Predicting drug sensitivity and resistance: Profiling ABC transporter genes in cancer cells

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Summary

For analysis of multidrug resistance, a major barrier to effective cancer chemotherapy, we profiled mRNA expression of the 48 known human ABC transporters in 60 diverse cancer cell lines (the NCI-60) used by the National Cancer Institute to screen for anticancer activity. The use of real-time RT-PCR avoided artifacts commonly encountered with microarray technologies. By correlating the results with the growth inhibitory profiles of 1,429 candidate anticancer drugs tested against the cells, we identified which transporters are more likely than others to confer resistance to which agents. Unexpectedly, we also found and validated compounds whose activity is potentiated, rather than antagonized, by the MDR1 multidrug transporter. Such compounds may serve as leads for development.

Introduction

The ABC (ATP binding cassette) family of membrane transport proteins includes the best-known mediators of resistance to anticancer drugs. In particular, MDR (multidrug resistance) pumps ABCB1 (MDR1-P-gp), ABCC1-MRP1, and ABCG2-MXR (Gottesman et al., 2002; Cole et al., 1992; Borst et al., 2000; Deeley and Cole, 1997; Litman et al., 2001) actively extrude many types of drugs from cancer cells, thereby conferring resistance to those agents. More broadly, the ABC transporters appear to have evolved to defend the cell through recognition and energy-dependent removal of a large variety of natural toxic agents (Sarkadi et al., 1996). Their functional significance is suggested by the observation that they form one of the largest protein families, found in various cellular membranes of organisms from bacteria to mammals. Based on sequence homology, 48 different ABC transporters (grouped into seven subfamilies ranging from A to G) have been defined in the human genome. Their functions range from export of cholesterol (ABCA1) to regulation of chloride current (ABCC7-CFTR), and they play roles in the absorption, distribution, and excretion of pharmacological compounds.

Despite these generalizations, relatively little is known about the functions of most members of the family. Given the high degree of similarity of ABC sequences, however, it seems plausible that additional members may also be drug exporters and may thus be associated with decreased sensitivity of cancer cells to chemotherapy. To explore that proposition, we wanted to characterize ABC gene expression in a set of cancer cells whose responses to a large number of compounds are known and whose molecular characteristics have been cataloged. The natural choice was a panel of 60 human cancer cell lines (the NCI-60) used by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) to screen >100,000 chemical compounds since 1990. Included among the 60 are leukemias, melanomas, and cancers of ovarian, breast, prostate, lung, renal, colon, and central nervous system origin. 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 (Paull et al., 1989; Weinstein et al., 1992). In addition to this pharmacological characterization, the NCI-60 cells have been more extensively profiled at the DNA, mRNA, protein, and functional levels than any other set of cells in existence. Therefore, if we were to measure their expression of ABC transporters, it would be possible to link ABC transport function to

SIGNIFICANCE

Multidrug resistance of tumors is frequently associated with decreased cellular accumulation of anticancer drugs and elevated expression of ABC transporters such as MDR1. At present, relatively little is known about the substrate specificities of most ABC transporters. Here, we present a pharmacogenomic approach, in which we correlate expression profiles of all 48 human ABC transporters with patterns of drug activity in the NCI-60 cell lines. The findings are used to identify candidate substrates for several ABC transporters, as well as compounds whose toxicities are potentiated by ABCB1-MDR1. The gene expression database will serve as a high-quality "time capsule" that can be mined to generate new hypotheses and to illuminate additional features of ABC transporters and their functional relationships with other molecules.

a variety of other molecular, physiological, and pharmacological features of the cells.

We wished to focus, in particular, on the relationship between ABC expression levels and sensitivity to drugs or drug candidates, asking which of the transporters do (and which do not) confer resistance or sensitivity to various classes of agents. Previous transcript expression profiling of the NCI-60 using cDNA arrays of >9000 elements (Scherf et al., 2000) and Affymetrix Hu6800 oligonucleotide chips (Staunton et al., 2001) proved useful for identification of patterns and molecular biomarkers. However, those studies had three major limitations with reference to the ABC transporters: (1) only 15 and 11 of the 48 transporters were represented in the cDNA and oligonucleotide arrays, respectively; the total combined coverage was only 17 of 48, and the mean correlation between the two sets for the 9 transporters represented in both was only +0.43; (2) in part because of limited sensitivity, 64.2% and 58.5% of the values were undetectable above background for the cDNA and oligonucleotide arrays, respectively; and (3) crosshybridization among close family members could be expected, especially with the cDNA arrays, but also with the oligonucleotide arrays insofar as they were not designed with central focus on the ABC transporters. Since reproducible, quantitative correlations between expression and sensitivity were required for our study, we chose to measure transcript expression by the "gold standard" method, quantitative real-time RT-PCR, rather than the less sensitive, less specific microarray technology. Finding such correlations (and confirming them in follow-up studies) demonstrates the important role of ABC transporters in the drug resistance of cancer cells. One striking result was the identification of at least one compound whose toxicity appears to be potentiated, rather than antagonized, by ABCB1 (MDR1).

