Generation of a Drug Resistance Profile by Quantitation of *mdr*-1/P-Glycoprotein in the Cell Lines of the National Cancer Institute Anticancer Drug Screen

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Abstract

Identifying new chemotherapeutic agents and characterizing mechanisms of resistance may improve cancer treatment. The Anticancer Drug Screen of the National Cancer Institute uses 60 cell lines to identify new agents. Expression of *mdr*-1/P-glycoprotein was measured by quantitative PCR. Expression was detected in 39 cell lines; the highest levels were in renal and colon carcinomas. Expression was also detected in all melanomas and central nervous system tumors, but in only one ovarian carcinoma and one leukemia cell line. Using a modified version of the COMPARE program, a high correlation was found between expression of *mdr*-1 and cellular resistance to a large number of compounds. Evidence that these compounds are P-glycoprotein substrates includes: (*a*) enhancement of cytotoxicity by verapamil; (*b*) demonstration of cross-resistance in a multidrug-resistant cell line, (*c*) ability to antagonize P-glycoprotein, increasing vinblastine accumulation by decreasing efflux; and (*d*) inhibition of photoaffinity labeling by azidopine. Identification of many heretofore unrecognized compounds as substrates indicates that P-glycoprotein has a broader substrate specificity than previously recognized. This study confirms the validity of this novel approach and provides the basis for similar studies examining a diverse group of gene products, including other resistance mechanisms, putative drug targets, and genes involved in the cell cycle and apoptosis. (*J. Clin. Invest.* 1995. 95:2205–2214.)
Key words: P-glycoprotein • drug resistance • multidrug resistance • polymerase chain reaction • drug development

Introduction

The success of chemotherapy in a large number of malignancies has stimulated efforts to identify new agents and improve existing therapies. The identification of new agents is being pursued with both an expanded screening in established models and rational drug design (1, 2). The National Cancer Institute's (NCI) Anticancer Drug Screen seeks to identify new agents with potential antitumor activity using a panel of 60 cell lines from a broad range of malignancies. Natural products as well as semisynthetic and synthetic compounds are screened, and a sensitivity pattern is determined. Using this information and a computer-based pattern identification program designated COMPARE, potential targets of unknown compounds have been predicted (3, 4).

The recognition of drug resistance as a major reason for the failure of chemotherapy has stimulated efforts to identify mechanisms of drug tolerance. Success in this search offers the possibility that therapies may be designed to overcome mechanisms of resistance. This approach has been pursued aggressively in vitro with models of multidrug resistance. Recognition of the role of P-glycoprotein as a drug efflux pump by Bech-Hanson et al. (5) and Juliano and Ling (6) and by the subsequent work by Tsuruo and co-workers (7–9) demonstrating reversal of drug tolerance by verapamil has stimulated efforts to antagonize the function of this membrane phosphoglycoprotein. Expression of P-glycoprotein has been documented in a variety of human cancers, and in some malignancies it has been shown to be a negative prognostic factor (10–14). Although precise quantitation has not been obtained, the sensitivity of the methods and the standard cell lines used for comparison suggest that a range of levels can be found. Several studies to date have documented increases in P-glycoprotein expression after treatment with natural products, supporting a role for this protein in clinical drug resistance (15–17).

With the ultimate goal of using the Anticancer Drug Screen to identify and characterize P-glycoprotein antagonists and to compare the efficacy of these antagonists alone or in combination in a large number of malignancies, we sought initially to determine the expression of P-glycoprotein in the cell lines comprising the Anticancer Drug Screen. This offered the possibility to evaluate a quantitative PCR methodology previously described (18). Although an earlier study had documented expression in a large number of the cell lines in the Drug Screen, the occurrence of early plateau in the PCR-based methodology used in that analysis underestimated the level of expression in a number of the cell lines (19). In the present study we describe a quantitative PCR determination of *mdr*-1/P-glycoprotein expression and describe the correlations derived from these measurements. The reliability and accuracy of the quantitative PCR methodology are validated. The identification of a large number of putative P-glycoprotein substrates is presented. The potential use of the Drug Screen to characterize and understand mechanisms of resistance is discussed.

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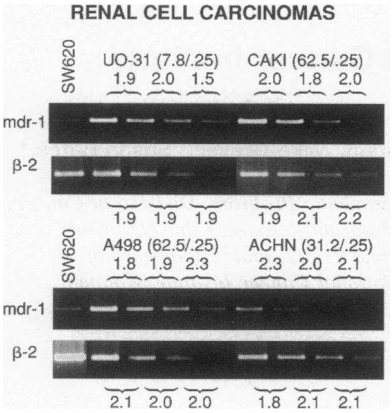

Figure 1. Quantitative PCR of RNA from renal cell carcinoma cell lines. This composite demonstrates the PCR results of serial RNA dilutions. SW620 to the left of each panel is the control cell line used in all experiments. The numbers in parentheses are the nanograms of RNA for *mdr*-1 and β-2 microglobulin, respectively, in the lowest dilution. Serial twofold dilutions from this amount were examined. The numbers above and below the brackets are the fold difference in the PCR products between two successive dilutions. A value of 2.0 is the theoretical difference in the exponential range from which reliable values can be obtained.

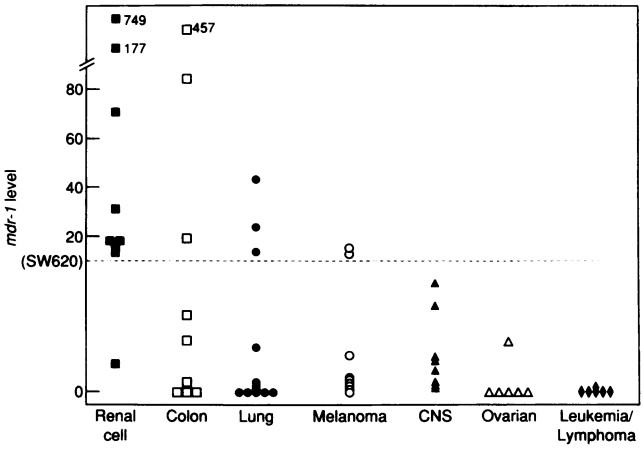

Figure 3. Graphic summary of *mdr*-1 levels in the 60 cell lines of the Anticancer Drug Screen Program. The SW620 reference cell line has a value of 10; the graph from 0 to 10 has been expanded to better demonstrate the range of expression.

