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Research Paper

Pharmacogenomics Approach Reveals MRP1 (ABCC1)-Mediated Resistance to Geldanamycins

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Purpose. Geldanamycin and its analogues belong to a new class of anticancer agents that inhibit the molecular chaperone heat shock protein 90. We hypothesized that membrane transporters expressed on tumor cells may contribute at least in part to cellular sensitivity to these agents. The purpose of this study is to identify novel transporters as determinant for sensitivity and resistance to geldanamycins.
Methods. To facilitate a systematic study of chemosensitivity across multiple geldanamycin analogues, we correlated mRNA expression profiles of majority of transporters with anticancer drug activities in 60 human tumor cell lines (NCI-60). We subsequently validated the gene–drug correlations using cytotoxicity and transport assays.
Results. The GA analogues displayed negative correlations with mRNA expression levels of the multidrug resistance protein 1 (MRP1, ABCC1). Suppressing MRP1 efflux using the inhibitor MK-571 and small interfering RNA in cell lines with intrinsic and acquired MRP1 overexpression (A549 and HL-60/ADR) and in cell lines stably transduced with MRP1 (MCF7/MRP1) increased intracellular drug accumulation and increased tumor cell sensitivity to geldanamycin analogues.
Conclusions. These results suggest that elevated expression of MRP1, like the alternative efflux transporter MDR1 (ABCB1, P-glycoprotein), can significantly influence tumor cell sensitivity to geldanamycins as a potential chemoresistance factor.

KEY WORDS: geldanamycin; membrane transporter and chemoresistance; MRP1; pharmacogenomics.

INTRODUCTION

Geldanamycin (GA, NSC 122750), a naturally occurring benzoquinone ansamycin antibiotic produced by yeast, exhibits a potent anti-proliferative activity against tumor cells (1,2). The target of geldanamycin is heat shock protein 90 (Hsp90), a molecular chaperone that maintains stability of multiple "client proteins" implicated in tumor growth and survival, including protein kinases, transcription factors and mutated oncogenic proteins (3). Treatment of tumor cells with GA blocks ATP binding to the Hsp90 and results in proteasome-mediated degradation of Hsp90 client proteins (4). The novel anti-tumor mechanism of GA has stimulated a strong interest in the development of analogues with reduced systemic toxicity and increased water solubility. Two analogues, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG, NSC 330507) and 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin (17-DMAG, NSC 707545), showing reduced liver toxicity without losing their cytotoxic potency of GA against tumor cells, have yielded encouraging results in clinical trials (5).

GA as well as its analogues 17-AAG and 17-DMAG show various anti-tumor activity against different tumor types and individual patients (6). Identifying genetic determinants of sensitivity or resistance to these drugs will be important for optimizing cancer therapy. Since a prerequisite for any anticancer drug to inhibit tumor growth is sufficient intracellular drug accumulation, membrane transporters may determine the sensitivity to GA analogues (7). To identify transporters potentially associated with response to GA analogues, we have used DNA microarray to analyze mRNA expression of a majority of human membrane transporters in the NCI-60, a panel of 60 diverse

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ABBREVIATIONS: ABC, ATP-binding cassette; DTP, Developmental Therapeutics Program; GA, geldanamycin; Hsp90, heat shock protein 90; Hsp90, heat shock protein 90; MOAs, mechanism of action; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2Htetrazolium, inner salt); NCI, the National Cancer Institute; SRB, sulforhodamine B; 3-([3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl-(3-dimethylamino-3-oxopropyl)-thio-methyl]thio)propanoic acid, MK-571.