Results and discussion

Forty-eight ABC proteins are coded by the human genome (see http://nutrigene.4t.com/humanabc.htm for a comprehensive database). Figure 1 shows a clustered image map ("heat map") (Weinstein et al., 1997) that offers a visual summary of the patterns of ABC transporter expression across the 60 cell lines. The complete RT-PCR results on the 48 ABC transporters (47 determined in this work and one previously reported [Prades et al., 2002]) are presented in Supplemental Table S1 at http://www.cancercell.org/cgi/content/full/6/2/129/DC1. A 49th ABC gene, ABCC13, is predicted to encode a nonfunctional protein (Yabuuchi et al., 2002) and therefore is not included in this study. Quantitative analysis showed that the pattern of expression is most characteristic of tissue of origin for melanoma (9 of the 10 melanoma cells cluster together on the dendrogram). The one melanoma line not found in the melanoma cluster (LOX-IMVI) is amelanotic and undifferentiated and has been shown to lack transcripts characteristic of melanoma (Stinson et al., 1992). MDA-MB435 and MDA-N were originally thought to be from breast cancer, but their appearance within the melanoma cluster is consistent with strong molecular profile evidence that they are melanoma-derived or at least melanoma-like (Scherf et al., 2000; Ellison et al., 2002; Ross et al., 2000). MDA-N is an ERBB2 transfectant of MDA-MB435. CNS (5/6), renal (5/8), and ovarian (4/6) cells tend to form clusters, whereas the leukemia, colon, lung, breast, and prostate cancer cell lines do not cluster well by tissue of origin. Overall, the coherence by tissue of origin is moderate (see Supplemental Table S2), as indicated by a κ statistic of 0.46 (with two-tailed 95% confidence interval = 0.33–0.60). Interestingly, the two lumenal, estrogen receptor-positive breast lines (T47D and MCF7) cluster together.

This database provides valuable information on the expression patterns of both known and currently uncharacterized ABC transporters. Some of them are expressed ubiquitously (e.g., ABCC1), whereas others are selectively expressed in particular cell types (e.g., ABCB5 in melanoma-derived cells; see inset in Figure 1 and Supplemental Table S3). Langmann et al. (2003) found high expression of ABCA2 in brain, ABCA3 in lung, and ABCB1 and ABCC4 in kidney. When analyzed by Monte Carlo permutation t test, our data show that ABCA2 is ubiquitously expressed throughout the 60 lines ($p > 0.61$ for each of the nine tissues of origin), whereas ABCA3 is selectively expressed ($p = 0.039$) in H522M, A549, and EKVX (all of them lung cancer lines). ABCB1 is indeed selectively expressed in the renal cancer cell lines ($p = 0.0059$). However, ABCC4 is only moderately expressed in those cells ($p > 0.145$ for each of the nine tissues of origin). This apparent discrepancy with respect to the results of Langman et al. may be due to heterogeneity of the human tissue samples used in that study, or may reflect distinctive characteristics of the cancer cells. The distribution of ABC transporters on the gene dendrogram appears to be independent of sequence-homology categories. ABCB2 and ABCB3, known to function as heterodimeric components of the ER transport system for peptide antigen presentation, are found in different clusters, suggesting that their reported coordinate expression is disrupted in the cancer cells. Conversely, ABCG5 and ABCG8, which also form a heterodimer, show the expected concordance in expression pattern across the 60 cells (see Figure 1).

Correlation of ABC transporter mRNA levels with drug resistance

In a previous study using cDNA microarrays, the 60 cell lines were found to cluster reasonably well by tissue of origin on the basis of expression patterns determined for a broad range of genes, but they did not cluster as well on the basis of patterns of drug sensitivity (Scherf et al., 2000). Furthermore, there was only a modest correspondence between the two clusterings. Hence, cell clusters in the present study that appear similar for both ABC transporter expression and drug activity patterns are particularly interesting. Clusters such as that consisting of ACHN, UO-31, HCT15, and NCI-ADR-RES fall into that category. Not surprisingly, ABCB1 (i.e., MDR1) is highly expressed in those cells.

Since ABCB1 (MDR1-Pgp) extrudes molecules from the cell, the activity patterns of its substrates across the 60 cell lines are expected to be negatively correlated with its pattern of expression (Shoemaker, 2000; Lee et al., 1994). Figure 2 indicates that such is indeed the case for a set of 118 compounds with putatively known mechanisms of action (Weinstein et al., 1992). Reported substrates (e.g., geldanamycin, paclitaxel and its analogs, doxorubicin and vinblastine, and bisantrene [Lee et al., 1994]), indicated by blue bars, show striking inverse correlations, whereas compounds not transported by MDR1 (e.g., hydroxyurea, camptothecins, methotrexate, and 5-fluorouracil) are invariably found to be noncorrelated or positively correlated (red bars). Of the 118 compounds, only two inversely correlated drugs, an anthrapyrazole-derivative (NSC 355644, $r = -0.36$) and Baker's soluble antifol (NSC 139105, $r = -0.3$), have not

