

Using the National Cancer Institute Anticancer Drug Screen to Assess the Effect of *MRP* Expression on Drug Sensitivity Profiles

M. ALVAREZ, R. ROBEY, V. SANDOR, K. NISHIYAMA, Y. MATSUMOTO, K. PAULL, S. BATES, and T. FOJO

Departamento Hematología-Oncología, Universidad Católica de Chile, Santiago, Chile (M.A.), Medicine Branch, Division of Clinical Sciences, National Cancer Institute (R.R., V.S., K.N., Y.M., S.B., T.F.), and Information Technology Branch, Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 (K.P.)

Received April 1, 1998; Accepted August 11, 1998

This paper is available online at <http://www.molpharm.org>

ABSTRACT

The *MRP* gene contributes to one form of multidrug resistance. To identify drugs interacting with *MRP*, we measured *MRP* mRNA expression by quantitative PCR in 60 cell lines of the National Cancer Institute Anticancer Drug Screen. Expression was detected in all cell lines (highest in lung carcinomas and central nervous system tumors) with a range of 14-fold. A mean graph of *MRP* mRNA levels was constructed to determine Pearson correlation coefficients (PCCs) with mean graphs of >40,000 compounds using the COMPARE analysis. Only 20 compounds had PCCs of ≥ 0.500 . The PCCs for VP-16, doxorubicin, and vincristine were 0.008, 0.13, and 0.257, respectively. Initially, 36 compounds with PCCs of ≥ 0.428 were analyzed using two *MRP*-overexpressing cell lines; low levels of cross-resistance was demonstrated for 23 compounds (1.3–

9.4-fold). Twenty-four compounds also were available for further studies. Using a fluorescence activated cell sorter assay to measure competition of calcein efflux from *MRP*-overexpressing cells, 10 compounds were found to increase calcein retention by ≥ 2 -fold. Ten compounds also were able to reduce ATP-dependent [3 H]LTC₄ transport into vesicles from *MRP*-overexpressing cells. These results contrast with previous studies with *MDR-1* in which high correlations were found and confirmed for a large number of compounds. Although other assays may be more revealing, in these unselected cell lines, *MRP* mRNA expression was a poor predictor of drug sensitivity. This raises the possibility that other factors, including conjugating enzymes, glutathione levels, or other transporters, confound the *MRP* effect.

Studies of drug-resistant cells have identified numerous putative mechanisms, including alterations in drug target, drug disposition, and drug efflux (Beck *et al.*, 1995; Brock *et al.*, 1995; Hasegawa *et al.*, 1996). Although these mechanisms often have been identified in cell lines with acquired resistance, studies with unselected cell lines and clinical samples often have found correlations between drug sensitivity and expression of drug-resistance genes, suggesting these mechanisms most likely contribute not only to acquired resistance but also to intrinsic drug sensitivity (Bordow *et al.*, 1994; Baldini *et al.*, 1995; Leith *et al.*, 1995).

The mechanism of *MRP*-mediated resistance is not fully clarified. Although increased drug efflux and reduced accumulation have not been demonstrated consistently in all sublines overexpressing *MRP*, it generally is accepted that this protein functions to export drugs (Zaman *et al.*, 1995). Less clear, however, is the requirement for prior drug me-

tabolism/conjugation and how essential these modifications are in the drug-resistant phenotype. Studies demonstrating *MRP*-mediated transport of a variety of GSH, glucuronide, and sulfate conjugates, most notably, the cysteinyl leukotriene LTC₄ support the thesis that prior drug modification must occur; although the evidence is less clear that for most chemotherapeutic agents such metabolism occurs (Jedlitschky *et al.*, 1994, 1996; Leier *et al.*, 1994; Muller *et al.*, 1994; Loe *et al.*, 1996a, 1996b; Shen *et al.*, 1996). Although the ability to metabolize drugs may be acquired, it also is possible that such mechanisms exist in unselected cells and that these may be in excess. The latter may be especially important if one considers *MRP*-mediated transport as a mechanism of intrinsic resistance because expression is almost universal.

To better understand the role of *MRP* in mediating intrinsic resistance and with the hopes of identifying additional agents whose transport can be mediated by *MRP*, we characterized *MRP* expression in the 60 cell lines of the National

M.A. was supported in part by a grant from Fondecyt (Project 1960475). The order of the first two authors is to be considered arbitrary.

ABBREVIATIONS: *MRP*, multidrug resistance-associated protein; GSH, glutathione; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; DPBS, Dulbecco's phosphate-buffered saline; *MDR-1*, multidrug resistance-1; calcein AM, calcein acetomethoxy ester; PCC, Pearson correlation coefficient; LTC₄, leukotriene C₄.

Cancer Institute Anticancer Drug Screen. Expression was documented in all 60 cell lines, with a range of expression of 14-fold. When these values were used as a "seed" in a COMPARE analysis, only low correlations were observed, suggesting that expression of MRP alone could not predict drug sensitivity. In the current report, we describe the results of these studies and discuss possible explanations for the observed results.

Experimental Procedures

Materials. Calcein AM was obtained from Molecular Probes (Eugene, OR). Doxorubicin was purchased from Adria Laboratories (Columbus, OH). Probenecid, ATP, creatine phosphate, creatine kinase, neuraminidase from *Clostridium perfringens*, and PMSF were purchased from Sigma Chemical (St. Louis, MO). [14,5,9,20-³H]LTC₄ was obtained from Dupont NEN Life Science Products (Boston, MA). Compounds identified through analysis of the drug screen data as potential MRP-interacting drugs were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (National Institutes of Health, Bethesda, MD).

Cell lines and cell culture. The cell lines comprising the National Cancer Institute Anticancer Drug Screen panel were obtained and processed as described previously (Monks *et al.*, 1991). 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 addition, six multidrug-resistant cell lines and their respective parental cells were used in the validation studies. [Some of these were kindly provided by Dr. Austin Doyle (UMCC-1/VP) (Doyle *et al.*, 1995); Dr. A. Hindenburg (HL-60/AR) (Bhalla *et al.*, 1985); and Drs. Erasmus Schneider and Ken Cowan (MCF7/VP) (Schneider *et al.*, 1994).] These cell lines were used to determine the relative cytotoxicity of compounds either in MRP-overexpressing cells and the respective parent or in the calcein efflux assay described below. The parental cells included the small cell lung carcinoma cell line UMCC-1, the human leukemia cell line HL-60, estrogen receptor-positive MCF7 and ZR-75B human breast carcinoma cells, and estrogen receptor-negative MDA-MB231 human breast carcinoma cells. The resistant cell lines included three [UMCC-1/VP (4 μ M), MCF7/VP (4 μ M), and HL-60/AR (1 μ M)] that have been described previously (Bhalla *et al.*, 1985; Schneider *et al.*, 1994; Doyle *et al.*, 1995). The other three cell lines were initially isolated as single clones from their respective parental cells, and the clones then were exposed to increasing concentrations of VP-16 as a population. They are designated MCF7/VP17 (1500 ng/ml), ZR/VP13 (900 ng/ml) and MDA/VP7 (3000 ng/ml). The parental cells and the resistant sublines were grown in monolayers or in suspension in Eagle's minimum essential medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin in 5% CO₂ at 37°. The resistant sublines have been maintained continuously in VP-16 or adriamycin at the concentrations indicated in parentheses.

RNA extraction and electrophoresis. RNA was isolated from the 60 cell lines over a period of 8 weeks. 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 MRP was performed using the forward primer ⁷⁹²⁵'-CGGAACCATCCACGACCCTA-ATC-3' and the reverse primer ¹⁰⁸⁶⁵'-ACCTCCTCATTCGCATC-CACCTTGG-3' (GenBank Accession no. L05628). In an initial experiment, 125 ng of RNA was reverse transcribed and amplified for

30 cycles to obtain an estimate of MRP expression. With 125 ng of RNA, MRP expression was detected in the 60 cell lines. Precise quantification then was performed using serially diluted samples. RNA isolated from SW620 human colon carcinoma cells cultured in the laboratory was used as a reference standard. Expression of MRP could be readily detected in 125 ng of RNA and in serial dilutions around 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 SW 620 cell line was arbitrarily assigned a value of 10, and all other values were determined relative to this. All quantifications were performed by densitometry.

Cytotoxicity assays. Cytotoxicity assays were performed as described previously (Skehan *et al.*, 1990). Briefly, 300-1000 cells/well plated onto 96-well dishes were incubated overnight, after which drug was added and the cells were incubated for an additional 5 days. At the end of the incubation period, cells were fixed with trichloroacetic acid and stained with 0.4% sulforhodamine B dissolved in acetic acid. Unbound dye was removed by washing four times with 1% acetic acid, and protein bound dye was extracted with 10 mM unbuffered Tris base. Absorbance was determined with a 96-well microtiter plate reader.