human cancer cell lines from the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) for anticancer compound screening (8). Next, we applied a pharmacogenomics approach in which transporter gene expression profiles were correlated with anticancer potency of drugs tested against the NCI-60. A strong negative correlation between expression of a transporter gene and drug potency means that cells expressing higher levels of this transporter are less sensitive to this compound, suggesting a chemoresistance mechanism. In contrast, a strong positive transporter–drug correlation indicates that cells with higher expression of this transporter are more sensitive to this compound. Using this approach, we have successfully identified and validated a number of novel drug–transporter interactions (for review of our findings and detailed methods, see references (7,9)). As an extension of these studies, we conducted analysis focusing on multiple GA analogues and transporter gene expression in the NCI-60. Significant negative correlations were detected between the GAs and transporter genes encoding two of the membrane efflux pumps, multidrug resistance transporter 1 (MDR1, ABCB1 or P-glycoprotein) and multidrug resistance-associated protein 1 (MRP1 or ABCC1), indicating their contribution in resistance to these drugs (10). Further investigation confirmed that GA analogues are substrates as well as inhibitors of MDR1 (10).

Both MDR1 and MRP1 belong to the ATP-binding cassette (ABC) transporter protein family (11). Despite lack of amino acid sequence identity with MDR1 (approximately 19% overall), MRP1 is able to confer resistance to anthracyclines, vinca alkaloids and others, a resistance profile similar to that of MDR1 (12). Unlike MDR1, MRP1 transports glutathione and other drug conjugates (13). MRP1 also transports unconjugated compounds, although free glutathione may also be required for this process (12). In addition to its expression in drug-resistant tumor cells, MRP1 is ubiquitously expressed in normal tissues, including those important for drug absorption (lung and gut), metabolism and elimination (liver and kidney), and barrier sites (blood–brain barrier, blood–cerebral spinal fluid barrier, blood–testis barrier and the maternal–fetal barrier or placenta) (14).

Based on the results from pharmacogenomic analysis, we hypothesized that GA analogues may also be subject to the resistance mechanism mediated by MRP1-decreased accumulation in tumor cells through active drug efflux. In the present study, we aimed to validate the relationship between GA analogues and MRP1. Negative correlations occur between MRP1 gene expression and all the 25 GA analogues analyzed, with 16 drugs reaching statistical significance ($P<0.05$). Using cytotoxicity and transport assays, in the presence and absence of MRP1 inhibitor, small interfering RNA (siRNA) and stable MRP1 gene transduction, our results showed that elevated expression of MRP1 is associated with resistance to GA analogues and these compounds may be MRP1 substrates. These results contribute to our understanding of interactions between GAs and MRP1 efflux as a potential chemoresistance mechanism.

MATERIALS AND METHODS

Compound Potency Database for NCI-60

The September 2003 release of NCI antitumor drug screening database was obtained from the NCI's DTP website (Human Tumor Cell Line Screen: http://dtp.nci.nih.gov/docs/cancer/cancer_data.html), containing screening results and chemical structural data of GA analogues. For each compound and cell line, growth inhibition after 48 h of drug treatment had been assessed from changes in total cellular protein using a sulforhodamine B (SRB) assay (15,16). The data provide GI_{50} values for each compound–cell line pair (GI_{50}, the concentration causing 50% growth inhibition).

Gene Expression Databases of NCI-60

A customized oligonucleotide microarray containing probes targeting 461 transporter and 151 channel genes, as well as 100 probes for unrelated genes was used to measure transporter gene expression in the NCI-60. Array hybridization, data analysis and database were described in a previous study (8). A second gene expression database, the Novartis microarray dataset, was also employed for comparison and validation. This data set contains the average of triplicate expression measurements for 59 NCI cell lines based on 12,626 oligonucleotide probes from Affymetrix U95Av2 arrays, available at NCI/DTP's website (http://dtp.nci.nih.gov/mtargets/download.html).

Correlation of Gene Expression Profiles with Compound Potency Patterns

Pearson correlation coefficients were calculated to correlate gene expression profiles with patterns of compound potency across the NCI-60 as described previously (17). To identify transporter genes associated with GA, correlation analysis was initially performed for expression profiles measured with microarray against the potency of six GA analogues. Unadjusted p values were obtained using Efron's bootstrap resampling method (18), with 10,000 bootstrap samples for each gene–drug comparison. To identify the correlation between MRP1 and 25 GA analogues, P values for the correlation coefficients were obtained by fitting a linear regression model. In the presence of missing values the correlation between each pair of variables is computed using all complete pairs of observations on those variables.