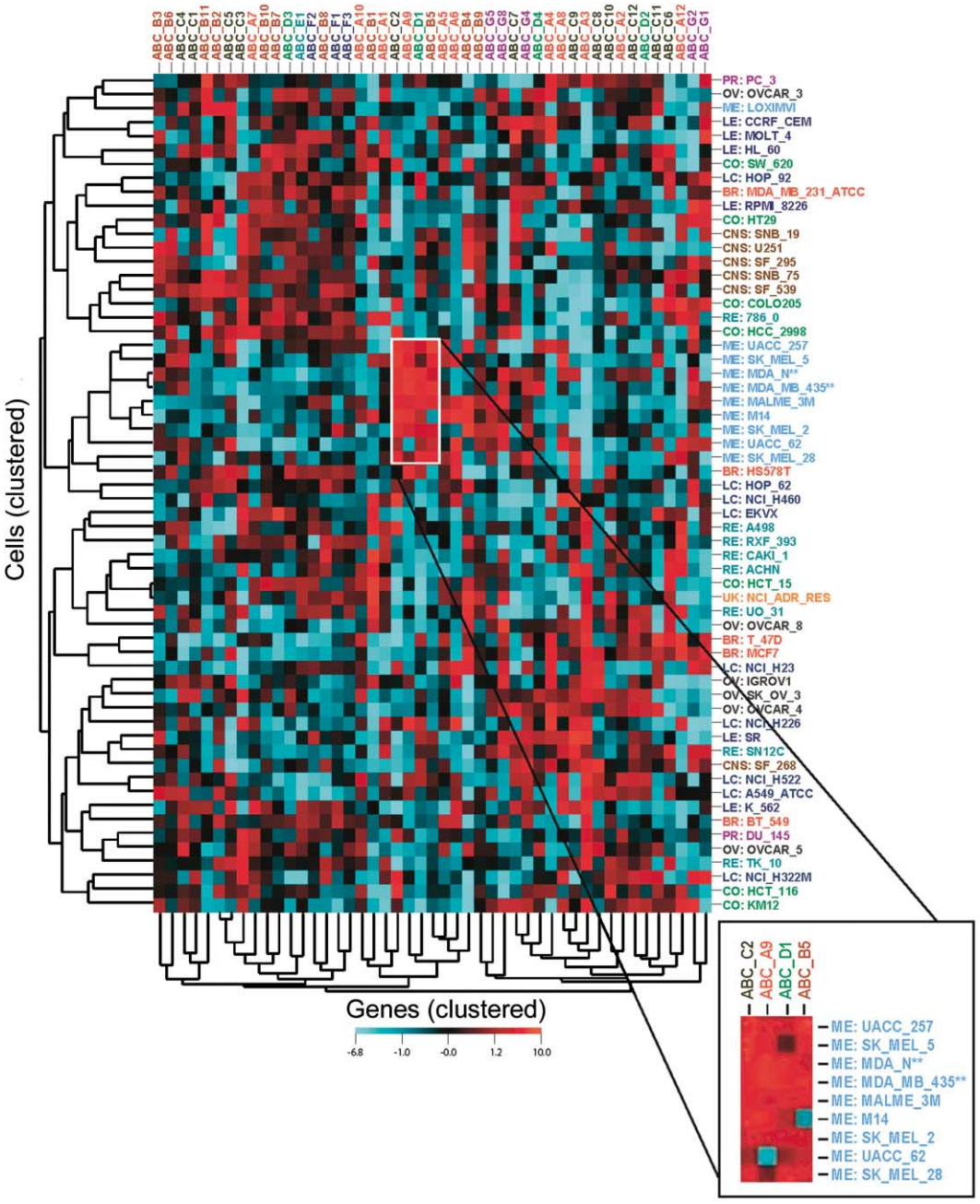


Figure 1. ABC transporter gene expression in the NCI-60 human cancer cell panel

The clustered image map shows patterns of gene expression assessed by real-time 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 row and column means. The inset highlights ABC transporters characteristically expressed in melanoma cells. For more details, see Supplemental Table S3.

previously been established as MDR1 substrates (black bars). However, resistance to Baker's antifol is reversed by verapamil, a potent inhibitor of MDR1 transport, suggesting that it is indeed an MDR1 substrate (Gupta et al., 1988).

To identify additional compounds that show significant inverse correlation with the expression of ABCB1, we extended the analysis to a larger data set containing the activity patterns of 1,429 compounds (Scherf et al., 2000). Since the measured distribution of ABCB1 was heavily right-skewed (i.e., a small number of the cell lines expressed high levels of ABCB1 transcript), we were concerned that the correlations obtained might be based on the relationship of drug activity to MDR1 expression in only a subset of the cell types. Therefore, we also computed bootstrap distributions (based on 10,000 bootstrap samples) of all correlations to obtain estimates of variability and confidence intervals with stringent significance levels. The 18 compounds

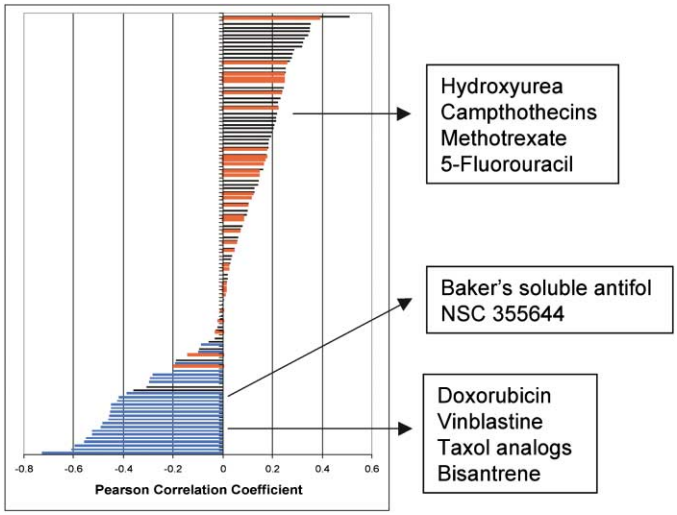


Figure 2. Relationship between drug sensitivity and ABCB1 expression in the NCI-60 for a set of 118 drugs of putatively known mechanism of action

Blue bars indicate known ABCB1 substrates; red bars indicate compounds shown in previous studies not to be substrates of ABCB1; black bars indicate compounds for which data were not available from the literature. The drug names listed at the top and bottom are commonly used, representative agents from the classes shown by red and blue bars.

that survived this statistical screening share structural features (large size, polyaromatic backbone, amphipathic character) with the well-known MDR1 substrates (Rabow et al., 2002; Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/6/2/129/DC1). NSC 328426 (phyllanthoside), NSC 259968 (Bouvardin), and NSC 156625 (Coralyne) have been tested in various laboratories and shown to interact with MDR1 (Lee et al., 1994; Gupta et al., 1988). The rest have not previously been implicated in MDR1-mediated resistance.