Methods

Materials. Deoxycytidine 5′-[α-^{32}P]triphosphate (3,000 Ci/mmol, 1 Ci = 37 GBq) was obtained from DuPont-New England Nuclear (Boston, MA). ^{3}H-azidopine (44.7 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Compounds identified through analysis of the Drug Screen data as potential P-glycoprotein substrates were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (National Institutes of Health).

Cell lines. The cell lines comprising the National Cancer Institute's Anticancer Drug Screen Panel were obtained and processed as previously described (2). Briefly, after an initial acquisition, in vitro expansion was followed by cryopreservation of a large number of master stock samples, for serial rethawing at 20-passage intervals.

In vitro assay method. Anticancer drug screening was accomplished as previously described using a sulforhodamine-B assay to determine growth inhibition/cell kill (2). Nine cell lines with a broad range of *mdr*-1 expression were selected to evaluate the effect of verapamil on a series of potential *mdr*-1 substrates. Cells were inoculated onto 96-well tissue culture plates at densities ranging from 5,000 to 20,000 cells/well. The culture plates were incubated at 37°C in 5% CO_2 for 24 h, then verapamil was added at 5 or 12.5 μg/ml, and the plates were incubated for a further 4 h. After this time the test agents were added in 10 threefold dilutions and incubated for a further 48 h.

RNA extraction and electrophoresis. RNA was isolated from the 60 cell lines over a period of 8 wk. Attempts were made to maintain similar conditions for all cell lines; however, variability was inherent in growth characteristics, with some cells growing as diffuse monolayers and others as expanding clones. Total cellular RNA was extracted from cells by homogenization in guanidine isothiocyanate followed by centrifugation over a cesium chloride cushion. After isolation, careful measurements of RNA concentrations were made. Comparability of quantities and RNA quality were determined by electrophoresis on a 6% formaldehyde gel, followed by ethidium bromide staining. Where a discrepancy was observed, quantitation and electrophoresis were repeated.

Quantitative PCR. Quantitative PCR for *mdr*-1 and β-2 microglobulin expression was performed using published primers as previously described (18). Briefly, in an initial experiment, 125 ng of RNA was reverse transcribed and amplified for 30 cycles to obtain an estimate of *mdr*-1 expression. With 125 ng of RNA, *mdr*-1 expression was detected in 28 of the 60 samples. Precise quantitation was then performed for the 28 positive cell lines using serially diluted samples. Expression was examined in the 32 negative cell lines by using 1 μg of RNA. If PCR was negative at 30 cycles, the number of cycles was increased to 40. RNA isolated from SW620 human colon carcinoma cells cultured in the laboratory was used as a reference standard. Expression of *mdr*-1

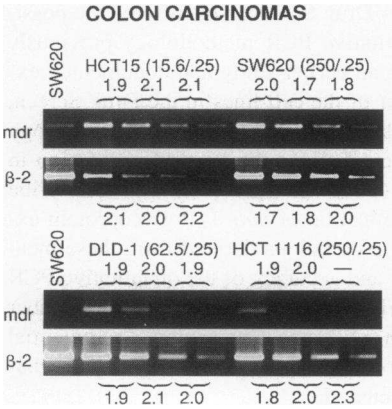

Figure 2. Quantitative PCR of RNA from colon carcinoma cell lines. This composite is organized as described in Fig. 1. The SW620 on the right side of the upper panel is the cell line in the drug screen program; the one on the left is the control cell line carried in the laboratory. Their *mdr*-1 values differ by about twofold.

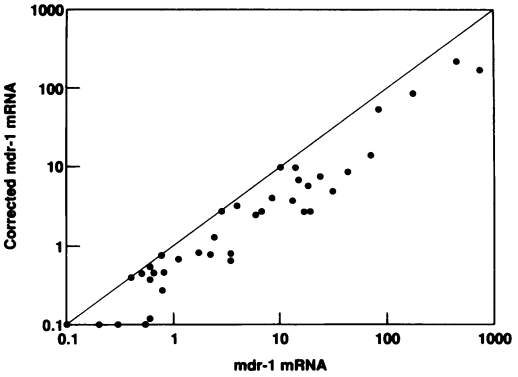

Figure 4. Comparison of *mdr*-1 results standardized by measured RNA concentration with those standardized using β-2 microglobulin expression as control. The *mdr*-1 values determined using the measured RNA concentration provide the x-coordinates. The *mdr*-1 values using β-2 microglobulin as internal control yield the y-coordinates. The line presents the results expected if the two were identical.