COMPARE analysis and determination of PCCs. The version of COMPARE used in this work differs from the original version of COMPARE that made comparisons based on calculated mean differences (Δ) (Paull *et al.*, 1989). The current version of COMPARE is configured to calculate pairwise correlations with the $-\log_{10}$ of one of the specific National Cancer Institute cell line activity parameters GI₅₀, TGI, or LC₅₀ (Boyd *et al.*, 1992). The GI₅₀ is the National Cancer Institute designation for a time zero-corrected IC₅₀ value and is defined as the concentration of an agent that causes a 50% growth inhibition. Thus, $-\log_{10}$ (GI₅₀ values) for a seed or probe compound are correlated with the corresponding data from each compound in a database. In this study, the GI₅₀ data were used in the COMPARE studies, with the MRP mean graph or fingerprint data derived from the MRP expression values. The correlation coefficients used were the PCCs output by the SAS procedure PROC CORR (SAS Institute, Cary, NC) using the out = output option. Previous studies had demonstrated that with these calculations, PCCs differed little from Spearman correlation coefficients. In contrast to the original use of COMPARE where the cytotoxicity profile of a compound whose activity had been determined in the 60 cell lines was configured as a mean graph or fingerprint and used as the probe in the COMPARE analysis, the probe used in this study was the fingerprint or mean graph generated from the data of expression of MRP in the 60 cell lines. The larger the level of MRP, the greater the potential drug resistance of that cell line to those agents whose activity is modulated by MRP. 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 (more negative) values than those from drug-sensitive cell lines. Therefore, by taking the negative of the MRP values, the highest positive correlations should be obtained with database compounds whose drug resistance was best correlated with MRP expression. Taking the positive of the MRP values would be expected to result in the highest positive correlations with compounds that are active in cell expressing high levels of MRP. Finally, to make the operation of this special application of COMPARE more easily visualized, we invoked the paradigm of the mean graph (Paull *et al.*, 1989) and constructed a mean graph of the MRP seed values.

Northern blotting. Total RNA (20 μ g/lane) was separated on a 6% formaldehyde gel and transferred to Hybond N⁺ with 10 \times standard saline citrate. The membrane was hybridized at 42° overnight with radiolabeled probe in Hybrisol I (Oncor, Gaithersburg, MD). Synthetic oligonucleotides corresponding to the published cDNA sequence of human MRP were used to isolate by RT-PCR specific

products for direct cloning into pGEM 3z vectors (Promega). The identity of the cDNA clone was confirmed by direct sequence analysis before use as a probe for Northern blot analysis. The sequences of the oligonucleotides used in the RT-PCR were $^{251}5'$ -AATGTCACGTG-GAATACCAGC-3' and $^{965}5'$ -AACAGGCACGACTTGTTC-3'.

Immunoblotting. Immunoblotting for MRP was performed using detergent-solubilized membrane proteins resolved on a 6% sodium dodecyl sulfate-polyacrylamide gel. Immunoblotting was performed with a 1:2000 dilution of a monoclonal MRP antibody, kindly provided by Dr. Rik Schepers (Free University Hospital, Amsterdam, Netherlands). After three washes in Tris-buffered saline/Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody at a dilution of 1:1000 for 1 hr. Five washes then were performed, and immunolabeled protein was detected by chemiluminescence.

Calcein efflux by flow cytometry. Single-cell suspensions were prepared from log phase cells either by trypsinization (MCF7 and MCF7/VP) or by vigorous agitation (UMCC-1 and UMCCVP). The suspensions were aliquoted into round-bottomed 96-well plates. The cells were sedimented and resuspended in complete media (phenol red-free Eagle's minimum essential medium with 10% fetal calf serum) containing 0.25 μ M calcein AM with or without 5 mM probenecid and incubated at 37° in 5% CO₂ for 30 min. After incubation, the cells were again sedimented at 300 \times g and washed once in DPBS. Cells that were to be analyzed for calcein accumulation were washed a second time, resuspended in DPBS, and kept on ice in the dark until analyzed. The remaining cells were resuspended in calcein AM-free complete medium with or without probenecid and incubated at 37° in 5% CO₂ for a 1-hr efflux period. After the efflux period, the cells were sedimented and washed twice with DPBS. The samples were resuspended in DPBS and kept on ice in the dark until analyzed.

When testing drugs potentially interacting with MRP, cells were resuspended in complete medium containing calcein AM with or without 10 or 100 μ M drug and incubated at 37° in 5% CO₂ for 30 min. The cells were sedimented, washed once with DPBS, resuspended in medium with or without 10 or 100 μ M drug, and incubated for 1 hr at 37° in 5% CO₂. The cells were sedimented and washed twice with DPBS. A FACSort flow cytometer (Becton Dickinson, San Jose, CA) with a 488-nm argon laser was used to analyze the samples. Calcein fluorescence was collected after a 530-nm bandpass filter. A minimum of 10,000 events were collected per sample, and the samples were gated on forward scatter versus side scatter to exclude clumps and debris.

Preparation of membrane vesicles. Membrane vesicles were prepared by nitrogen cavitation from HL-60, HL-60/AR, MCF7, and MCF7/VP cells, as described previously (Cornwell et al., 1986). Briefly, 10⁹ cells were washed by centrifugation at 1,000 \times g for 5 min with DPBS containing 1% aprotinin and then with vesicle buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl₂, 250 mM sucrose, and 2 mM PMSF). Cell pellets were resuspended into vesicle buffer and equilibrated at 4° under a nitrogen pressure of 500 psi for 60 min. EDTA was added to the lysed cell suspension to a final concentration of 1 mM. The lysed cell suspension was diluted 1:4 with dilution buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 2 mM PMSF). To remove nuclei and unlysed cells, the lysed cell suspensions were centrifuged at 4,000 \times g for 10 min. The supernatant was layered onto a 35% sucrose cushion (10 mM Tris-HCl, pH 7.5, 35% sucrose, and 1 mM EDTA) and ultracentrifuged at 16,000 \times g for 30 min. The interface was collected, diluted with dilution buffer, and then ultracentrifuged at 100,000 \times g for 45 min. The vesicle pellet was resuspended in dilution buffer using a 23-gauge needle. Vesicles were stored at -70° until the time of use. The protein levels were determined by the method of Bradford (1976). Sialidase accessibility for the determination of inside-out vesicles was examined as described (Ishikawa, 1989; Ishikawa and Ali-Osman, 1993).

[³H]LTC₄ uptake by membrane vesicles. Uptake of [³H]LTC₄ by vesicles was measured by filtration as described previously (Ish-

ikawa, 1989; Ishikawa and Ali-Osman, 1993). The incubation medium contained membrane vesicles (50 μ g of protein), [³H]LTC₄ (0.025 μ Ci; 0.189 pmol), 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM creatine phosphate, 100 μ g/ml creatine kinase in a final volume of 100 μ l with or without 1 mM ATP, and various concentrations of the test drugs. The reaction was carried out at 37° and stopped with 4 ml of ice-cold stop solution (250 mM sucrose, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.4). The diluted samples were passed through Millipore (Bedford, MA) filters (GVWP, 0.22- μ m pore size) under light suction to measure the trapped [³H]LTC₄ in the vesicles. The filters were washed with ice-cold stop solution and air dried. Each filter was placed in scintillation fluid, and radioactivity was determined by liquid scintillation counting. To confirm whether the amount of [³H]LTC₄ accumulated by membrane vesicles was actual transport into vesicles or binding to membranes, experiments were conducted to determine the uptake of [³H]LTC₄ in different concentrations of extravesicular sucrose.

Results

Previous experience measuring *MDR-1* and other molecular targets in the cell lines of the National Cancer Institute Anticancer Drug Screen had demonstrated that the quantitative PCR methodology used provided accurate measurements of expression (Alvarez et al., 1995). These observations, combined with the demonstration that in a number of unselected cell lines antibodies could not detect MRP protein expression (Matsumoto Y, Nishiyama K, Bates S, and Fojo T, unpublished observations and experiments described in legend to Fig. 6), were catalysts for measuring MRP mRNA expression using the quantitative PCR methodology described previously (Alvarez et al., 1995). Fig. 1 shows representative results for lung and colon carcinoma cell lines. Varying amounts of RNA were analyzed in serial 2-fold dilutions to ensure that the exponential range of amplification was assayed. In all cases, RNA from SW620 cells was included as a reference control to control for variations in reaction efficiency from experiment to experiment, as well as technical variations, and to provide a reference value. Previous studies had demonstrated that the primers used could sustain exponential amplification over the range studied (Zhan et al., 1997). A 2-fold increase is evidence that the amplification is in the exponential range and that a valid result can be derived. Using this approach, the *MRP* level was determined in the 60 cell lines at least twice. These levels were calculated using the input RNA as the standard. Previous studies using these same samples in the analysis of *MDR-1* expression demonstrated that standardization with β_2 -microglobulin did not significantly change the results (Alvarez et al., 1995). The results in the 60 cell lines are depicted graphically in Fig. 2, according to the tissue of origin. Although there was considerable overlap among the different tissue types, the highest levels were observed in lung carcinoma- and central nervous system tumor-derived cell lines; whereas the lowest levels were found in colon carcinoma- and melanoma-derived cell lines. Unlike previous experience with *MDR-1* where expression was detected in only 39 of the cell lines, MRP expression was detected in all cell lines over a 14-fold range (2.6–36). Fig. 2 also depicts the mean graph or fingerprint generated using values of calcein efflux as discussed below.