Evidence that correlations predict drug resistance due to ABC transporters

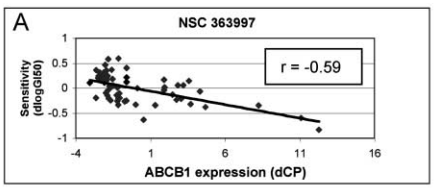
To test whether our approach using the NCI-60 does, in fact, identify new substrates, we used the MTT assay to test all top-scoring compounds that were available from DTP for follow-up experiments. KB-3-1, a human carcinoma cell line, and KB-V1, a multidrug resistant derivative of KB-3-1 that overexpresses MDR1-P-gp (Shen et al., 1986), were used for the tests. Figure 3 shows a typical result. When compared with the parental line, KB-V1 cells proved resistant to NSC 363997. PSC 833, an MDR1 antagonist, reversed the resistance, providing evidence that the observed resistance was linked to P-gp function. Further experiments showed that KB-V1 cells were 30- to 300-fold less sensitive than KB-3-1 cells to all 6 compounds available for study. This resistance of KB-V1 cells was invariably reversible by PSC 833. The intrinsic fluorescence of one of the compounds, NSC 634791, allowed us to measure the effect of MDR1 activity on its export from cells. Following incubation with NSC 634791 for 10 min at 37°C, MDR1-positive cells contained less of the fluorescent compound than did the parental KB-3-1 cell line (Figure 3). The decreased accumulation was completely reversible by addition of PSC 833 (which had no effect on the parental cells), further corroborating the hypothesis that NSC 634791 is an MDR1 substrate.

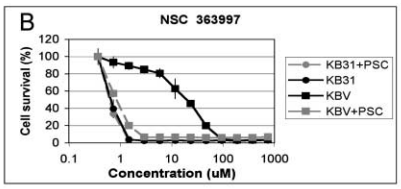
These results support the idea that correlation of ABCB1 expression with activity patterns in the 60 cell lines can be used to identify potential ABCB1 substrates among the >100,000 compounds tested at the NCI (Alvarez et al., 1995; Izquierdo et al., 1996; Bates et al., 1994; Wu et al., 1992). The chemical properties of inversely correlated compounds may also help define the "common pharmacophore" of the structurally dissimilar MDR1 substrates (Scala et al., 1997; Pajeva and Wiese, 2002; Blower et al., 2002).

ABC proteins transport a wide variety of compounds through the lipid bilayer, and identification of transported molecules should help us understand the physiological mechanisms and specificities of the transporters. To identify which ABC transporters and substrates may play roles in drug resistance of cancer cells, we calculated Pearson's correlation coefficients for a total of 68,592 relationships (48 genes × 1429 compounds; Supplemental Table S5) using bootstrap analysis with 10,000 iterations. The analysis yielded 131 highly inverse-correlated gene-drug pairs (Supplemental Table S6), sufficiently highly correlated in the negative sense that none of their 10,000 bootstrap samples were positively correlated. This list suggests that several ABC transporters of unknown function can, in fact, influence response of cells to treatment. Assuming functional relationship, the compounds are predicted to be substrates of the respective ABC transporters. To verify this hypothesis, we performed independent followup experiments in defined systems for the most interesting correlative findings. As examples, we will describe here the results for two transporter drug pairs, one involving ABCC2-MRP2, the other involving ABCC11.

The ABCC (MRP) subfamily is comprised of nine members that transport structurally diverse lipophilic anions and function as drug efflux pumps (Kruh and Belinsky, 2003). ABCC2-MRP2 is a canalicular efflux pump with a role in the hepatobiliary excretion of bilirubin glucuronide as well as numerous pharmaceuticals. ABCC11, a recently identified member of the superfamily, has been shown to mediate the ATP-dependent transport of cyclic nucleotides and confer resistance to certain nucleotide analogs (Guo et al., 2003). Of the 1429 compounds analyzed in this study, 14 were shown by the stringent bootstrap criterion described above to be less active in cells that expressed large amounts of ABCC2. One of these compounds was available from DTP for followup testing (Figure 4A). One compound was less active in the presence of large amounts of ABCC11 (Figure 4C, Supplemental Figure S1, and Supplemental Table S6). To verify whether these highly significant negative correlations indicate functional relationships, in which ABCC2 and ABCC11 protect the cells by exporting the related compounds, we compared control cells with ABCC2-transfected or ABCC11-transfected derivatives in MTT assays. In sharp contrast to the control (sham-transfected) cells, the ABCC2-overexpressing MDCKII cells proved extremely resistant to NSC 641281, reinforcing the suggestion that NSC 641281 is an ABCC2-MRP2 substrate (Figure 4B). Similarly, ABCC11-transfected LLC-PK1 cells were 2- to 3-fold more resistant to NSC 671136 than were control, sham-transfected cells (Figure 4D), suggesting that ABCC11-mediated resistance can extend to types of compounds other than nucleotide analogs.