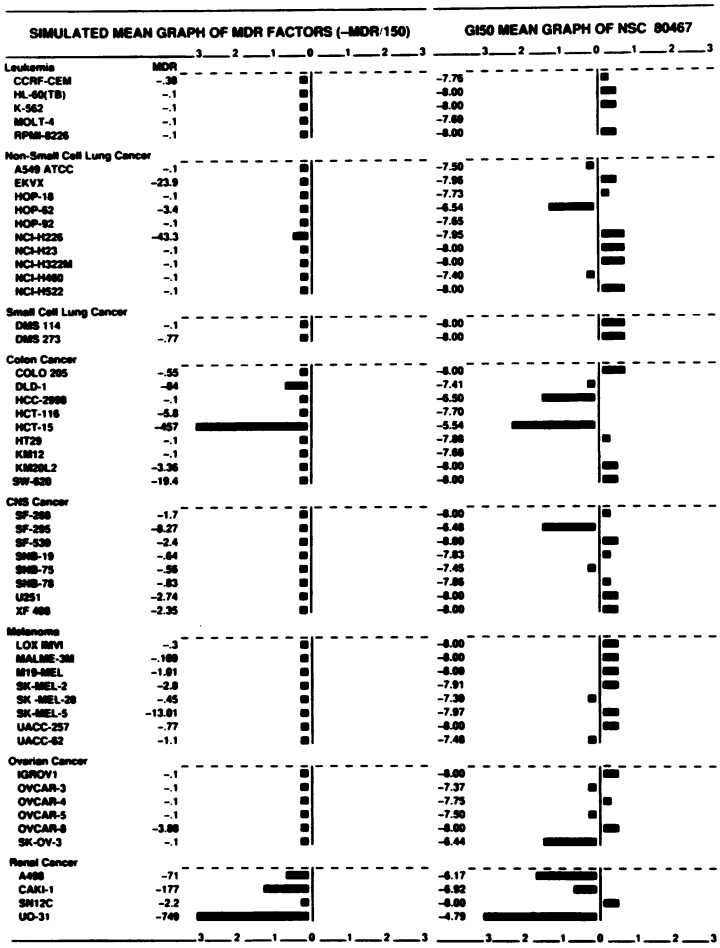

Figure 5. Comparison of a simulated mean graph of the "ideal" P-glycoprotein compound with the GI50 mean graph of compound NSC 80467. The simulated mean graph was derived by using the *mdr*-1 values shown in Fig. 3. The vertical line in the graph for compound NSC 80467 is the mean inhibitory concentration (mean GI50) for all cell lines tested. This mean is assigned a value of zero and all the GI50s of cell lines are plotted relative to this. The bars representing cell lines that require concentrations higher than the mean for inhibition point to the left; those representing cell lines more sensitive to compound NSC 80467 point to the right. In the simulated mean graph of the ideal P-glycoprotein compound, a value of zero has been assigned for a cell line without P-glycoprotein expression. All other cell lines have values greater than zero, with the magnitude proportional to the *mdr*-1 level. No bars extend to the right since values less than zero are not possible. Visual inspection demonstrates similarities between the ideal graph and that of compound NSC 80467.

can be readily detected in 125 ng of RNA and in serial dilutions below this. At these dilutions, PCR is in the exponential range. RNA from the SW620 cell line was serially diluted and amplified in every experiment, and a sample was included in every gel. Thus, in every experiment, the reaction conditions were internally controlled, and in every gel a reference standard was included. The level in the SW620 cell line was arbitrarily assigned a value of 10 and all other values were determined relative to this. A similar approach was taken for determination of β-2 microglobulin levels, again using SW620 cells grown in the laboratory for reference. In this case, the level in the SW620 cell line was arbitrarily assigned a value of 1 and all other values were determined relative to this. This allowed for quantitation based upon either measured RNA or β-2 microglobulin levels. In the latter, the *mdr*-1 values obtained from the exponential range of amplification were divided by the β-2 microglobulin values similarly obtained; the *mdr*-1 level of the SW620 cell line was 10 (*mdr*-1/β-2 = 10/1 = 10). All quantitations were performed by densitometry.

Drug accumulation and efflux studies. Drug accumulation and efflux studies were performed as previously described (20). Vinblastine accumulations were carried out for 1 h; efflux studies were performed for 10 min after a 2-h loading period.

In vitro cytotoxicity assay. Cytotoxicity assays with parental SW620 cells and multidrug-resistant SW620 Ad 300 cells were done in 96-well microtiter plates using the colorimetric methods described by Skehan et al. (21). Cells were seeded at a density of 1,000/well in 100 μl of growth medium. Cytotoxic agents diluted in 100 μl of the growth medium were added 24 h after seeding, and the cells were incubated for 96 h. Cells were fixed with TCA, stained with 0.4% (wt/vol) sulforhodamine-B dissolved in 1% acetic acid, and rinsed with 1% acetic acid to remove unbound dye. Bound dye was solubilized and the OD at 564 nm was measured.

Photoaffinity labeling with ^{3}H-Azidopine. 4×10^6 cells were photolabeled in 100 μl of PBS with 4 μCi of ^{3}H-azidopine, as previously described (22).

COMPARE analysis and determination of Pearson and Spearman correlation coefficients. The version of COMPARE used in this work differs from the original version of COMPARE that made comparisons based on a calculated mean difference in "deltas" (3). The current version of COMPARE is configured to calculate pairwise correlations with the $-\log_{10}$ (minus $\log_{10}$) of one of the specific NCI cell line activity parameters: GI50, TGI, or LC50 (23). For instance, the GI50 is the NCI designation for a time 0–corrected IC_{50}, the concentration of agent causing a 50% growth inhibition. Thus, $-\log_{10}$ (GI50)s for a "seed" or probe compound are correlated with the corresponding data from each compound in a database. In this paper, the GI50 data were used in the COMPARE studies, and the seed data were derived from a different source as described below. The correlation coefficients used are generally the Pearson correlation coefficients output by the SAS® procedure PROC CORR (SAS Institute Inc., Cary, NC) using the out=outp option. We also calculated Spearman correlation coefficients using the out=outs option for PROC CORR for these correlations employing a small subset of the available seed values. The Spearman correlations were investigated because of a concern that, for these small subsets, the seed data deviated too much from a normal distribution to use the Pearson method. In fact, the Spearman correlation coefficients differed little from the Pearson correlation coefficients.

This application of COMPARE differs from all previously reported applications in that the seed data are not generated from actual screening

Table I. List of Compounds Identified by COMPARE Analysis When mdr-1 Values of All 60 Cell Lines Were Used as Seed