Chemicals

Geldanamycin (GA), 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG), 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), FITC-GA, 17-(2-(pyrrolidin-1-yl) ethyl)amino-17-demethoxygeldanamycin (17-AEP-GA) and 17-(dimethylaminopropylamino)-17-demethoxygeldanamycin (17-DMAP-GA) were purchased from InvivoGen (San Diego, California). Macbecin II, NSC 658514 and 661581 were obtained from NCI/DTP. 3-([3-(2-[7-Chloro-2-quinolinyl] ethenyl)phenyl-(3-dimethylamino-3-oxopropyl)-thio-methyl] thio)propanoic acid (MK-571) was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Others were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Cell Culture

The cell lines were cultured in RPMI 1640 medium containing 5 mM L-glutamine, supplemented with 10% fetal bovine serum (FBS), 100 U/mL sodium penicillin G and

100 μg/mL streptomycin. Cells were grown in tissue culture flasks at 37°C in a 5% CO_2 atmosphere. HL-60/ADR cell line was a gift from Dr. Kenneth K. Chan, The Ohio State University (Columbus, Ohio). MCF7/MRP1 and MCF7/pLNCX cells were kindly provided by Dr. Charles Morrow at the Wake Forest University School of Medicine (Winston-Salem, North Carolina). Other cell lines were obtained from the Division of Cancer Treatment and Diagnosis at NCI.

Cytotoxicity Assay

Growth inhibitory potency for attached cells was tested using SRB assay (Sigma) (8, 19). Cells growing in suspension were assessed with the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2Htetrazolium, inner salt) assay (Promega, Madison, WI). Three thousand to 4,000 cells/well for SRB assay, 5,000–10,000 cells/well for MTS assay were seeded in 96-well plates and incubated for 24 h. Before exposure to test compounds, cells were treated individually with MK-571 or medium (as control) for 10 min. Test compounds were added in a dilution series in three replicated wells for 4 days. To determine IC_{50} values, the absorbance of control cells without drug was set at 1. Dose–response curves were plotted using Prism software (San Diego, California). Each experiment was performed independently at least twice. Student's *t* test was used to determine the degree of statistical significance.

FACS Analysis—FITC-GA Efflux Assay

HL-60/ADR cells (1×10^6) were pre-incubated for 5 min at 37°C in RPMI 1640 medium, with or without 50 μM MK-571. FITC-GA was then added to a final concentration of 5 μM, and the cells were incubated for 10 min at 37°C, then sedimented by centrifugation, and resuspended in cold PBS. Green fluorescence intensity was measured using a flow cytometer equipped with a 488 nm argon laser (Becton Dickinson Biosciences, San Jose, California). Acquisition of events was stopped at 12,000 counts.

HPLC Analysis and GA Uptake Study

To confirm that GA is the substrate of efflux pump MRP1, HPLC analysis was used to investigate GA uptake in HL-60/ADR cells. Reversed-phase HPLC analysis was conducted on a Hewlett-Packard 1050 HPLC system (Avondale, PA) employing a 50×4.6 mm Vydac 218TP3405 C18 column (Deerfield, IL). Isocratic elution was carried out using 44% acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 1 ml/min. The detection was made at 308 nm. Under this condition, the retention time of GA was 2.5 min. Following addition of 2.5 μM of GA, in the presence or absence of 50 μM of MRP1 inhibitor MK-571, samples of 1×10^6 cells in 200 μl of serum-free RPMI 1640 medium were incubated at 37°C. The drug concentration was measured in the medium over time for up to 60 min. Before measuring drug concentration, the samples were centrifuged to remove cells. The supernatant (100 μl) was injected to the column for the determination of GA remaining in the medium.