The real-time RT-PCR database and analytical approach presented here thus provide an unbiased method for discovering the substrate specificities of known, as well as yet uncharacterized, members of the superfamily. Activity profiles





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NSC number	r	KB-3-1 (MDR1-)	KB-V1 (MDR1+)	DMF
NSC359449	-0.71	0.3 (0.3)	100 (0.3)	333
NSC646946	-0.71	0.1 (0.1)	20 (0.1)	200
NSC618757	-0.61	1 (1)	150 (1)	150
NSC363997	-0.59	0.5 (0.5)	20 (0.5)	40
NSC694268	-0.59	0.5 (0.5)	50 (0.5)	100

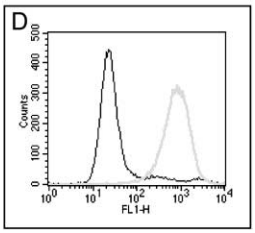

Figure 3. Verification of novel ABCB1 substrates by studies following up on NCI-60 correlations

A: Scatter plot showing the correlation (r) of ABCB1 expression with sensitivity of the 60 cells to NSC 363997 (r = −0.59; 99.99% two-tailed bootstrap confidence interval −0.8488 to −0.1130).

B: MTT assay dose-response curves for treatment of KB-3-1 parental cancer cells and the selected resistant variant KBV1 with increasing concentrations of NSC 363997. The dashed lines indicate the same, but in the presence of 2 μM inhibitor PSC 833 (for KB-3-1, the solid and dashed lines overlap). Values are means ± SEM for representative experiments performed in triplicate.

C: Summary of further, analogous cytotoxicity assays performed using the other five available compounds. Concentrations resulting in 50% cell death (IC_{50}) in the absence and presence (values in parentheses) of 2 μM PSC 833 are shown in μmoles/liter. The effect of the MDR1-P-gp inhibitor PSC 833 on IC_{50} values in KB-V1 cells is expressed as a dose modifying factor, DMF = (IC_{50}/[IC_{50}]$_{PSC833}$), where (IC_{50})$_{PSC833}$ is the value obtained in the presence of the inhibitor.

D: Accumulation of the intrinsically fluorescent compound NSC 634791 in MDR1-overexpressing KB-V1 cells. Representative histograms show the green fluorescence of cells after incubation with 1.74 μM NSC 634791 for 10 min at 37°C in the presence (gray) or absence (black) of 2 μM PSC 833.

of the 18 ABCB1 substrates were very strongly correlated with expression of the transporter. In contrast, the other two molecules considered to be "MDR transporters," ABCC1 (MRP1) and ABCG2 (MXR or BCRP), were more weakly correlated with a smaller number of compounds, which did not include known substrates. The lack of the expected correlations may partially be explained by the statistical limitations of the correlative approach: the sample size (n = 60), the experimental uncertainty, and the fact that multiple tests of significance are being done simultaneously may contribute to false negative predictions (hence, the "multiple testing" corrections we describe in the text and Experimental Procedures). 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. As indicated in Supplemental Table S8 and Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/6/2/129/DC1, a comparison of the mRNA and protein expression profiles for ABCB1 and ABCG2, respectively, indicates that, at least for those two transporters, the real-time RT-PCR and protein expression data correlate quite well. Second, correlation does not imply causality. Third, there might be functionally relevant differences between the RNA messages that are not detected by RT-PCR measurements. That is, the 60 cells may contain splice variants or SNPs that influence the substrate specificities of the transporters (Imai et al., 2002; Honjo et al., 2002). Fourth, cofactors (e.g., conjugating enzymes) or transporters (e.g., ABCC1) may be expressed discordantly in the 60 cells. Fifth, the ubiquitous and consistent expression of certain ABC transporters (e.g., ABCC1) makes it difficult to detect functional relationships through correlations. Finally, the presence of MDR1 may mask the contribution of less potent transporters of the same compounds. The somewhat surprising correlation of compounds with the expression of ABCD1, a peroxisomal half-transporter associated with adrenal leukodystrophy (Mosser et al., 1993) (Supplemental Table S6), is probably a consequence of the inverse correlation between the expression of ABCD1 and ABCB1 (r = −0.48, see also Figure 1 and Supplemental Table S1). Compounds showing inverse correlation with ABCD1-expression are therefore likely to be positively correlated with ABCB1 expression (Supplemental Table S5).

Positive correlations identify compounds potentiated by ABCB1

The positive correlation between activity and ABCB1 expression for some of the compounds in Supplemental Table S5 suggests that those compounds can inhibit growth of the cancer cells more strongly if MDR1 is overexpressed. For some transporters, including MDR1, several high positive correlations between gene expression and drug sensitivity are higher than would be expected by random sampling from a distribution of no real underlying correlations (in the case of MDR1, for the top 10 positive correlations, the Benjamini-Hochberg procedure [Reiner et al., 2003] estimates on average 3 false positive predictions). Thus the toxicity of at least some of the compounds increases systematically with higher MDR1 expression in the NCI-60. To investigate that possibility, we used the MTT assay and the KB-3-1/KB-V1 cell pair to test the top-scoring compound that was available from DTP (Figure 5A). Figure 5B shows that KB-V1 cells are 4- to 5-fold more sensitive than the parental KB-3-1. The finding that PSC 833 completely reversed sensitivity of KB-V1 cells to NSC 73306 strongly suggests that the increased sensitivity is due to the function of MDR1, not to other, nonspecific properties of the KB-V1 cells.