	Pearson correlation coefficient	NSC number or name (description)
A	0.949	Deoxybouvardin
	0.881	S3-Desacetylphyllanthoside
	0.880	Trioxacarcin A
	0.844	Chromomycin A3
	0.830	Bruceantin
	0.825	Bisantrene hydrochloride
	0.821	Olivomycin
	0.819	NSC 645806 (8-nitro-9-aminoacridine derivative)
	0.803	Quassinoid
	0.781	Phyllanthoside
	0.776	NSC 640085 (aniline mustard derivative)
	0.770	Olivomycin
	0.764	(−) Cephaeline dihydrochloride
	0.757	Bouvardin
	0.751	NSC 80467 (fused naphthquinone imidazolium derivative)
	0.745	Bisantrene hydrochloride
	0.733	NSC 355644 (anthrapyrazole derivative)
	0.728	NSC 353076 (ellipticinium derivative)
	0.718	NSC 637905 (pyrrolo pyrrole derivative)
B	0.499	Taxol
	0.463	Vinblastine
	0.457	Actinomycin-D
	0.409	Adriamycin
	0.353	Daunomycin
	0.269	Teniposide (VM-26)
	0.153	Etoposide (VP-16)
	0.139	Cisplatinum
	0.128	BCNU
	0.109	Tetraplatin
	0.099	Melphalan
	0.095	CCNU
	0.083	Thiotepa
	0.077	Nitrogen mustard
	0.061	Chlorambucil
	0.038	Amsacrine
	0.035	Cytosine arabinoside
	0.021	6-Mercaptopurine
	−0.022	Hydroxyurea
	−0.052	Methotrexate
	−0.085	5-Fluorouracil
C	0.639	Deoxydoxorubicin
	0.409	Adriamycin
	0.353	Daunomycin
	0.191	Morpholino-adriamycin
	0.031	Cyanomorpholino-adriamycin

data. Instead, the seed data represent measurements of a multidrug resistance–related property of the cell lines used in the NCI screen. The larger the values of this multidrug resistance–related property for a particular cell line, the greater the potential drug resistance of that cell line to those agents subject to the particular multidrug resistance property (by being a good substrate for P-glycoprotein, for instance). The COMPARE databases store the screening data as −log(GI50) for historical reasons related to the mean graph sign conventions. This means that data from more drug-resistant cell lines are stored with smaller values than those from drug-sensitive cell lines. Therefore, by taking the negative of the multidrug resistance property measurements (the negative of the *mdr*-1 values), the highest positive correlation coefficients should be obtained with database compounds whose drug resistance was best correlated with the multidrug resistance property. Finally, to make the operation of this special application of COMPARE more easily visualized, we invoked the paradigm of the mean graph (3) and constructed a mean graph of the multidrug resistance property's seed values.

Results

Previous experience measuring *mdr*-1 expression in both cell lines and tissue samples suggested that, in a large fraction of the cell lines in the Anticancer Drug Screen, expression would be detectable only with use of the polymerase chain reaction. Figs. 1 and 2 present representative results for renal cell and colon carcinoma cell lines. Varying amounts of RNA were analyzed in serial twofold dilutions, ensuring that amplification was in the exponential range. A twofold increase indicated that the amplification proceeded in the exponential range and that an accurate value could be obtained. Similar measurements were carried out using *mdr*-1 and β-2 microglobulin primers in separate reactions. The level of *mdr*-1 in all cell lines was determined at least twice in two independent serial dilutions and in some cases four times. These results are summarized in Fig. 3. The values used in this figure were obtained by using carefully measured RNA concentrations to standardize for input RNA. Expression was detected in 39 of the 60 cell lines: in 28, with 125 ng or less of input RNA; in an additional 11, with 1 μg amplified 30–40 cycles. The other 21, which had undetectable expression even after amplifying 1 μg of RNA 40 cycles, were assigned values of 0.1 u. The 39 cell lines with detectable expression included 9/9 renal cell carcinoma cell lines, 6/9 colon carcinoma cell lines, 5/13 lung carcinoma cell lines, 9/9 melanoma cell lines, 8/8 cell lines established from central nervous system tumors, 1 ovarian carcinoma cell line, and 1 cell line in the leukemia/lymphoma category.

Similar results were obtained when *mdr*-1 values were calculated using β-2 microglobulin as the internal standard. This is graphically depicted in Fig. 4, which demonstrates the results when all cell lines are plotted individually. In this graph the x-coordinates are the *mdr*-1 values determined using the measured RNA concentration for standardization; the y-coordinates are the *mdr*-1 values calculated using β-2 microglobulin expression as an internal control. The line presents the ideal results that would be obtained if both *mdr*-1 values were identical. The distribution around this ideal presents graphically the similar results obtained with both standardizations, validating the use of either *mdr*-1 value for further studies.

It has been previously established that the sensitivity profile or fingerprint of a given drug can predict its mechanism of action using the COMPARE program (4). Having established the level of expression in the 60 cell lines, we sought to determine the correlation of these values with the sensitivity profile of drugs previously screened, with the dual goal of validating the results of the quantitative PCR analysis and determining whether a drug's fingerprint can predict mechanism(s) of resistance. To determine any correlations, a modified version of the COMPARE program was devised as described in Methods. In this modified program, the seed or probe was defined as the set