Small Interfering RNA (siRNA)-Mediated Down-Regulation of ABCC1 Gene Expression

siRNA duplexes for human ABCC1 gene were predesigned and chemically synthesized by QIAGEN, Inc. (Valencia, CA), available as four siRNA for a single gene, Hs_ABCC1_2 HP, Hs_ABCC1_3 HP, Hs_ABCC1_4 HP and Hs_ABCC1_5 HP (FlexiTube siRNA). Chemically synthesized mock siRNA (fluorescein-labeled, nonsilencing) was also purchased from QIAGEN. Transfection was performed with HiPerFect Transfection Reagen (QIAGEN) using 20 nM of siRNA in 60-mm culture plates. To measure cytotoxic drug potency, 24 h after siRNA transfection, cells grown in 60-mm plates were subcultured into 96-well plates. After 24 h of incubation, the cells were further incubated with the test drugs for 3 days before SRB assay.

Real-Time Quantitative Reverse Transcription-PCR

Total RNA was prepared by using the TRIZOL reagent (Invitrogen), following the protocol of the manufacturer. Two micrograms of total RNA was incubated with RNase I and reverse transcribed with oligo(dT) with Superscript III RT-PCR kit (Invitrogen). One microliter of reverse transcription product was amplified by primer pairs specific for MRP1. GAPDH was used as a normalizing control. Primers used for MRP1 were 5′-GGA CCT GGA CTT CGT TCT CA-3′ (forward) and 5′-CGT CCA GAC TTC TTC ATC CG-3′ (reverse). The primers for GAPDH were 5′-AGC CAC ATC GCT CAG ACA C-3′ (forward) and 5′-GCC CAA TAC GAC CAA ATC C-3′ (reverse). Relative gene expression was measured with the Applied Biosystems 7300 Real-Time PCR System. All amplification controls and samples were performed in triplicate.

RESULTS

MRP1 Gene Expression Negatively Correlated with Anticancer Potency of GA Analogues in NCI-60

The mRNA expression was measured in the NCI-60 cell lines with a customized microarray containing oligonucleotide probes targeting the majority of transporter genes presently known to be relevant to drug transport (8). To identify genes potentially involved in sensitivity to GA analogues, we performed correlation analysis between microarray gene expression profiles and growth inhibitory potency of six representative GA analogues across the NCI-60. MRP1 (ABCC1) was one of the five transporter genes showing significant correlations with these compounds from the pharmacogenomic screening (10). We next performed correlation analysis between gene expression of MRP1 and growth inhibitory potency of 25 GA analogues across the NCI-60. The GA analogues were identified according to the common chemical substructures, the benzoquinone ansamycin moiety (see Table II in reference (10)). This yielded Pearson correlation coefficients for each MRP1–drug pair. All of the GA analogues showed negative MRP1 correlations, and the mean correlation coefficient was −0.33, suggesting that the majority of these compounds are substrates of MRP1 (Fig. 1A). Sixteen out of 25 compounds showed statistically significant correlations with MRP1 expression. The parent compound GA (NSC 122750) showed the strongest correlation