It has been previously established that the drug sensitivity profile of a given drug depends on the drug's mechanism(s) of

action or target(s) and mechanism(s) of resistance (Paull *et al.*, 1992, 1995; Lee *et al.*, 1994; Alvarez *et al.*, 1995). Thus, having measured the expression of MRP in the 60 cell lines, we sought to determine the PCCs between the MRP values and the drug sensitivity profiles of ~40,000 compounds previously analyzed and stored in the National Cancer Institute Drug Screen database. To determine this correlation, we used a modified version of COMPARE that was used previously in a similar analysis with *MDR-1* values. In this modified version, the fingerprint or mean graph of the MRP values was used as the probe in the COMPARE analysis, after multiplication by -1 . In this way, the MRP COMPARE should rank higher those compounds that are less potent against cells with high MRP expression and more potent against cells with low MRP expression. An example of this analysis is shown in Fig. 3, which depicts the fingerprint or mean graph of the MRP values, compared with the mean graph of NSC 626482, a compound with one of the highest PCC (0.529). By convention, bars pointing to the left in the mean graph represent a resistant cell line or in the case of MRP expression, a high level of expression, which would be predicted to confer drug tolerance. The poor correlation observed with this compound is evident from the figure. The highest PCC was 0.615, but only 20 of the nearly 40,000 compounds had PCCs above 0.500. PCCs for drugs whose activity has been previously shown to be modulated in MRP-overexpressing cell lines, as well as several commonly used anticancer agents, were also very low. The PCCs for VP-16, doxorubicin, and vincristine were 0.008, 0.13, and 0.257, respectively. The chemical properties of the compounds was unremarkable. In a previous analysis, we observed an average molecular weight of 325 for 452,084 compounds in the National Cancer Institute chemical database. A more recent analysis of 93,831 compounds gave marginally higher average molecular weight values (mean, 399; median, 346; mode, 296). In addition, the mean molecular weight for compounds with high correlations with *MDR-1* expression or rhodamine efflux (the majority were confirmed as P-glycoprotein substrates) was 600 (Lee *et al.*, 1994). By comparison, the compounds in the MRP COMPARE had a mean molecular weight of 444, a median of 374, and a mode of 373. The percentage of compounds with at least one positively charged atom at physiologic pH was 40%, less than but not dissimilar to the 44%

cationic substrates found previously for *MDR-1* substrates. There was one anionic compound among the 50 compounds with the highest PCCs, but this compound also had a quaternary nitrogen positive charge; so there really were no anionic compounds. Similar results were obtained whether the data for MRP expression were entered as the calculated value or the log of the calculated value.

Although the low PCCs and the lack of common structural features suggested that the analysis had failed to identify compounds whose toxicity might be consistently significantly modulated by MRP expression, we nevertheless sought to verify the results. Initially, we determined the cytotoxicity of 36 compounds with PCCs of ≥ 0.428 using the MRP-overexpressing cell lines MCF7/VP and ZR/VP13 and parental MCF7 cells and ZR-75B (Schneider *et al.*, 1994; Matsumoto *et al.*, 1997). These 36 were chosen because sufficient quantity was available for the preliminary studies. It should be emphasized that the choice was based solely on availability and that no bias was introduced into the selection. The 36 included 21 of the 30 compounds with PCCs above 0.481 (for the other nine compounds, no material or insufficient material was available). Of these 36 compounds, 23 were found to be more toxic to the parental cells than to the resistant sublines (1.3–9.4-fold more toxic). The cytotoxicity curves for three of these compounds are shown in Fig. 4, where their relative potency is compared with the results obtained with VP-16. The chemical structures of 11 of the compounds are depicted in Fig. 5. Of these 36 compounds, larger quantities were available for further studies for 24 compounds. The data for these 24 compounds that were evaluated further as described below are summarized in Table 1.

To further characterize potential interactions of these compounds with MRP, competition of calcein efflux in MRP-overexpressing cell lines was evaluated. Previous studies have shown that calcein efflux is increased in MRP-overexpressing cell lines compared with parental controls (Feller *et al.*, 1995). As a first step to validate this assay, we compared calcein efflux in six drug-selected cell lines overexpressing MRP, with calcein efflux in parental cells. Fig. 6A demonstrates the expression of MRP mRNA in these cell lines as determined by Northern analysis of total RNA and the level of MRP protein detected by immunoblotting. Using a fluorescence activated cell sorter-based assay, calcein efflux was

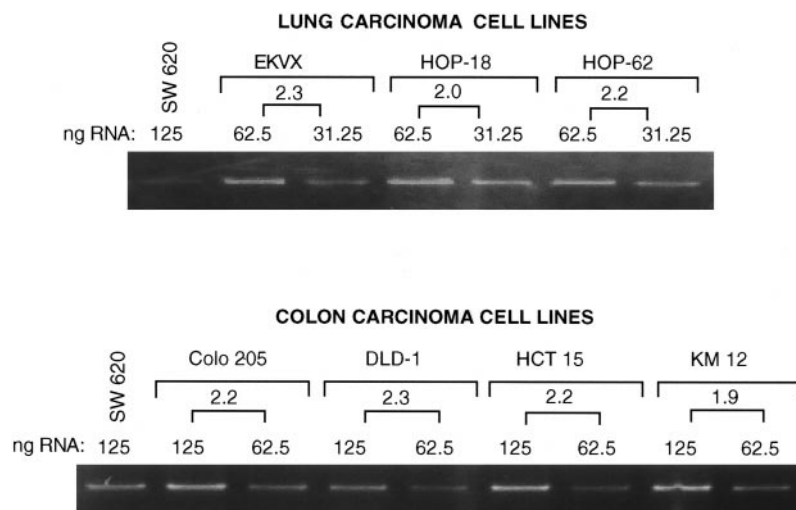


Fig. 1. Quantitative PCR of RNA from lung and colon carcinoma cell lines. This composite demonstrates the PCR results of serial 2-fold dilutions of RNA. RNA from the SW620 human colon carcinoma cell line was used as the reference standard in all experiments. The nanograms of input RNA used are identified. Numbers above the brackets, 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.

found to be increased in all six cell lines compared with their respective parental cells, and the efflux could be modulated by 5 mM probenecid, a drug previously reported to compete MRP-mediated calcein efflux (Versantvoort *et al.*, 1995). Fig. 6B demonstrates representative histograms in parental MCF7 cells and the MRP-overexpressing subline MCF7/VP. Calcein fluorescence after a 30-min accumulation period (control) is compared with that after a 1-hr efflux in the absence of an added antagonist (efflux) or in the presence of 5 mM probenecid (probenecid efflux). The extent of efflux was calculated as either the difference (in mean channel numbers) between the control value and the value obtained after efflux in the absence of probenecid or the difference between efflux in the presence of probenecid and that in the absence of probenecid. When these two values were calculated, a high correlation was observed. Furthermore, as shown in Fig. 6, C and D, when the values representing the difference between efflux in the presence of probenecid and efflux in the absence of probenecid (shift in mean channel numbers) were plotted against the levels of MRP mRNA determined by the Northern analysis or the protein level was determined by immunoblotting, significant correlations were obtained. Similar high correlations were obtained when the shift in mean channel number was calculated using the control value and the value obtained after efflux in the absence of probenecid.

Having established a significant correlation between MRP expression and calcein efflux in the six drug-resistant cell lines, studies were designed to measure the ability of the compounds identified in the MRP COMPARE to modulate calcein efflux. One compound (NSC 627991) was fluorescent and consequently could not be used in the calcein efflux assay. However, its fluorescent properties allowed for its accumulation to be quantified, and as shown in Fig. 7A, accumulation in MRP-overexpressing UMCC-1/VP cells was lower than in parental UMCC-1 cells and was ATP dependent with higher accumulation in the absence of ATP. Twenty-three other compounds without significant fluorescence were evaluated by quantifying inhibition of calcein efflux from MRP overexpressing cells (MCF7/VP and UMCC-1/VP).

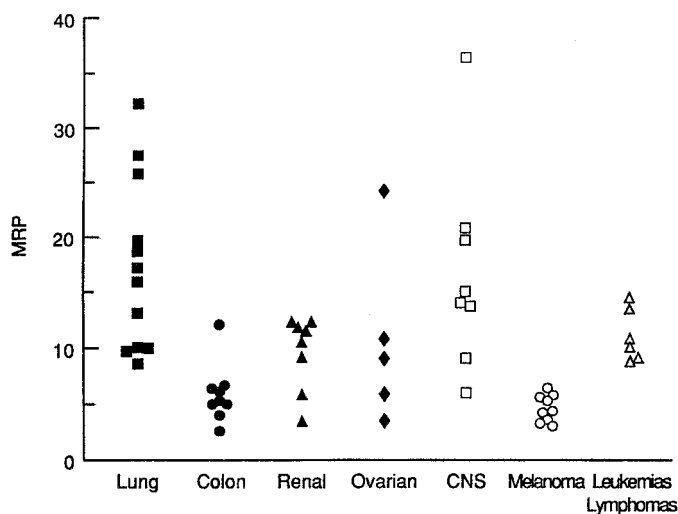


Fig. 2. Graphic summary of the MRP levels in the 60 cell lines of the National Cancer Institute Anticancer Drug Screen Program. SW620 human colon carcinoma cells maintained in the laboratory were used as the reference for all measurements. This cell line was arbitrarily assigned a value of 10.