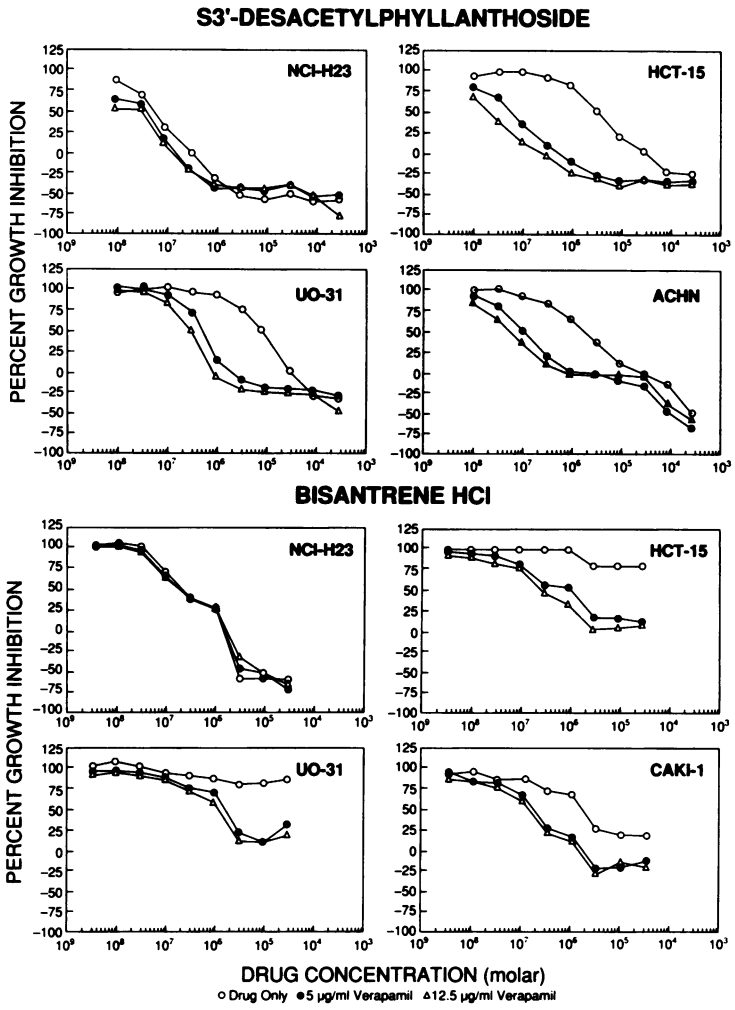

Figure 6. Results of cellular response to two of the agents with high Pearson correlation coefficients, and the effect of verapamil. Results with S3'desacetylphyllanthoside and bisantrene HCL are shown. Effect of 5 and 12.5 μg/ml verapamil on the sensitivity of four cell lines. NCI-H23 does not express *mdr*-1; the other cell lines express detectable levels. Negative values for the percent growth inhibition represent less number of cells at the end of the experiment than plated at the start.

of *mdr*-1 values multiplied by -1 for reasons outlined in Methods. This allowed comparisons to be made between the *mdr*-1 levels of the 60 cell lines and the fingerprint obtained for any previously tested drug.

The operation of this modified COMPARE can be easily visualized using the paradigm of the mean graph as in Fig. 5. The left panel of Fig. 5 shows a mean graph with *mdr*-1 values plotted to the left (by convention bars pointing to the left in a mean graph depict resistant cell lines). Compounds with good correlation coefficients using the *mdr*-1 values as the seed should have mean graphs like this model. The GI50 mean graph of one such compound, NSC 80467, is shown in the right panel

Table II. Fold Sensitization Observed with the Addition of 12.5 μg/ml Verapamil for the Compounds Listed in Nine Cell Lines with a Broad Range of mdr-1 Expression

Drug	MCF-7 ADR RES	U0-31	HCT-15	CAKI-1	DLD-1	ACHN	SW620	NCI-H23	MCF-7
Vincristine	33	9.8	173	29	37	6	10	8	4.1
Deoxybouvardin	109	49	270	55	16	26	—	1.1	—
S3'Desacetylphyllanthoside	47	30	176	42	9	32	3.9	1.6	1.4
Chromomycin A3	29	11	16	4.4	3	3.1	1.2	1.2	0.9
Bruceantin	14	4.2	6.2	3	2.4	1.8	1.1	1	1
Bisantrene HCL	13	25	117	12	14	15	2	1.2	3.4
Olivomycin	2388	110	311	35	18	19	1.1	—	—
An 8-nitro-9-aminoacridine	12	5.3	3.1	1.6	1.6	1.3	0.7	1.4	1
Quassinoid	67	11	44	12	5.6	4.1	1.8	—	—
Fused naphthquinone imidazolium derivative	34	37	268	26	31	50	—	5.4	—

of Fig. 5. Using the *mdr*-1 values to construct the seed and then correlating these 60 seed values with over 30,000 compound data sets in the NCI COMPARE database, we found a Pearson correlation coefficient of 0.779 for NSC 80467 (52 of the 60 possible cell lines were considered in the correlation). Many compounds showed even better correlations, and similar high correlations were confirmed when Spearman correlation coefficients were calculated. In this manner, many potential P-glycoprotein substrates were identified.

The 19 compounds with the highest Pearson correlation coefficients are listed in part *A* of Table I. The Pearson correlation coefficients for commonly used drugs are shown in part *B*. Higher values were obtained for known P-glycoprotein substrates when compared to other agents that are not part of the multidrug resistance phenotype, such as the platinum compounds, alkylating agents, and antimetabolites. In addition, a correlation was also observed for various anthracycline analogues, as shown in part *C*. The morpholino derivatives, to which P-glycoprotein–expressing cells have been shown to be sensitive, had lower correlation coefficients.

The observation that a majority of the 19 compounds with the highest Pearson correlation coefficients were large natural products suggested the results were valid. However, further documentation was sought. To do this, the effect of verapamil on the cytotoxicity of nine of these compounds and vincristine was tested in vitro using a group of nine cell lines with a broad range of *mdr*-1 expression. These included seven of the cell lines in the NCI Drug Screen (UO-31, HCT-15, CAKI-1, DLD-1, ACHN, SW620, and NCI-H23) as well as parental MCF-7 cells and the Adriamycin-resistant subline, MCF-7/ADR. Representative results for two compounds (S3'desacetylphyllanthoside and bisantrene HCl) are shown in Fig. 6. The effect of 5 and 12.5 μg/ml verapamil on the sensitivity of four cell lines is shown. Sensitization by verapamil was not observed in the NCI-H23 cell line, which does not express *mdr*-1, but it was seen in the three other cell lines, all of which express *mdr*-1. For the nine compounds tested, similar results were obtained, with variations among the different compounds in the degree of sensitization. These results are summarized in Table II, which tabulates the dose-modifying factors for the 12.5 μg/ml experiments. The dose-modifying factors were calculated by dividing the IC_{50} in the absence of drug by the IC_{50} in the presence of verapamil. Verapamil sensitized the *mdr*-1–expressing cell lines to these nine compounds to a similar or greater degree than it sensitized the cell lines to vincristine. The relative degrees of sensitization vary among the various cell lines, consistent with the diversity of the drugs and the diversity of the cell types. These results suggested that these compounds were likely P-glycoprotein substrates and provided indirect evidence that the *mdr*-1 measurements were accurate.