To substantiate further that the observed potentiation of NSC 73306 was not due to nonspecific factors arising during the generation of KB-V1, we repeated the MTT assays using HeLa-transfectants in which human *MDR1* is under tetracycline control. In these cells, addition of tetracycline suppresses transcription of *MDR1* mRNA, and, over a period of a few days, MDR1 disappears from the cells, providing a near-isogenic model for well-controlled experiments (Aleman et al., 2003). Figure 5C shows that the MDR1-expressing cells (MDR1-On) are 2- to 4-fold more sensitive than are MDR1-Off cells, providing strong evidence that the increased sensitivity to NSC 73306 is mediated by MDR1 function. Interestingly, NSC 73306 does not block MDR1-mediated transport of other molecules (data not shown), suggesting that it might avoid the well-documented

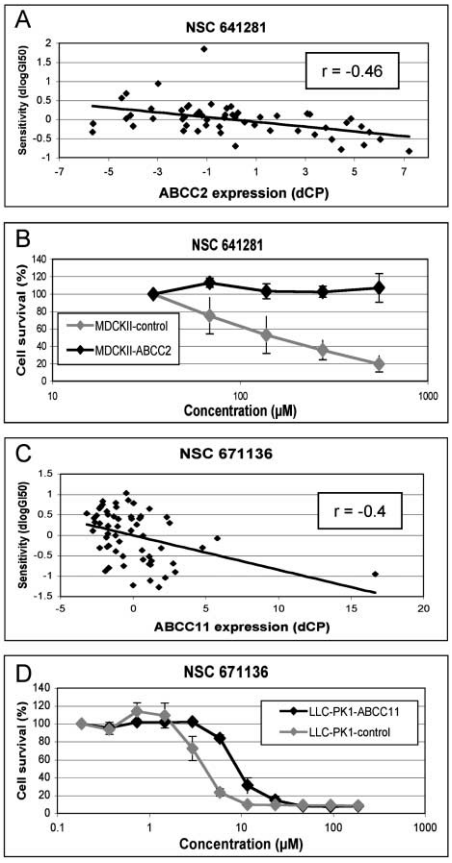

Figure 4. Prediction from the NCI-60 data of new substrates for ABCC2-MRP2 and ABCC11-MRP8, then validation of the predictions by MTT assay

A: Scatter plot showing the correlation (r) of ABCC2 expression with sensitivity of the 60 cells to NSC 641281 (r = −0.46; 99.99% two-tailed bootstrap confidence interval −0.7987 to −0.0440).

B: Dose-response curves for treatment of sham-transfected and ABCC2-transfected MDCKII dog kidney cells with NSC 641281. The ABCC2-expressing cells showed no signs of toxicity even at maximal concentrations.

C: Scatter plot showing the correlation (r) of ABCC11 expression with sensitivity of the 60 cells to NSC 671136 (r = −0.4; 99.99% two-tailed bootstrap confidence interval −0.6726 to −0.0141). Note: removal of the single, high-expressing cell line (T47D) from the analysis does not significantly reduce the observed correlation (r = −0.38; 99.99% confidence interval −0.7233 to −0.03915).

D: Dose-response curves for treatment of sham-transfected and ABCC11-transfected LLC-PK1 non-small cell lung cancer cells with NSC 671136.

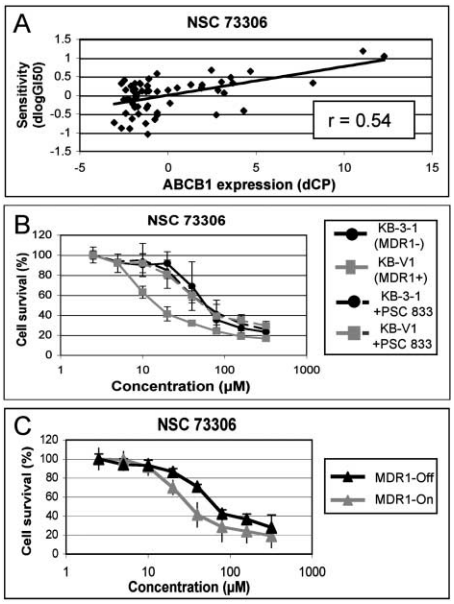

Figure 5. Prediction from the NCI-60 data, followed by independent verification, that the toxicity of NSC 73306 is potentiated, rather than inhibited, by expression of ABCB1

A: Scatter plot showing positive correlation (r = +0.54; 95% confidence interval 0.259 to 0.713) of ABCB1 expression with sensitivity of the 60 cell lines to NSC 73306.

B: Dose-response curves indicating that selected resistant KBV1 cells are approximately 4-fold more sensitive to NSC 73306 than are the parental KB-3-1 cells in an MTT assay. Dashed lines indicate the corresponding results in the presence of 2 μM PSC 833, which completely abolished the heightened sensitivity of KBV1.