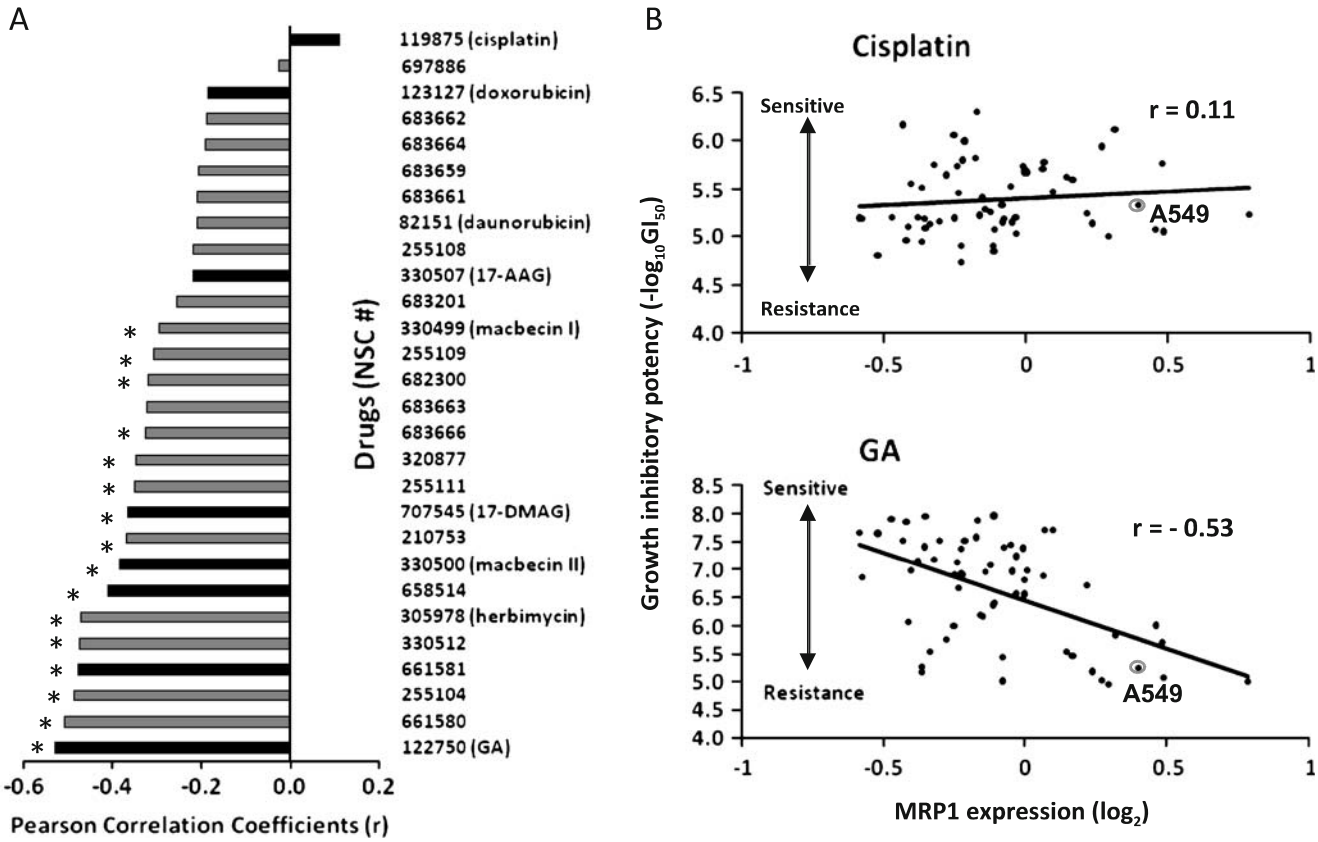

Fig. 1. Relationship between drug sensitivity and MRP1 expression in the NCI-60 for a set of 25 GA analogues. **A** Plot of sorted Pearson correlation coefficients (r) between MRP1 gene expression and cytotoxic activities of 25 GA analogues, plus cisplatin, daunorubicin and doxorubicin for comparison. NSC # of compounds is shown for each drug. *Black bars* indicate compounds which are available for experimental validation. *P value <0.05; **B** Scatter plot showing the correlation (r) of MRP1 expression with sensitivity of the 60 cells to cisplatin (NSC 119875) (r=0.11, P>0.05) and GA (NSC 122750) (r=0.53, P<0.0001). The *circle* indicates the location of A549 cells which have relatively high expression MRP1 and are relatively more resistant to GA.

coefficient, −0.53 (P<0.0001). 17-AAG (NSC330507) and 17-DMAG (NSC 707545) also showed negative correlation, −0.22 (P>0.05) and −0.36 (P<0.05), respectively. These results suggest that MRP1 may function as an efflux pump for GA and its analogues. For comparison, Fig. 1A also showed the MRP1 correlations for daunorubicin (r=−0.21, P>0.05), doxorubicin (r=−0.18, P>0.05) and cisplatin (r=0.11, P>0.05). Although the correlation was not significant, daunorubicin and doxorubicin have been known to be MRP1 substrates (20), while cisplatin is not a MRP1 substrate (21). Fig. 1B shows a representative relationship between MRP1 expression level and growth inhibitory potency for GA and cisplatin. This pharmacogenomic approach has biological limitation, in that the relationships established between compounds and genes are merely correlative, not causal (22). In addition, the P values for the Pearson correlation coefficients gave false negative prediction for daunorubicin and doxorubicin. Therefore, the hypotheses generated on the association between MRP1 and multiple GA analogues need experimental validation.

