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 chemoresponsiveness 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, nitrobenzylmercapturine 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 ABCC5) 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 ABCC5 by small interfering RNA increased sensitivity to several drugs in melanoma cells, implicating ABCC5 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 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.
**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. 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 discordant 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.3

**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.4 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 R statistical software package R.6 The plot of $M$ versus $A$ plot for spots on the $p^6$ grid of each slide, and $q_i$ is the scale factor for the $p^6$ grid (to obtain equal variances among 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 ($G_{50}$ values for 60 human tumor cell lines) were those obtained by the Developmental Therapeutics Program. 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. 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 $P$ 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’-AAGCGGAGCAGTGGTTCAGGT-3’, beginning from nt 2113 of the $ABCB1$ mRNA sequence NM_000927, as recommended. 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’-AAGAGGGCTCTAAATGGTGG-3’ ($ABCB5_{754}$); 5’-AAGGGAGAACTCGCTGACCT-3’ ($ABCB5_{930}$); and 5’-AAGCAATTGTCTCG-AGTGCCCTG-3’ ($ABCB5_{1114}$), which are located at nt 745, 930, and 1114 of the $ABCB5$ mRNA sequence NM_000927, 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 surforhodamine 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 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, MO) 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% surforhodamine 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) was 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 isosine-glycoidaldehyde and azacytidine was assessed with the 2.3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-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 drug.
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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+, Ca2+, 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 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. 1A), 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. 1A and 2A). Fig. 2A 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 > 3-fold in both cell types (Table 2), indicating that SLC29A1 (equilibrative nucleoside transporter 1) plays an important role 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. 1B, 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. 1C 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 transporter for the native substrates aspartate and glutamate.
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 transporter system), and both showed negative correlation with multiple drugs, including cisplatin (Supplementary Table 1). The amino acid transport system Xc− 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...
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. 2B). 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 antifolate (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. 3A). 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, in an increase in potency was 3.5- and 43.8-fold after verapamil treatment. Therefore, application of RNA interference gene silencing and ABCB1 antagonist verapamil, for GA and BAF, respectively, in an increase in potency was 3.5- and 43.8-fold after verapamil treatment.

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effective in down-regulating mediated gene silencing, we synthesized siRNA duplexes against chemoresistance. To identify suitable expressed in melanoma cells, suggesting a tissue-specific role in transfected with mock siRNA (Table 2, Supplementary Fig. 3). ABCC5_930 were 2–3-fold more sensitive to several drugs, including camptothecin, 10-OH camptothecin, and 5FU, than were control cells.

ABCB5 mRNA level). SK-MEL-28 cells transfected with siRNA-ABCB5 showed strong negative correlation with camptothecin, 7-Cl AQP1, and Cl– leakage currents tend to polarize cells, whereas AQP9, aquaporins involved in transport of water, urea, and glycerol, correlated positively with several 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.

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, ATP1A3, ATP1B1, ATP1B3, and ATP1G1 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.

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. 3B). 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 ErbB2 transfectant of MDA-MB-435, 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.

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, ATP1A3, ATP1B1, ATP1B3, and ATP1G1 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

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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, \(\text{CACAID}\), which encodes the alpha 1D subunit of the \(I_{\text{Ca}}\) type channel, showed negative correlation with several drugs, including deoxycorticosterone (Table 1). Interestingly, \(I_{\text{Ca}}\) type calcium channel antagonists block \(ABCB1\) and thereby are thought to overcome drug resistance (43). It remains to be seen whether blocking \(\text{CACAID}\) 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.

### 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.
REFERENCES


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

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