all of the useful information in them. Others who have domain expertise with respect to particular transport molecules or drugs will see and focus on relationships that are not apparent—or not apparently important—to us.

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