MRP1 Reduced Intracellular GA Accumulation

We used the HL-60/ADR cells as an experimental model to investigate whether overexpression of MRP1 could decrease intracellular accumulation of GA analogues. HL-60/ADR, a subline of HL-60 promyelocytic leukemia cells, have been isolated *in vitro* by subculturing in progressively higher concentrations of doxorubicin (23,24). This cell line exhibited a multidrug-resistant phenotype, defective in the cellular accumulation of MRP1 substrate drug, and contain elevated levels of MRP1. Despite the multidrug-resistant properties, these cells do not overexpress ABCB1 (MDR1) and do not contain detectable levels of P-glycoprotein (25). We firstly used the fluorescent derivative of GA, FITC-GA, to investigate the role of MRP1 on transport and efflux of GA from the HL-60/ADR cells. Following incubation with FITC-GA for 10 min at 37°C, HL-60/ADR cells contained less of the fluorescent compound than did the HL-60 cells. The decreased accumulation in HL-60/ADR was reversible by addition of the MRP1 inhibitor MK-571 (50 μM) (which had no effect on the parental cells), supporting the hypothesis that GA is a MRP1 substrate (Fig. 2A). To exclude the influence of the fluorescein-5-isothiocyanate dye linked to GA at the C17 position on the FITC-GA molecule (26), we used an alternative method to confirm the contribution of MRP1 to GA transport and uptake. GA (2.5 μM) was incubated with HL-60/ADR cells for various time points up to 60 min. The drug remaining in the culture media was measure using a HPLC method. GA showed a time-dependent uptake/metabolism by the HL-60/ADR cells (Fig. 2B). MRP1 inhibition by

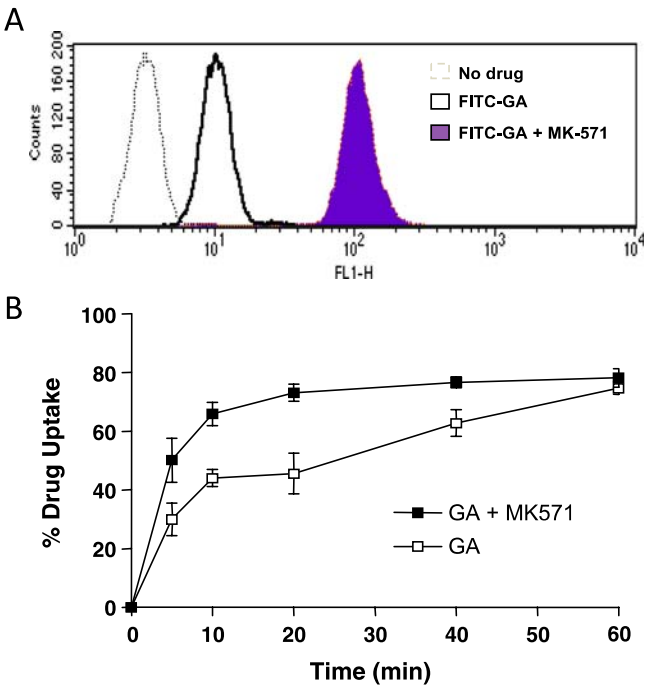

Fig. 2. GA uptake in HL-60/ADR cells. **A** Accumulation of the fluorescent compound FITC-GA in MRP1-overexpressing HL-60/ADR cells after incubation with 5 μM FITC-GA for 10 min at 37°C in the presence or absence of 50 μM MK-571. **B** Analysis of GA uptake by HL-60/ADR cells by HPLC method after incubation with 2.5 μM GA at 37°C in the presence or absence of 50 μM MK-571. The cell uptake was followed over time up to 60 min. Data is reported as percent of drug removal (uptake) from the medium.

50 μM MK-571 in the HL-60/ADR cells significantly increased the rate of GA uptake/metabolism by the cells from the medium. All together, these results indicate that GA is the substrate of MRP1.