Encouraged by these results, we performed a similar COMPARE analysis using only the eight cell lines with *mdr*-1 levels > 20. It was reasoned that a higher correlation could be observed if the large number of cell lines with undetectable or very low levels were not included in the analysis. When a similar comparison was made, a large number of compounds with high Pearson correlation coefficients were identified, including 59 with correlation coefficients > 0.9. Table III lists the 19 compounds with the highest correlation coefficients. As in Table I, the correlation coefficients for commonly used chemotherapeutic agents and five anthracyclines are also listed. Fig. 7 provides an indication of the diversity of structure of these

Table III. List of Compounds Identified by COMPARE Analysis When mdr-1 Values of 8 Cell Lines with the Highest Levels Were Used as Seed

	Pearson correlation coefficient	NSC number or name (description)
A	1.000	NSC 618094 (Pyrrolo thiopyran benzoxepin derivative)
	1.000	NSC 624332 (Dihydro dimethyl phenylphosphorin derivative)
	1.000	NSC 646428 (Triaryl phosphonium ion salt)
	0.984	NSC 649087 (Tetramethoxyflavone)
	0.980	NSC 376128 (Dolastatin 10)
	0.980 (0.776)	NSC 640085 (Analine mustard derivative)
	0.980	NSC 648785 (Semi-synthetic thiocolchicine derivative)
	0.978	NSC 325319 (Didemnin-B)
	0.978	NSC 626852 (Mercaptophenyl ethanonato copper complex)
	0.973	NSC 633320 (Small, neutral, synthetic nitrogen heterocycle)
	0.971 (0.764)	NSC 32944 (Cephaeline dihydrochloride)
	0.966	NSC 172946 (3-desmethyl colchicine)
	0.965	NSC 620308 (Dolastatin 10 isomer)
	0.963	NSC 648114 (Quinolone with basic side chain)
	0.961	NSC 346243 (Aminoacridine)
	0.955	NSC 626316 (Podophyllotoxin derivative)
	0.953	NSC 643179 (Trimethoxybenzylidene pinacolone)
	0.952	NSC 526417 (Echinomycin A)
	0.948	NSC 344003 (Anthracenedione with aminoalkyl substitution)
B	0.826	Taxol
	0.753	Vinblastine
	0.729	Actinomycin-D
	0.706	Adriamycin
	0.573	Etoposide (VP-16)
	0.558	Daunomycin
	0.530	Teniposide (VM-26)
	0.273	Nitrogen mustard
	0.240	Amsacrine
	0.160	Thiotepa
	0.113	Melphalan
	0.088	Chlorambucil
	0.047	CCNU
	0.042	Cisplatinum
	−0.026	Hydroxyurea
	−0.064	Cytosine arabinoside
	−0.100	BCNU
	−0.170	Tetraplatin
	−0.325	Methotrexate
	−0.351	5-Fluorouracil
	−0.363	6-Mercaptopurine
C	0.856	Deoxydoxorubicin
	0.706	Adriamycin
	0.558	Daunomycin
	0.439	Morpholino-adriamycin
	0.099	Cyanomorpholino-adriamycin

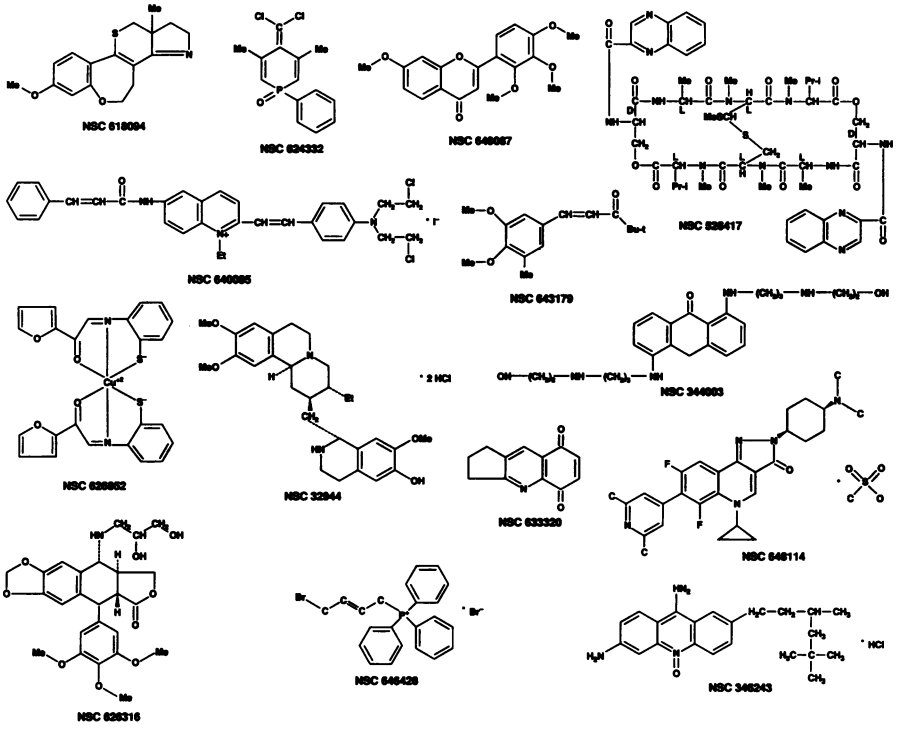

Figure 7. Chemical structures of 14 compounds chosen from among the 19 with the highest Pearson correlation coefficients when the program was seeded with the *mdr*-1 values of the 8 cell lines with the highest levels, and this was compared with relative resistance.

compounds. Although this analysis identified a large number of compounds that were not natural products, examination of the results suggested that potential P-glycoprotein substrates had been identified. Specifically, this analysis identified a large number of the compounds selected in the previous analysis. The 100 compounds with Pearson correlation coefficients > 0.867 included 14/19 from Table I and 8/9 for which cytotoxicity had been modulated by verapamil.