Using this approach, inhibition of calcein efflux (defined as a 200% increase of calcein fluorescence) could be demonstrated for 10 of the 23 compounds with concentrations of 10–100 μ M. Representative experiments are shown in Fig. 7B and are summarized numerically in Table 1. The degree of inhibition was comparable to that observed after the addition of 5 mM probenecid, performed simultaneously as a positive control. Of the 10 compounds, significant cross-resistance had not been demonstrated in the cytotoxicity assays for four. This

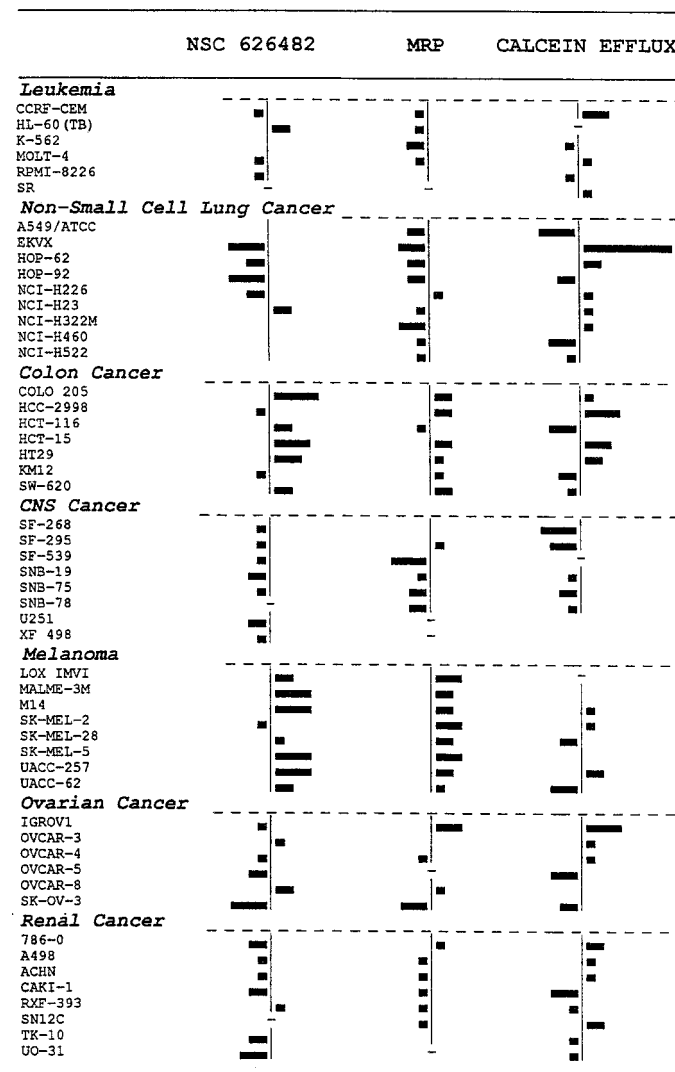


Fig. 3. Comparison of the simulated mean graph of MRP expression with the GI_{50} mean graph of compound NSC 626482 ($PCC = 0.529$) and the mean graph of calcein efflux. The simulated mean graph was derived by using the MRP values shown in Fig. 2 or calcein efflux value discussed in the text. Vertical line in the MRP profile (or fingerprint), mean level of MRP expression in the 60 cell lines relative to this mean value. The size of the bar is proportional to the extent of deviation from this mean. Bars pointing to the left, cell lines whose MRP levels are greater than the mean. Bars pointing to the right, cell lines whose MRP levels are less than the mean. This presentation was chosen because by convention, the mean graphs of all compounds tested in the drug screen present cell lines that are more resistant with bars pointing to the left. A similar presentation was chosen for the calcein efflux values. As shown for NSC 626482, bars representing cell lines that require concentrations higher than the mean for inhibition point to the left; whereas those representing cell lines more sensitive to NSC 626482 than the mean point to the right. Higher MRP levels (bars pointing to the left) would confer resistance to an MRP compound, as would high levels of calcein efflux.

difference in assay results was not unexpected and is considered further in the Discussion.

To further document an interaction of these compounds with MRP, membrane vesicles were prepared from HL-60 and HL-60/AR cells, and inhibition of LTC₄ uptake was measured as shown in Fig. 8. These cells were chosen for these studies because of extensive previous reproducible experience both in the literature and in our laboratory (Jedlitschky *et al.*, 1994). The percentage of inside-out membrane vesicles were 43.2% for the HL-60 and 50% for the HL-60/AR membrane vesicle preparations. Fig. 8A shows the time course of [³H]LTC₄ uptake by membrane vesicles. The uptake of [³H]LTC₄ was enhanced by the presence of ATP, with vesicles prepared from HL-60/AR cells accumulating more [³H]LTC₄ than vesicles from HL-60 cells in the presence of ATP. Furthermore, as shown in Fig. 8B, the uptake of [³H]LTC₄ by HL-60/AR vesicles in the presence of ATP was osmotically sensitive, indicating that most of the [³H]LTC₄ accumulated was actually transported into the intravesicular space of the HL-60/AR membrane vesicles, in contrast to that accumulated by HL-60 vesicles in the presence of ATP, which reflects binding to membrane vesicles and not transport. Fig. 8C compares the effect on [³H]LTC₄ uptake of 22 of the compounds evaluated in the current study. As can be seen, 10 of the compounds were able to reduce [³H]LTC₄ uptake by ≥25%, with some at concentrations as low as 10 μM (data shown are for 100 μM for all compounds).

The inability of the MRP mRNA analysis to identify more drugs whose cytotoxicity was significantly affected by MRP expression or to identify higher correlations prompted an

attempt to characterize the 60 cell lines using an assay of MRP function. Having validated the calcein efflux assay in the selected cell lines, as described above, calcein efflux was measured in the 60 cell lines of the Anticancer Drug Screen. The efflux of calcein was measured under normal culture conditions, after a 30-min loading period and after an efflux period of 1 hr. Fig. 3 shows the mean graph that was constructed with the data for calcein efflux in 58 cell lines. The values graphed are the difference between the value at the conclusion of a 1-hr period of efflux in the presence of 5 mM probenecid minus the value after a 1-hr efflux period in the absence of probenecid. As shown above, a strong correlation between this value and MRP expression had been shown in the drug-selected cell lines. In contrast to the results obtained with the MRP mRNA measurements, a narrower range of values were found with this assay, and the tissue-specific distribution observed with the MRP mRNA measurements was not as apparent. When the efflux values were compared with the mRNA values, a correlation of only 0.005 was found. Because the latter could reflect the poor predictive value of the mRNA measurements, a fingerprint or mean graph was constructed using the efflux values, and this fingerprint was used as the probe in a COMPARE analysis. As with the MRP mRNA analysis, only low correlations were obtained, with the highest PCC being 0.57, and only 26 compounds had values above 0.5. Again, examination of the structures of the compounds with the highest PCCs failed to identify common structural attributes. Because this approach did not result in a higher correlation, a decision was

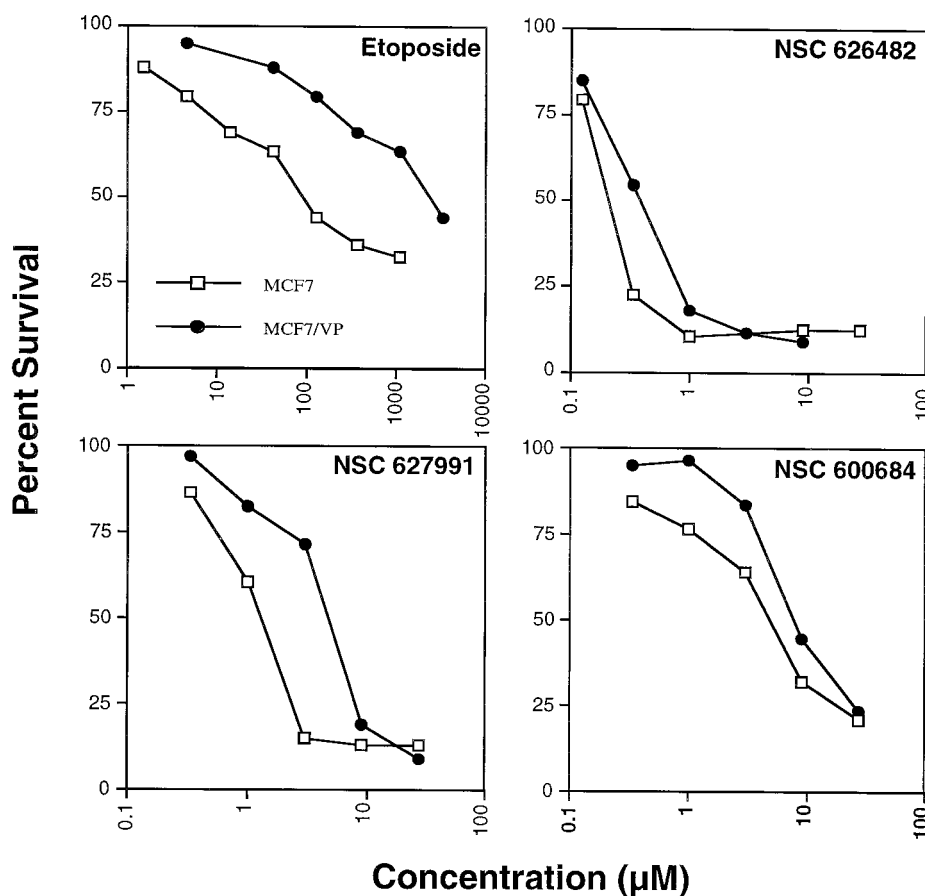


Fig. 4. Results of cytotoxicity studies of VP-16 and three of the compounds with the highest PCCs. In this experiment, cytotoxicity for parental MCF7 human breast carcinoma cells and the MRP-overexpressing subline MCF7/VP was determined.

made to not further pursue validation of the compounds ranked highest.