MRP1 Overexpression in Intrinsic and Acquired Drug Resistant Cell Lines Mediated Resistance to GA Analogues

To test whether high expression of MRP1 confers resistance to the GA analogues, we used cytotoxicity assays on A549, HL-60 and HL-60/ADR cell lines. A549 cells were used due to its high level of MRP1 expression among the NCI-60 (Fig. 1B). A pair of sensitive and resistant cell lines, HL-60 (MDR1(−)/MRP1(−)) and HL-60/ADR (MDR1(−)/MRP1(+)) were also included in the cytotoxicity study. These cell lines were treated with increasing concentration of GA compounds, with and without the presence of MK-571 (50 μM). As shown in Fig. 3A, MK-571 significantly sensitized the A549 cells to the GA and 17-AAG. In Fig. 3B, when compared with HL-60, HL-60/ADR cells proved much more resistant to GA and 17-AAG. MK-571 reversed the resistance to GA and 17-AAG in HL-60/ADR cells. These results provide evidence that the observed resistance to GA analogues was linked to the MRP1 function. Further experiments were conducted for other available GA analogues, including 17-DMAG (NSC 707545), macbecin II (NSC 330500), NSC 658514, NSC 661581, and the novel GA derivative 17-AEP-GA and 17-DMAP-GA. Our results showed that resistance in HL-60/ADR cells to these GA analogues was reversible by MK-571 in each case (Table I). In A549 cells, which express lower levels of MRP1 than HL-60/ADR, MK-571 had modest sensitization effects on macbecin II and doxorubicin ($P>0.05$, Table I), suggesting that they are weaker MRP1 substrates. MK-571 did not have effect on the non-substrate drug cisplatin in A549 and HL-60/ADR cells (Table I).

MRP1 Overexpression in MRP1-Transducted Cell Line Conferred Resistance to GA Analogues

A549 and HL-60/ADR are cells with intrinsic resistance or acquired resistance by drug selection. Other genetic or phenotypic changes may have occurred during resistance development or drug selection, such as altered expression of other transporters. To unequivocally attribute GA resistance to MRP1, we used cell line derived from parental breast cancer cell line MCF7 stably transduced with MRP1 gene (27). MCF7/MRP1 cells showed a high MRP1 protein level comparable to drug-selected MRP1-overexpressing cells (27). The vector control MCF7/pLNCX cells were more sensitive to GA and 17-AAG than the MCF7/MRP1 cells, but showed no difference to cisplatin (Fig. 4A). In the presence of MK-571, MCF7/MRP1 cells were sensitized to GA and 17-AAG, but not to cisplatin. To rule out the role of other genes in these cell lines, we performed RT-PCR analysis for genes