C: Dose-response to NSC 73306 of KB HeLa cells expressing MDR1 under tetracycline control. Cells were grown in the absence (MDR1-On) or presence (MDR-Off) of 2 μg/ml tetracycline for at least seven days before starting the MTT assay. Cell surface expression and function of MDR1 were verified prior to the assay by staining with anti-MDR1 monoclonal antibody (MRK-16) and by a performing a functional assay based on MDR1-controlled accumulation of the fluorescent dye Calcein (Homolya et al., 1996) (data not shown). The MTT assay showed an approximately 2-fold higher sensitivity to NSC 73306 with upregulation of MDR1-P-gp. Values are means ± SEM of triplicate measurements.

side effects observed in clinical trials of "classical" MDR1 inhibitors (Kellen, 2003). Possible explanations for the increased (collateral) sensitivity of otherwise resistant cells include ATP depletion (Kabanov et al., 2003) and altered activity of enzymes that influence the toxicity of the compound (Bergman et al., 2003). The results reported in this section suggest that the pharmacogenomic approach presented here can be exploited to discover such MDR1-potentiated compounds, which may serve as leads for development of novel anticancer agents to treat resistant disease. Studies are underway to characterize other positively correlated compounds and to elucidate their mechanisms of action.

Conclusions

This work demonstrates correlations between expression of ABC transporters and response to cytotoxic drugs, confirmed in all tested cases by evidence of resistance or sensitivity in cell lines transfected with the appropriate ABC transporters. Although in some cases the levels of resistance were relatively low, ample evidence indicates that even low (2- to 4-fold) levels of drug resistance can have a significant impact on the clinical efficacy of anticancer treatment (Gottesman et al., 2002). Animal models have also demonstrated decreased responsiveness to chemotherapy in xenografts of a KB line selected for low levels of in vitro resistance (Horton et al., 1988). For many anticancer drugs, toxic-to-therapeutic ratios are low and, therefore, even a small increase in cell-based resistance can hamper chemotherapy.

The importance to cancer therapy of circumventing MDR is evident (Sikic, 1999). Even precisely targeted anticancer drugs are subject to mechanisms of resistance. The real-time RT-PCR database presented here provides a means to identify (1) ABC transporters whose expression confers drug resistance, (2) compounds that retain activity in cells that express MDR proteins, (3) compounds that are exported by MDR proteins, and

(4) drugs that exploit hidden vulnerabilities of MDR cells. Here, we have summarized some initial conclusions from analyses to date. More importantly, however, the database will serve for many years as a high-quality "time capsule" that can be mined by ourselves and others to illuminate additional features of the ABC transporters and their complex functional relationships with other molecules. In particular, these gene expression profiles will aid studies of the physiological function of the many ABC transporters whose mechanisms remain to be characterized.

Experimental procedures

Purification of RNA

Using the RNeasy kit (Qiagen) according to the manufacturer's instructions, as described previously (Scherf et al., 2000), we purified total RNA from stocks of the cell lines used in the DTP screen. Aliquots of the RNA were stored at −70°C. The quality (purity and integrity) of the RNA samples was assessed using an Agilent 2100 Bioanalyzer with the RNA 6000 NanoLabChip reagent set (Agilent Technologies) and by assessing the ribosomal RNA bands on a native agarose gel. The RNA was quantitated using a spectrophotometer.

Quantitative RT-PCR

Expression levels of 47 ABC transporters were measured by real-time quantitative RT-PCR using the LightCycler RNA Amplification SYBR Green kit and a LightCycler machine (Roche Biochemicals, Indianapolis, IN). Specific oligonucleotide probes were designed for each of the ABC transporters using DNAStar Primer Select (DNASTAR Inc.), and they were then synthesized at Lofstrand Laboratories (Gaithersburg, MD). When possible, the amplicons were designed to encompass exon-intron boundaries to avoid amplification of genomic DNA. The sequence of ABCA13 was not known when we assembled the PCR primers. Data reporting ABCA13 expression (Supplemental Table S1 at http://www.cancercell.org/cgi/content/full/6/2/129/DC1) were taken from Prades et al., 2002. Since the Syber Green assay detects accumulation of double-stranded DNA, we selected those primers (from a battery consisting of about 200 primers) that amplified a single product of the correct size. A list of the primers and corresponding gene reference/accession numbers for the ABC proteins is shown in Supplemental Table S7. RT-PCR was carried out on 150 ng total RNA, in the presence of 250 nM specific primers. Following reverse transcription (20 min at 55°C), the PCR reaction consisted of 45 cycles of denaturation (15 s at 95°C), annealing (30 s at 58°C), and elongation (30 s at 72°C). 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 by the LightCycler. Crossing point values were calculated, using the LightCycler software package, by the Fit Points analysis method, with baseline fluorescence set at 1. The SyberGreen assay measures accumulation of double-stranded products, and the appearance of primer dimers limits quantitation at high cycle numbers. The specificity of amplified products was verified by melting-curve analysis and agarose gel electrophoresis (not shown). 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 artifacts, selected cell lines were assessed in replicate. The average pairwise correlation of replicate expression profiles was 0.96. The reproducibility of the measurements was further confirmed by cluster analyses, which showed that replicates clustered tightly together (data not shown).

Since the expression levels of housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH], Porphobilinogen Deaminase [PBGD], tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, and zeta polypeptide [YWHAZ]) were found to be highly variable among the 60 cell lines (not shown; however, see Vandesompele et al., 2002), they were not used as controls, and data were normalized with respect to the mean expression of the transporters (excluding ABCA13). Finally, the values were mean-centered and multiplied by −1 to indicate expression values with reference to the mean expression of each ABC transporter across the 60 cell lines.