Discussion

The current study describes the characterization of *MRP* expression in the 60 cell lines of the National Cancer Institute Anticancer Drug Screen and an attempt to evaluate the

role of this protein in modulating drug sensitivity in unselected cell lines. *MRP* mRNA expression was measured by quantitative PCR; and a functional assay was attempted using calcein efflux to quantify *MRP*-mediated transport. Although low correlations were observed between cytotoxicity and either *MRP* expression or calcein efflux, some evidence of interaction with *MRP* was found for more than half of the compounds with the highest PCCs, validating the

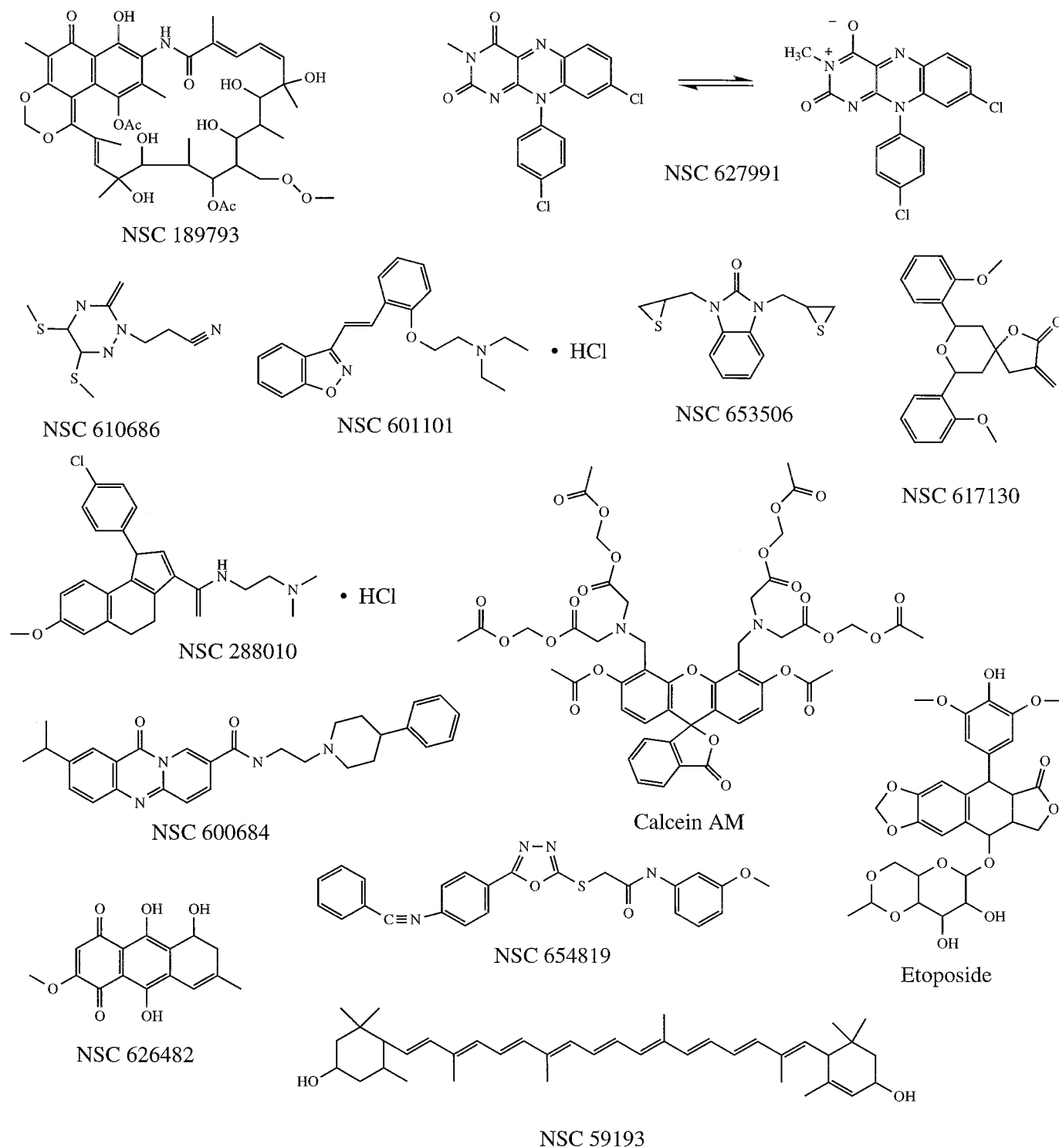


Fig. 5. Chemical structures of 11 of the compounds from the *MRP* Compare that were found by at least one assay to interact with *MRP* in experiments described in the manuscript. Also shown for comparison are the structures of calcein-AM and etoposide.

analysis. However, compared with previous studies measuring other molecular markers, including *MDR-1*, MRP expression alone was a poor predictor of drug sensitivity in the 60 unselected cell lines of the National Cancer Institute Drug Screen, suggesting that other factors confound the MRP effect.

Unlike molecular markers that are expressed in only a subset of cell lines, MRP mRNA expression was demonstrated in all 60 cell lines of the drug screen panel (Alvarez *et al.*, 1995; Freije *et al.*, 1997; O'Connor *et al.*, 1997; Wosikowski *et al.*, 1997). Using quantitative PCR, the range of expression was found to be ~14-fold; with some differential based on the tissue of origin of the carcinoma. The highest levels were observed in lung and central nervous system carcinomas, and the lowest in melanomas and colon carcinomas. Thus, the fingerprint for MRP demonstrates "tissue-determined" distribution. The fingerprint data then were used as the probe in a COMPARE analysis to identify compounds whose activity profile is highly correlated with the expression of MRP. Correlations were obtained with >40,000 compounds in the database. Surprisingly, unlike *MDR-1*, epidermal growth factor receptor, aldehyde dehydrogenase, and other molecular targets, high correlations were not found with any drugs (Lee *et al.*, 1994; Alvarez *et al.*, 1995; Fitzsimmons *et al.*, 1996; Freije *et al.*, 1997; Wosikowski *et al.*, 1997). Only two compounds had a PCC above 0.6 and only 20 were above 0.500. Among the 176 standard agents, the highest correlation was only 0.375, including poor correlations with VP-16 (0.008), adriamycin (0.13), and vincristine (0.257), agents previously identified as composing part of the MRP-resistance phenotype. Several possible explanations can be invoked for the low correlations compared with those obtained with other molecular targets, and these are discussed below.

Although the PCCs were low, we sought to determine whether the compounds with the highest PCCs could interact with MRP. The initial screening was performed with two etoposide-selected sublines (MCF/VP and ZR/VP13), previously shown to overexpress MRP (Schneider *et al.*, 1994; Matsumoto *et al.*, 1997). Using these models, modest cross-resistance was found to 23 of 36 compounds with PCCs of >0.428. A sensitivity of 63% (23 of 36) perhaps was not surprising given the low PCCs and contrasts with a 100% predictive value of the higher PCCs obtained when a similar analysis was performed with *MDR-1* (Lee *et al.*, 1994; Alvarez *et al.*, 1995). To further confirm an interaction of these compounds with MRP, we sought to determine whether these compounds could function as antagonists of MRP-mediated drug transport. To do this, we adapted published observations of calcein transport by MRP-overexpressing cell lines and developed an assay to measure inhibition of calcein efflux. Calcein is available commercially as calcein AM, a cell-permeant P-glycoprotein substrate. After uptake, the ester is cleaved, generating calcein, a negatively charged compound that cannot diffuse across membranes but is a substrate for transport by MRP (Feller *et al.*, 1995). In six independent models composed of parental cells and etoposide- or adriamycin-selected MRP-expressing sublines, we showed a high correlation between MRP mRNA and protein expression, and calcein efflux, suggesting that calcein efflux could be used as a mirror of MRP expression. When 23 of the compounds were used as antagonists, 10 were found to increase calcein fluorescence 200% at concentrations of 10–100 μ M. One additional compound, NSC 627991, identified in the original screen as a potential substrate, could not be analyzed as an antagonist in the calcein efflux assay because of its intrinsic fluorescent properties. However, reduced accumulation could be demonstrated in cells overexpressing MRP compared with

TABLE 1

Summary of assays performed to evaluate the ability of a compound to interact with MRP

Pearson's correlation coefficient obtained by comparing the drug cytotoxicity profile with that of MRP expression as described in the text. Relative resistance is defined as the ratio of the IC₅₀ values (MCF7/VP:MCF7; ZR/VP13:ZR-75B). Inhibition of calcein efflux values are given as percentage change of calcein accumulation. Inhibition of LTC₄ uptake number represents the percent decrease with 100% maximum.

NSC number	Correlation coefficient	Relative resistance MCF7/VP	Relative resistance ZR/VP13	Inhibition of calcein efflux MCF7/VP	Inhibition of calcein efflux UMCC-1/VP	Inhibition of LTC ₄ uptake
26040	0.525	1	1			
59193	0.501	1.3	1.9		111	88
189793	0.489	1.5	3.9	289	286	73
288010	0.548	6.8	1	201	137 ^a	29
600684	0.487	2.3	9.4	195	262	34
601101	0.516	1	1	205 ^a	^a	
Discreet	0.513	1	1	^a	109 ^a	
610686	0.518	2.4	2.1	202	188	
617130	0.456	1	1	217	138	10
617131	0.489	1.2	4.3	139	153	
626482	0.529	2	1	429	195	83
626551	0.576	1	1.7	122		
627991	0.568	4	2.2	Fluorescent	Fluorescent	80
Discreet	0.486	1	2	Insoluble	Insoluble	Insoluble
639859	0.615	1	1.4	129 ^a	122	
Discreet	0.433	1	1	164 ^a	^a	90
643318	0.488	1	2.5	239	365	25
653506	0.494	1	1.3	186	131	
654819	0.481	1	1	343	319	86
654968	0.612	1	1	107		
656471	0.487	1	1.4			
657799	0.511	1	1	286	183	35
661418	0.511	1.8	1.3	^a	165 ^a	
Discreet	0.435	1	1	138	106	

^a Toxic at 100 μ M.

parental cells. Finally, we sought to further determine an interaction with MRP by determining the ability of a compound to block $[^3\text{H}]\text{LTC}_4$ uptake into membrane vesicles isolated from MRP-overexpressing drug-resistant cells. Using this analysis, 10 compounds were shown to inhibit $[^3\text{H}]\text{LTC}_4$ uptake, including seven of the 10 compounds with an effect on MRP-mediated calcein transport and NSC

627991. It should be emphasized that some of the compounds that blocked both $[^3\text{H}]\text{LTC}_4$ uptake and calcein efflux (e.g., NSC 654819) had very low levels of cross-resistance demonstrated in the MRP-overexpressing sublines compared with parental cells. These low levels of cross-resistance would have likely been considered insignificant; however, the evidence from the other assay suggests that these compounds do