14 compounds in Table III for which sufficient quantities were available were evaluated further. Using four different approaches, all 14 were determined to be P-glycoprotein substrates. These four methods of analysis were chosen because they measure different properties of potential P-glycoprotein substrates and complement each other. The results are depicted in Figs. 8 and 9. First, the various compounds were tested for their effect on the accumulation and efflux of vinblastine in a multidrug-resistant cell line. For these studies, an Adriamycin-selected subline, SW620 Ad 300, derived from SW620 cells by continuous exposure to Adriamycin and expressing high levels of P-glycoprotein, was used. Previous characterization has demonstrated a predominant role for P-glycoprotein in the resistance of this subline (24). Vinblastine accumulation in the Adriamycin-selected SW620 Ad 300 cell line is ~ 5% that in parental SW620 cells. The results of this analysis are depicted in *A* and *B* of Fig. 8. Two known P-glycoprotein "substrates" (vincristine and paclitaxel) and the antagonist verapamil are included for comparison. The concentration of all compounds was 10 μm. Under these conditions, 12 of the 14 compounds were able to increase vinblastine accumulation above that in the SW620 Ad 300 cells. (For NSC 626852, background interference precluded a precise result from being obtained.) The 12 compounds tested inhibited vinblastine efflux. *C* shows the results obtained when cross-resistance was determined using parental SW620 cells and the multidrug-resistant subline SW620 Ad 300. Cross resistance was demonstrated for 10 of the 14 compounds; no cross-resistance was observed with 2 others (NSC 626852 and 643179); and a determination was not possible with 2 others (NSC 618094 and 649087), both of which were not toxic to either the parental or the resistant cells up to a concentration of 10 μM. The apparent discrepancies between the different measurements support the use of different approaches to determine whether a compound is recognized by P-glycoprotein, as discussed below.

In addition to these three approaches, the effect of the 14 compounds on ^{3}H-azidopine labeling was compared to that of vinblastine. Competition with 50 μm vinblastine or one of the 14 compounds was performed in live SW620 Ad 300 cells as shown in Fig. 9. Of the 14 compounds, 6 were as or more effective than vinblastine, while 8 others were able to decrease labeling to a lesser extent.

Discussion

The present study describes the quantitation of *mdr*-1 expression by PCR in the 60 cell lines of the National Cancer Institute's Anticancer Drug Screen Program. Expression of *mdr*-1/P-glycoprotein was detected in 39/60 cell lines. Renal cell carcinomas and colon carcinomas had the highest levels of expression, while the majority of ovarian carcinomas and leukemia/lymphomas were negative. Correlation of *mdr*-1 expression with sensitivity identified a large number of compounds that were demonstrated to be P-glycoprotein substrates by one or more of several approaches. These results validated the reliability of the quantitative PCR methodology used. The structural diversity of these compounds indicates that P-glycoprotein can recognize a spectrum of structures greater than that previously recognized. The widespread occurrence of P-glycoprotein in human cancer cell lines and its capability to transport a broad spectrum of compounds suggest that this protein can influence drug discovery. The present study confirms the validity of this

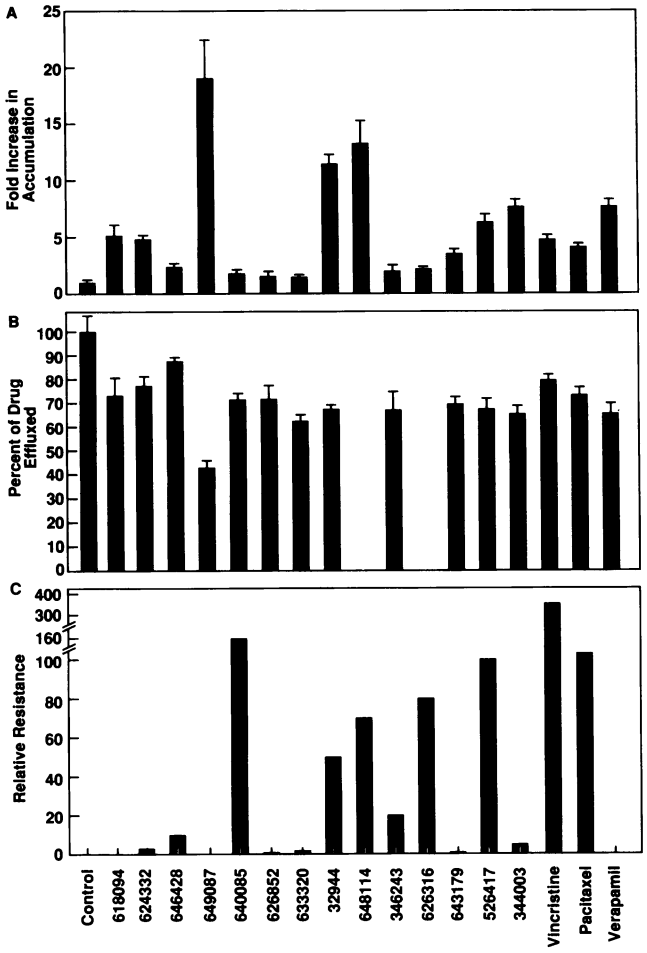

Figure 8. Evaluation of compounds with high Pearson correlation coefficients for potential as P-glycoprotein substrates in SW620 Ad 300 cells. Comparison with vincristine, paclitaxel, and verapamil. (*A* and *B*) The effect on vinblastine accumulation and efflux was determined using multidrug-resistant SW620 Ad 300 cells. The concentration of all compounds was 10 μM. Vinblastine accumulation was determined after a 1-h incubation. Increased accumulation was observed with 12 of the 14 compounds tested. Vinblastine efflux was determined as the percentage of drug effluxed in the first 10 min after a 2-h loading period. Decreased efflux was observed with the 12 compounds tested. (*C*) For the 14 compounds, the resistance of SW620 Ad300 cells relative to parental SW620 cells was compared. Multidrug-resistant SW620 Ad300 cells have high levels of P-glycoprotein and demonstrate cross resistance to the agents usually described as comprising the multidrug resistance phenotype. Cross resistance to 10 of the 14 compounds was demonstrated. For two compounds (NSC 618094 and NSC 649087), cross resistance could not be evaluated because of their lack of cytotoxicity at concentrations as high as 10 μM for both parental SW620 cells and the resistant subline.