Drug database

More than 100,000 chemical compounds have been tested in the NCI-60 screen by the Developmental Therapeutics Program. For this study, we focused on a subset consisting of 118 compounds whose mechanisms of action are putatively classifiable (Weinstein et al., 1992) and a larger set of 1400 compounds that have been tested multiple times and whose screening data met quality control criteria described elsewhere (Scherf et al., 2000). Both sets are available at http://discover.nci.nih.gov. The two were combined to form a joint dataset that included 1429 compounds.

Statistical analysis

The statistical analyses were performed using the SAS software package, v8.2 (SAS Institute Inc, Cary, NC), and the R package (www.r-project.org). Two-dimensional agglomerative hierarchical cluster analysis, with average linkage algorithm and distance metric $1-r$, where r is the Pearson correlation coefficient, was performed using the CIMminer tool (http://discover.nci.nih.gov) to group the 60 cell lines as well as 47 ABC transporters based on the expression profiles. The resulting matrix of numbers was displayed in clustered image map form (Weinstein et al., 1997) as Figure 1.

To determine quantitatively how well the 47 genes cluster the cell lines by their tissues of origin, we used a statistical method developed by S. Lababidi (unpublished data). In that method, the κ statistic is used to indicate how well the observed clusters correspond to the nine tissue-of-origin classifications. For that calculation, one cell line, UK: NCI-ADR-RES, was excluded because it did not clearly fit into any of the usual categories.

To identify which genes are, on average, significantly over- or underexpressed in cells from a given tissue of origin (in comparison with the rest of the cell lines), we used Monte Carlo permutation t tests with 10,000 iterations to compare, for each tissue, the within-tissue mean and the mean over all of the other tissue types (this approach avoids the assumption of normality and is suitable for small sample sizes).

To narrow down the list of candidates based on correlation of the gene expression data for 48 ABC transporters and the extended list of 1429 drug activities measured in 60 cell lines (both centered around zero across the cell lines as well as across the expression values or the drug activities, respectively), we calculated the 95% bootstrap confidence intervals of Pearson correlation coefficients for all of the possible relationships (a total of $48 \times 1429 = 68{,}592$ correlation coefficients). The bootstrap confidence intervals were calculated using the empirical percentiles method with balanced resampling of 10,000 iterations (Chernick, 1999). Balanced resampling forces each observation to appear exactly a number of times equal to the total number of iterations. By bootstrap resampling, we avoided parametric assumptions about the distributions of the variables and incorporated possible non-normal distributional characteristics. For 10,000 bootstrap iterations with 95% confidence interval, the component of resampling error has a standard error of no more than 0.002. In recognition of the multiple testing problem we preferred a stringent critical value of $p = 0.0001$. To control the overall false positive rate, we used both a step-down procedure (Westfall and Young, 1993) and a step-up procedure (Reiner et al., 2003) to adjust for multiple testing of all 47 genes simultaneously. In the Benjamini-Hochberg procedure, the p values are computed in the standard way by permutation, assuming that all distributions are exchangeable: the number of values in the permuted data with correlations over a threshold, divided by the number of compounds and by the number of permutations. We calculated the minimum FDR (q value) at which each compound would be declared using the step-up procedure for positively correlated test statistics (again true because all correlations being compared are computed against the same ABC gene). In this procedure, the first q value for the largest correlation is the Bonferroni-corrected p value for that gene; then further q values are calculated as $q_j = \max(p_j{}^*1429/j,\ q_{j-1})$. This procedure limits the expected proportion of false positives in the list 1,…j to at most q_j.

Drugs and chemicals

The compounds designated here by NSC numbers were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Colchicine and dimethyl sulphoxide (DMSO) were purchased from

Sigma Chemical Co. (St. Louis, MO), and PSC 833 was provided by Novartis Pharmaceuticals Corp. (East Hanover, NJ).

Analysis of drug sensitivity

Cell survival was measured by the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay. Cells were seeded in 100 μl medium at a density of 5000 cells/well in 96-well plates, and serially diluted drug (with or without 2 μM PSC 833) was added the following day in 100 μl medium to give the indicated final concentration. Cells were then incubated for 72 hr at 37°C in 5% CO_2, and the MTT assay was performed following the manufacturer's instructions (Molecular Probes, Eugene, OR).

Efflux assay

Trypsinized cells were washed twice in phosphate-buffered saline (PBS). 5×10^5 cells were preincubated for 5 min at 37°C in Iscove's Modified Dulbecco's Medium (Quality Biologicals, Gaithersburg, MD) with 0.5% dimethyl sulphoxide (DMSO), with or without 2 μM PSC 833. NSC 634791 was then added to a final concentration of 1.74 μM, and the cells were incubated for 10 min at 37°C, then sedimented by centrifugation, and resuspended in PBS. Green fluorescence intensity was measured using a FacsCalibur flow cytometer equipped with a 488 nm argon laser (Becton Dickinson Biosciences, San Jose, CA, USA). Acquisition of events was stopped at 10,000.

Acknowledgments

We thank Balázs Sarkadi for the MDCKII cell lines. We thank the staff of NCI DTP for generation of the pharmacological database used in this study and George Leiman for editorial assistance. This work was supported by the National Institutes of Health.

Received: March 12, 2004
Revised: June 7, 2004
Accepted: June 21, 2004
Published: August 23, 2004

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