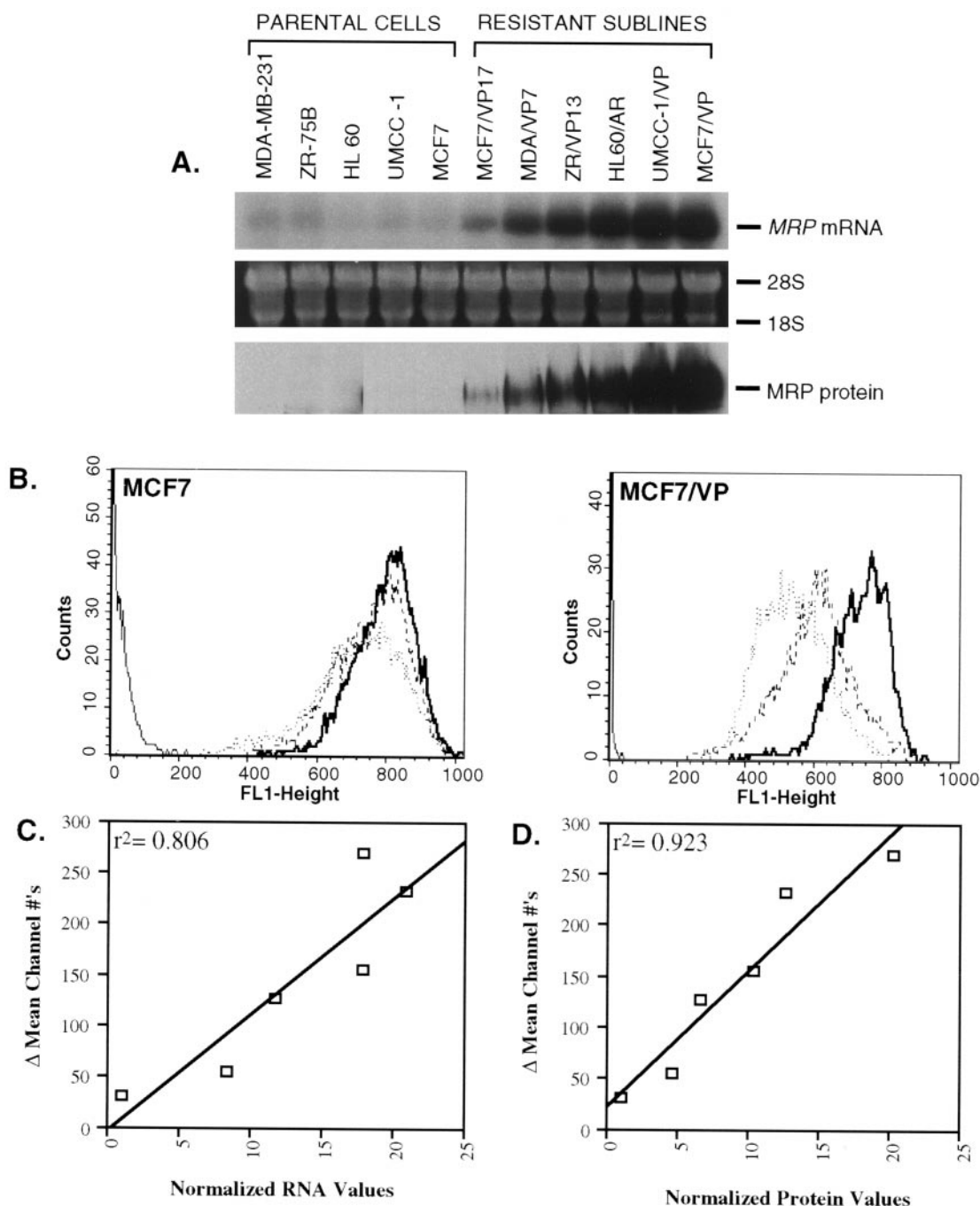


Fig. 6. Calcein efflux in MRP-overexpressing cell lines. A, MRP expression in the six multidrug-resistant cell lines used in this analysis. Northern blot and immunoblot are shown. B, Representative histogram of calcein accumulation and efflux in parental MCF7 cells and the MRP-overexpressing subline MCF7/VP. Accumulation after a 30-min incubation period in the MRP-overexpressing cell line is decreased compared with parental cells (dashed line), and the accumulation is further decreased after a period of efflux (dotted line). The latter can be abrogated by the addition of 5 mM probenecid, which maintains calcein levels high (solid line). C and D, Correlation in the six drug-resistant cell lines between MRP expression as determined by Northern analysis (C) or immunoblotting (D) and calcein efflux, measured as shift in mean channel numbers and representing the difference between the mean channel number after efflux in the presence of probenecid and the mean channel number after efflux in the absence of probenecid.

interact with MRP. These findings underscore the fact that not all the assays used have comparable sensitivities for all substrates. This can be explained by noting that the different assays developed to detect MRP interaction measure different things and can be affected differently. For example, in a given model, the cytotoxicity assays and the inhibition of calcein efflux may be affected by the presence or absence of specific conjugating enzymes necessary to confer cross-resistance. This possibility is supported by current evidence that suggests that MRP may transport drugs that have been modified to an anionic form by glutathione conjugation, glucuronidation, or sulfation (Jedlitschky *et al.*, 1994, 1996; Leier *et al.*, 1994; Muller *et al.*, 1994; Loe *et al.*, 1996b). On the other hand, the assay measuring inhibition of [3 H]LTC₄ uptake into vesicles requires that a drug interact directly with MRP, and in the cell-free system used, drug modification is unlikely to occur. Thus, this assay measures the interaction of unmodified drug with MRP. The differences in the various assays also can account for why two compounds can appear similar in one assay but differ in a second assay.

A comparison of the data from the various assays is summarized in Table 1.

As with MDR-1, we were able to identify additional compounds that interact with MRP. However, compared with MDR-1, fewer compounds were identified, and the degree of cross-resistance was substantially less. Several possible explanations have been considered to explain the low correlations obtained with the MRP expression pattern. The first is that unlike MDR-1, MRP is expressed in all cell lines, and the range of expression is not as great as that of MDR-1, making statistical correlations less powerful. Although statistically correct, it must be remembered that using MDR-1 as the "seed," with 28 cell lines having a value of zero, high correlations were obtained and confirmed. Although the highest correlations with MDR-1 were obtained with a subset of the cell lines expressing what was considered to be a significant level of MDR-1, high correlations exceeding 0.9 also had been obtained for numerous compounds using the MDR-1 expression data of the 60 cell lines in the Compare analysis. A second possibility is that because MDR-1 and MRP share