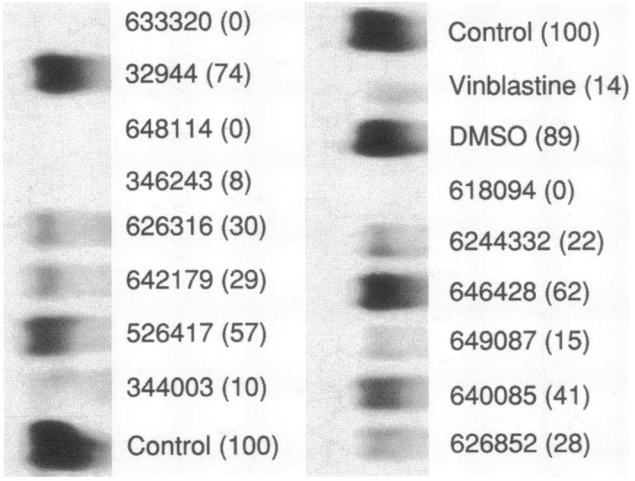

Figure 9. Competition of azidopine labeling by potential P-glycoprotein substrates. Intact multidrug-resistant SW620 Ad300 cells were incubated with ^{3}H-azidopine and photolabeled. Labeling was competed with 50 μM vinblastine or 50 μM concentration of one of the 14 potential P-glycoprotein substrates. The numbers in parentheses indicate the percent labeling compared to uncompeted control. Of the 14 compounds, 6 were at least as effective as vinblastine, while 8 others decreased labeling to a lesser extent, when compared to control.

novel approach and provides the basis for similar studies already under way, whose goal is to identify compounds whose activity is modulated by a diverse group of gene products, including other resistance mechanisms, putative drug targets, genes involved in regulation of the cell cycle, and genes with potential roles in apoptosis.

With the advent of clinical trials attempting to reverse multidrug resistance, we sought to characterize the expression of *mdr*-1/P-glycoprotein in the cell lines of the Drug Screen and to validate a quantitative PCR methodology previously described, with the ultimate goal of using this resource to characterize newly developed P-glycoprotein antagonists. To that end, we began by characterizing expression in the 60 cell lines using a precise method for quantitation by PCR as previously described (18). This quantitative approach, although labor intensive, does not depend on the addition of exogenous templates but relies on the kinetics of the amplification reaction and the knowledge that any measurement in the exponential range provides an accurate measurement of the amount of message. Its original description is extended in the present study and its utility and reliability are confirmed by the correlations established with drug resistance, which were in turn confirmed by one or more independent experiments. The control SW620 cell line has been previously determined to have 2.3 *mdr*-1 molecules/ng of total RNA. Assuming that the amount of RNA harvested per cell is similar for all the cell lines, then expression in the 60 cell lines ranges from 0 to > 1,700 *mdr*-1 molecules/ng of total RNA. The results in the present study differ from those obtained in a previous study, because the methodology used in that analysis resulted in early plateau and underestimated the level of *mdr*-1 (19). Thus, although similar results were obtained for cell lines with low levels of *mdr*-1 and those without *mdr*-1, much lower values were obtained in the previous analysis for the cell lines with higher *mdr*-1 levels. It is these higher values that are more valuable in an analysis such as the present one.

Several lines of evidence support the results obtained with the quantitative PCR methodology used to measure *mdr*-1 levels. The range and frequency of *mdr*-1 expression in the present study are consistent with previous reports and our unpublished experience indicating a high frequency and level of expression in renal cell and colon carcinomas, and lower levels in other malignancies (13, 25–31). Although levels were low, expression was documented in all melanoma cell lines and those established from central nervous system tumors as well as 5/13 lung

carcinoma cell lines. In addition, a high correlation has been found between the *mdr*-1 levels and those of a functional assay, rhodamine accumulation, in the same cell lines (our unpublished observations). Finally, the compounds identified as putative P-glycoprotein substrates were confirmed by one of several assays to be recognized by P-glycoprotein.

Previous analyses have used the COMPARE program and drug sensitivity values to identify patterns common to drugs that share a common target or mechanism of action (4). These fingerprints have also been used to identify putative targets of new or unknown compounds. The high correlations observed in these analyses have suggested that the target and mechanism of action are important determinants of drug sensitivity as determined in the Drug Screen assays. Thus, it was interesting to find that seeding the analysis with values of a mechanism of resistance (*mdr*-1) resulted in a high correlation for many compounds. The validity of these correlations was confirmed by experiments that indicated that these compounds were P-glycoprotein substrates. This result indicates that mechanism(s) of resistance can also influence the drug sensitivity profile. They indicate that similar analyses with other putative mechanisms of resistance may be very valuable.

Although the correlation coefficients for the drugs commonly identified as P-glycoprotein substrates (Adriamycin, vincristine, vinblastine, etoposide, and actinomycin-D) were not as high as those of other compounds, they were higher than commonly used drugs that are known not to be transported by P-glycoprotein, including platinum analogues, alkylating agents, and antimetabolites. In addition, it was interesting that among anthracyclines, the correlation was higher for Adriamycin and daunomycin than for analogues that are not part of the multidrug-resistant phenotype, morpholino and cyanomorpholino Adriamycin. Since multiple putative mechanisms of resistance have been proposed for Adriamycin, one could predict that a correlation with a single mechanism of resistance would be low. With regard to the vinca alkaloids, the 2-d assay used in the Drug Screen blunts the curves, and this may have affected the correlation coefficients.

The majority of the compounds identified were heretofore not regarded as P-glycoprotein substrates. For some, one might have made such a prediction, but for others, their identification broadens the spectrum of potential P-glycoprotein substrates. Although many of these drugs do not conform to the ideal pharmacophore described by Zamora et al. (32), the results should not be interpreted as contradictory. A compound without a specific target or with a nonmutatable target, for which P-glycoprotein is the sole mechanism of resistance, could have a higher correlation coefficient than another compound that is a better substrate. Similar comments could be made relating to the work of Ford et al. (33).

In summary, in the present study we have demonstrated the value of a previously described method for quantitating mRNA using PCR. In the 60 cell lines of the Drug Screen, expression of *mdr*-1 was found in 39. A correlation was demonstrated between *mdr*-1 expression and resistance for a large number of compounds, including many previously unrecognized P-glycoprotein substrates, validating the quantitative PCR methodology used. We conclude that P-glycoprotein has a broad specificity for transport and that its expression in a majority of human cancer cells can influence the identification of new agents. This type of analysis offers the possibility of learning more about other mechanisms of resistance and how genes can affect drug sensitivity and could help identify drugs potentially active against a target. A similar approach is currently being applied in studies with other gene products.

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