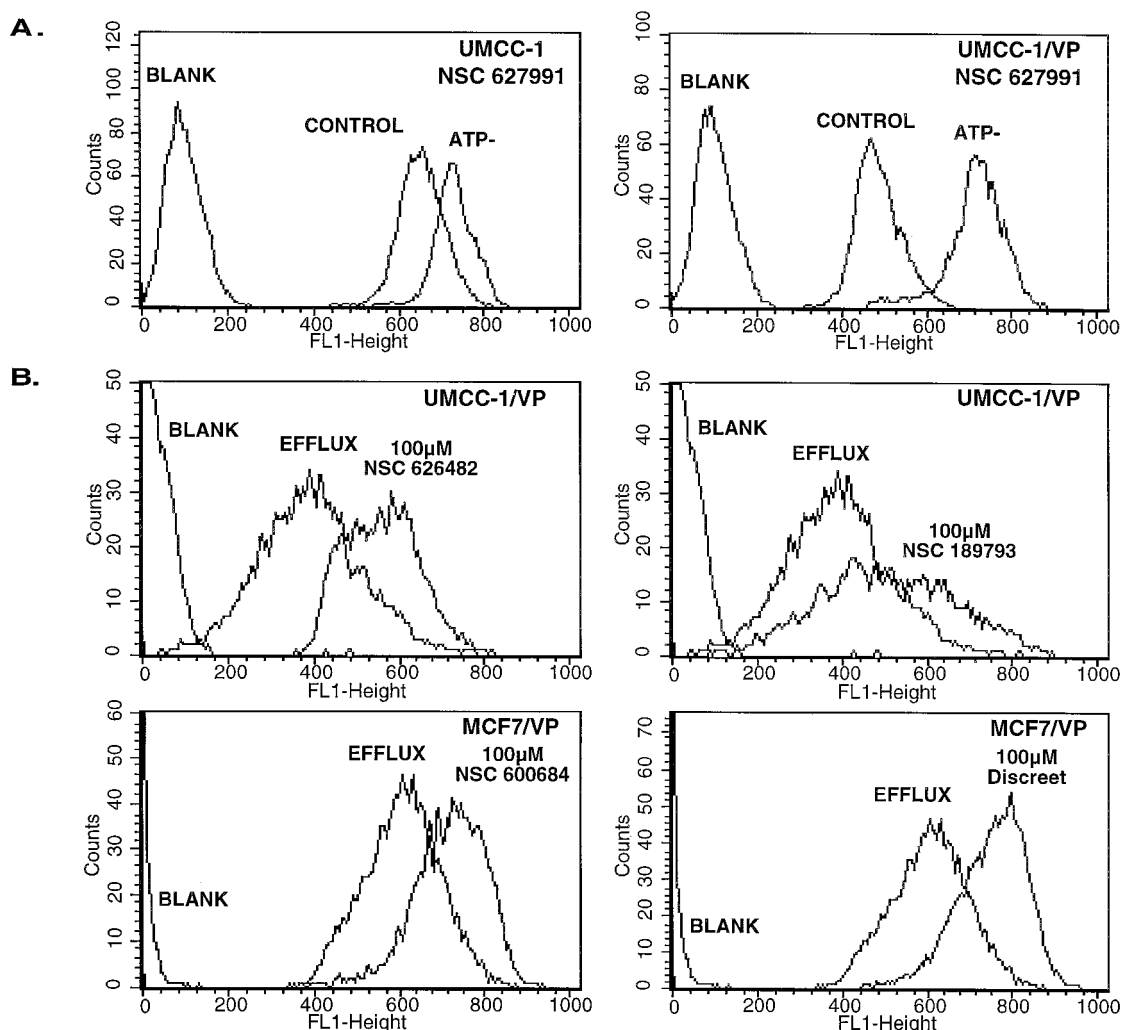


Fig. 7. A, Accumulation of NSC 627991 in parental UMCC-1 cells and MRP-overexpressing UMCC-1/VP cells in the presence of ATP (control) and in ATP-depleted conditions (ATP-). B, Effect of four compounds on calcein efflux from MRP-overexpressing cells. The accumulation of calcein and its efflux were measured in UMCC-1/VP or MCF7/VP cells. After a 30-min period of accumulation, efflux was allowed to proceed for 1 hr in the absence of any compound or in the presence of 100 μM NSC 626482, NSC 189793, NSC 600684, or a discreet compound. All four compounds could inhibit calcein efflux from the MRP-overexpressing cells.

some substrates, the correlations are “dominated” by MDR-1, obscuring the contribution of MRP. Alternately, MDR-1 is a better pump than MRP and exerts a greater influence on the sensitivity profile. If true, one would predict that higher correlations would emerge when the analysis was restricted to the 28 cell lines without MDR-1 expression or the 52 with lowest levels of MDR-1. Either of these subsets should give meaningful correlations because studies with MDR-1 and nm23 have shown previously that high correlations can be obtained with a subset as small as eight cell lines (Alvarez *et al.*, 1995; Freije *et al.*, 1997). However, when these analyses were performed, the MRP correlations did not improve (not shown). A third possibility was that resistance genes other than MDR-1 affect the results. Although this possibility cannot be excluded, the diversity of the compounds identified makes it unlikely that a single mechanism exists; instead,

multiple mechanisms may be invoked, a tenable hypothesis but a very difficult one to prove. A fourth possibility considered and addressed was that MRP mRNA does not accurately represent MRP protein or function. This possibility seemed unlikely, given the lack of evidence of a significant discordance among MRP mRNA expression, protein levels, and drug resistance in published models, including the six MRP-expressing models presented here that show a high correlation among MRP mRNA, protein expression, and calcein efflux. Nevertheless, we pursued it by measuring calcein efflux in the 60 cell lines. Calcein efflux was determined as a measure of “MRP activity” in much the same way rhodamine efflux has been used to measure P-glycoprotein function. Rhodamine efflux successfully identified P-glycoprotein substrates as well as, or even better than, measurements of MDR-1 mRNA expression when used as a seed in the Na-

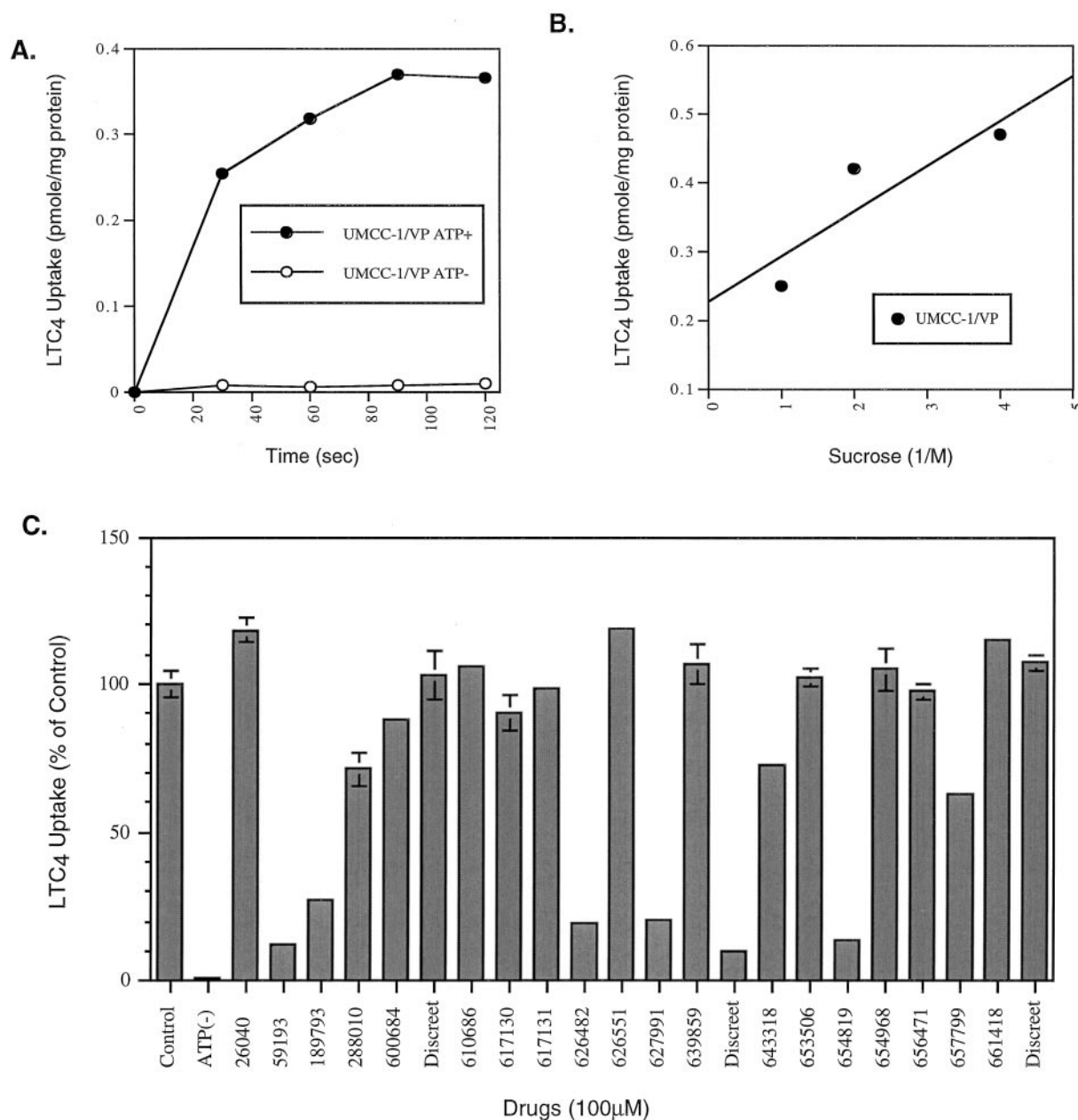


Fig. 8. $[^3\text{H}]\text{LTC}_4$ uptake and effect of various compounds. A, Time course of $[^3\text{H}]\text{LTC}_4$ uptake in membrane vesicles. B, Effect of extravesicular sucrose concentration. C, Effect of 22 compounds on $[^3\text{H}]\text{LTC}_4$.

tional Cancer Institute drug screen (Lee *et al.*, 1994). However, the failure to find higher correlations between the calcein efflux measurement and drug sensitivity suggested that this functional measurement did not improve the predictive potential of MRP, although the sensitivity of this assay was less than the PCR measurement. Indeed, it could be argued that this assay, which was validated in cells with higher levels of MRP, lacks sufficient sensitivity to be accurate in cell lines that have lower levels of MRP expression. In addition, as discussed below, we used an MRP substrate as the probe in a COMPARE analysis and were unable to increase the correlations. Thus, none of these explanations was satisfactory.

It must be emphasized that although the correlations obtained were low, they were considered statistically significant based on prior experience measuring and validating other molecular targets and from the knowledge of what *p* value a given PCC represents. For example, a PCC of 0.500 represents a *p* value of 2×10^{-5} . Even though the activity of >40,000 compounds was compared, statistically, only one in 200,000 ($p = 2 \times 10^{-5}$) would by chance give such a PCC, a number considerably less than the 20 compounds found to have a PCC of >0.500. That 23 of the 36 compounds with the highest PCCs could be demonstrated by one or more assays to interact with MRP suggests the correlations were valid. Nevertheless, the values were substantially lower than those obtained when other molecular markers were studied and overall supports the conclusions that the levels of MRP expression alone cannot predict drug sensitivity.

Current evidence suggests that MRP may preferentially or exclusively transport compounds that have undergone prior detoxification (Jedlitschky *et al.*, 1994, 1996; Leier *et al.*, 1994; Muller *et al.*, 1994; Loe *et al.*, 1996b). Although the extent to which chemotherapeutic agents undergo such modification and the capability of malignant cells to do this remain to be determined, such a requirement could confound the analyses performed. If drug sensitivity mediated by MRP depends on other factors, such as the level of GSH or specific drug-conjugating enzymes, analyses that ignore the contribution of these factors will give low correlations. This could provide at least a partial explanation for the results obtained in our analysis, although it must be recognized that some of these contributing factors, such as GSH, are likely to be present in excess in the majority of cells and thus not limit MRP function. We actually considered this possibility early in our studies and used NSC 627991, a quinone identified as a potential MRP substrate, as the probe in a COMPARE analysis (not shown). We reasoned that sensitivity to this compound, which was not an MDR-1 substrate, might more accurately reflect MRP function. We also hoped that unlike calcein, which does not require intracellular metabolism before transport, this compound might require such intracellular conversion and would provide an integrated measure of the "MRP pathway." Using this approach, compounds with higher correlations were identified (the majority were quinones), but these could not be confirmed as MRP substrates. This does not exclude the possibility that the low correlations result because factors other than those discussed above have not been considered; it is likely that the pathway is complex, with, for example, more than one conjugating enzyme contributing to the phenotype (Jedlitschky *et al.*, 1996). It does, however, speak to the potential complexity. Future efforts

will be directed at attempting correlations using several variables, including GSH levels and the expression and activity of several GSH transferases.

Finally, an analysis of the compounds with the highest PCCs revealed that quinones appeared more often than might have been expected. To establish a base-line, the percentage of quinone structures in the National Cancer Institute structure database (a group of 10,002 structures accessioned by the National Cancer Institute in 1976) was inventoried for quinone structures. This very large reference group contained 2.5% quinone structures. By contrast, among the top 50 compounds in the MRP COMPARE, there were 16 (32%) quinones, clearly more than would have been expected on a random basis. The reason or reasons for the high incidence of quinones are not certain at this time; it may have to do with quinone reactivity with —SH groups in molecules involved in detoxification processes. For example, anthraquinones such as mitoxantrone (hence, anthrapyrazoles and probably the anthracyclines) do react with glutathione via 1,4 addition reactions leaving an anionic conjugate.

In summary, we measured MRP expression in the 60 cell lines of the National Cancer Institute Anticancer Drug Screen. Low correlations were obtained when these values were used as a seed; this indicates that MRP expression is a poor predictor of drug sensitivity in these unselected cell lines. It is possible that other factors contribute to the MRP phenotype, and that until such factors are identified and characterized, a complete evaluation of the role of MRP in modulating drug sensitivity in these unselected cells and, by extrapolation, in untreated carcinomas will not be possible.

References

- Alvarez M, Paull K, Monks A, Hose C, Lee JS, Weinstein J, Grever MR, Bates SE, and Fojo AT (1995) Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the NCI Anticancer Drug Screen. *J Clin Invest* **95**:2205–2214.
- Baldini N, Scotlandi K, Barbanti-Brodano G, Manara MC, Maurici D, Bacci G, Bertoni F, Picci P, Sottili S, Campanacci M, and Serra M (1995) Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. *N Engl J Med* **333**:1380–1385.
- Beck J, Handgretinger R, Dopfer R, Klingebiel T, Niethammer D, and Gekeler V (1995) Expression of mdr1, mrp, topoisomerase II α /B, and cyclin A in primary or relapsed states of acute lymphoblastic leukaemias. *Br J Haematol* **89**:356–363.
- Bhalla K, Hindenburg A, Taub RN, and Grant S (1985) Isolation and characterization of an anthracycline-resistant human leukemic cell line. *Cancer Res* **45**:3657–3662.
- Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, and Norris MD (1994) Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. *Cancer Res* **54**:5036–5040.
- Boyd MR, Paull KD, and Rubinstein LR (1992) Data display and analysis strategies for the NCI Disease Oriented In-Vitro Antitumor Drug Screen, in *Cytotoxic Anticancer Drugs: Models and Concept for Drug Discovery and Development* (Valeriote FA, Corbett TH, and Baker LH, eds.) pp 11–34, Boston, Kluwer Academic Publishers.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–256.
- Brock I, Hipfner DR, Nielsen BS, Jensen PB, Deeley RG, Cole SP, and Sehested M (1995) Sequential coexpression of the multidrug resistance genes MRP and mdr1 and their products in VP-16 (etoposide)-selected H69 small cell lung cancer cells. *Cancer Res* **55**:459–462.
- Cornwell MM, Gottesman MM, and Pastan IH (1986) Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. *J Biol Chem* **261**:7921–7928.
- Doyle LA, Ross DD, Ordenez JV, Yang W, Gao Y, Tong Y, Belani CP, and Gutheil JC (1995) An etoposide-resistant lung cancer subline overexpresses the multidrug resistance-associated protein. *Br J Cancer* **72**:535–542.
- Feller N, Broxterman HJ, Wahrer DCR, and Pinedo HM (1995) ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): no inhibition by intracellular glutathione depletion. *FEBS Lett* **368**:385–388.
- Fitzsimmons SA, Workman P, Grever M, Paull K, Camalier R, and Lewis AD (1996) Reductase enzyme expression across the National Cancer Institute tumor cell line panel: correlation with sensitivity to mitomycin C and E09. *J Natl Cancer Inst* **88**:259–269.

- Freije JM, Lawrence JA, Hollingshead MG, De la Rosa A, Narayanan V, Grever M, Sausville EA, Paull K, and Steeg PS (1997) Identification of compounds with preferential inhibitory activity against low-Nm23-expressing human breast carcinoma and melanoma cell lines. *Nat Med* **3**:395–401.
- Hasegawa S, Abe T, Naito S, Kotoh S, Kumazawa J, Hipfner DR, Deeley RG, Cole SPC, and Kuwano M (1996) Expression of multidrug resistance-associated protein (MRP), MDR1 and DNA topoisomerase II in human multidrug-resistant bladder cancer cell lines. *Br J Cancer* **71**:907–913.
- Ishikawa T (1989) ATP/Mg²⁺ dependent cardiac transport system for glutathione S-conjugates: a study using rat heart sarcolemma vesicles. *J Biol Chem* **264**:17343–17348.
- Ishikawa T and Ali-Osman F (1993) Glutathione-associated cis-diamminedichloroplatinum (II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *J Biol Chem* **268**:20116–20125.
- Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, and Keppler D (1996) Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res* **56**:988–994.
- Jedlitschky G, Leier I, Buchholz U, Center M, and Keppler D (1994) ATP-dependent transport of glutathione S-conjugates by multidrug resistance-associated protein. *Cancer Res* **54**:4833–4836.
- Lee JS, Paull K, Alvarez M, Hose C, Monks A, Grever M, Fojo AT, and Bates SE (1994) Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute Drug Screen. *Mol Pharmacol* **46**:627–638.
- Leier I, Jedlitschky G, Buchholz U, and Keppler D (1994) Characterization of the ATP-dependent leukotrien C4 export carrier in mastocytoma cells. *Eur J Biochem* **220**:599–606.
- Leith CP, Chen IM, Kopecky KJ, Appelbaum FR, Head DR, Godwin JE, Weick JK, and Willman CL (1995) Correlation of multidrug resistance (MDR1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: identification of discordant MDR/efflux⁺ and MDR⁺/efflux⁺ cases. *Blood* **86**:2329–2342.
- Loe DW, Almquist KC, Cole SPC, and Deeley RG (1996a) ATP-dependent 17 β -estradiol 17-(β -D-glucuronide) transport by multidrug resistance protein (MRP). *J Biol Chem* **271**:9683–9689.
- Loe DW, Almquist KC, Deeley RG, and Cole SPC (1996b) Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles: demonstration of glutathione-dependent vincristine transport. *J Biol Chem* **271**:9675–9682.
- Matsumoto Y, Takano H, and Fojo T (1997) Cellular adaptation to drug exposure: evolution of the drug-resistant phenotype. *Cancer Res* **57**:5086–5092.
- Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J, and Boyd M (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* **83**:757–766.
- Muller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, deVries EGE, and Jansen PLM (1994) Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci USA* **91**:13033–13037.
- O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Weinstein JN, Friend S Jr, Fornace AJ, and Kohn KW (1997) Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer drugs. *Cancer Res* **57**:4285–4300.
- Paull KD, Hamel E, and Malspeis L (1995) Prediction of biochemical mechanisms of action from the in vitro antitumor screen of the National Cancer Institute, in *Cancer Chemotherapeutic Agents* (Foye WE, ed.) pp 9–45, Washington, DC, American Chemical Society.
- Paull KD, Lin CM, Malspeis L, and Hamel E (1992) Identification of novel antimetabolic agents acting at the tubulin level by computer-assisted evaluation of differential cytotoxicity data. *Cancer Res* **52**:3892–3900.
- Paull KD, Shoemaker RH, Hodes L, Monks A, Scudiero DA, Rubinstein L, Plowman J, and Boyd MR (1989) Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and Compare algorithm. *J Natl Cancer Inst* **81**:1088–1092.
- Schneider E, Horton JK, Yang C, Nakagawa M, and Cowan KC (1994) Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance. *Cancer Res* **54**:152–158.
- Shen H, Paul S, Breuninger LM, Ciaccio PJ, Laing NM, Helt J, Tew KD, and Kruh GD (1996) Cellular and in vitro transport of glutathione conjugates by MRP. *Biochem* **35**:5719–5723.
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, and Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82**:1107–1112.
- Versantvoort CHM, Bagrij T, Wright KA, and Twentyman PR (1995) On the relationship between the probenecid-sensitive transport of daunorubicin or calcein and the glutathione status of cells overexpressing the multidrug resistance-associated protein (MRP). *Int J Cancer* **63**:855–862.
- Wosikowski K, Schuurhuis D, Johnson K, Paull KD, Myers TG, Weinstein JN, and Bates SE (1997) Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *Cancer Res* **57**:4285–4300.
- Zaman GJR, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RPJ, Baas F, and Borst P (1995) Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. *Proc Natl Acad Sci USA* **92**:7690–7694.
- Zhan Z, Sandor VA, Gamelin E, Regis J, Dickstein B, Wilson W, Fojo AT, and Bates SE (1997) Expression of the multidrug resistance-associated protein gene in refractory lymphoma: quantitation by a validated polymerase chain reaction assay. *Blood* **89**:3795–3800.

Send reprint requests to: Dr. M. Alvarez, Departamento Hematología-Oncología, Universidad Católica de Chile, Lira 44, Santiago, Chile.