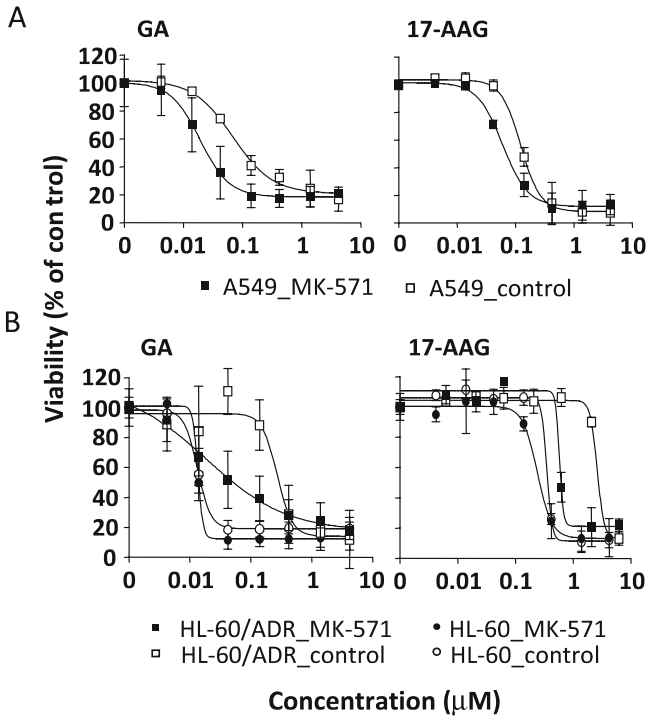

Fig. 3. Validation of MRP1-GA correlations by inhibiting MRP1 function with MK-571 in A549, HL-60 and HL-60/ADR cells. **A** Growth inhibition curves are shown for A549 cells, which expresses high levels of MRP1, in response to GA and 17-AAG, with or without treatment with MK-571 (50 μM). **B** Growth inhibition curves for HL-60 and HL-60/ADR cells in response to GA and 17-AAG, with or without treatment with MK-571 (50 μM). The cytotoxicity results are expressed as percentage survival of control cells without drug treatment (means + SD from three replicates).

Table I. Effects of Inhibition of MRP1 Activity on Cytotoxicity of GA Analogues in HL-60/ADR and A549 Cells

			IC_{50} (μM)			
			HL-60/ADR		*A549*	
NSC #	Name	*r*	(−) MK-571	(+) MK-571	(−) MK-571	(+) MK-571
122750	GA	−0.53	0.59±0.16	0.02±0.004* (35)	0.069±0.004	0.019±0.003* (3.7)
330500	Macbecin II	−0.39	7.3±1.1	3.6±0.7* (2.1)	0.12±0.005	0.06±0.003 (1.1)
330507	17-AAG	−0.22	2.4±0.004	0.58±0.01* (4.2)	0.12±0.005	0.06±0.003* (2.1)
707545	17-DMAG	−0.36	>5	0.40±0.13* (>13)	0.10±0.001	0.052±0.001* (1.9)
–	17-AEP-GA	–	1.4±0.04	0.08±0.05* (22)	0.043±0.008	0.017±0.001* (2.6)
–	17-DMAP-GA	–	>4.3	0.79±0.53* (>9.2)	0.12±0.005	0.079±0.004* (1.6)
658514		−0.41	10±2.1	3.7±0.21* (2.7)	2.1±0.057	0.56±0.12* (4.0)
661581		−0.48	3.9±0.48	0.73±0.08* (5.4)	0.41±0.085	0.13±0.024* (3.2)
123127	Doxorubicin	−0.18	> 5	2.9±0.16* (>1.8)	0.14±0.023	0.07±0.010 (2.1)
119875	Cisplatin	0.11	1.5±0.1	1.7±0.04 (0.93)	39±11	44±17 (1.1)

MK-571 (50 μM) was used to suppress MRP1 transport activity. *r* is the Pearson correlation coefficients between compound potency and MRP1 expression. IC_{50} is the concentration that produced 50% inhibition of cell growth compared to controls. Numbers in the parentheses represent fold-reversal, which is the IC_{50} for the cytotoxic drug in control cells divided by that in MK-571-treated cells. Doxorubicin, a MRP1 substrate, and cisplatin, a non-substrate, are included in the experiment for comparison. Results represent mean±SD of at least three experiments.
*$P<0.05$ *versus* controls without adding MK-571

possibly involved in GA response, MDR1, MRP1, SLC7A11 and SLC3A2, in A549, HL-60, HL-60/ADR, MCF7/pLNCX, and MCF7/MRP1 cells. As can be seen from Fig. 5, the expression levels of MRP1 in HL-60/ADR and MCF7/MRP1 cells were higher than other cells tested. In contrast, the expression levels of MDR1, SLC7A11 and SLC3A2 were either absent or not visibly different in these cells. These results support the conclusion that MRP1 mediates the resistance to GA analogues in MRP1 overexpressing cells.

MRP1 Small Interfering RNA Reverses Drug Resistance to GA Analogues

To specifically down-regulate MRP1 expression, we treated the cancer cells with four sets of siRNA duplexes predesigned by QIAGEN Inc. to target human MRP1 mRNA sequence. We selected cell lines HL-60/ADR and A549 expressing high levels of MRP1 for the siRNA study. The effects of gene down-regulation were detected by real-time

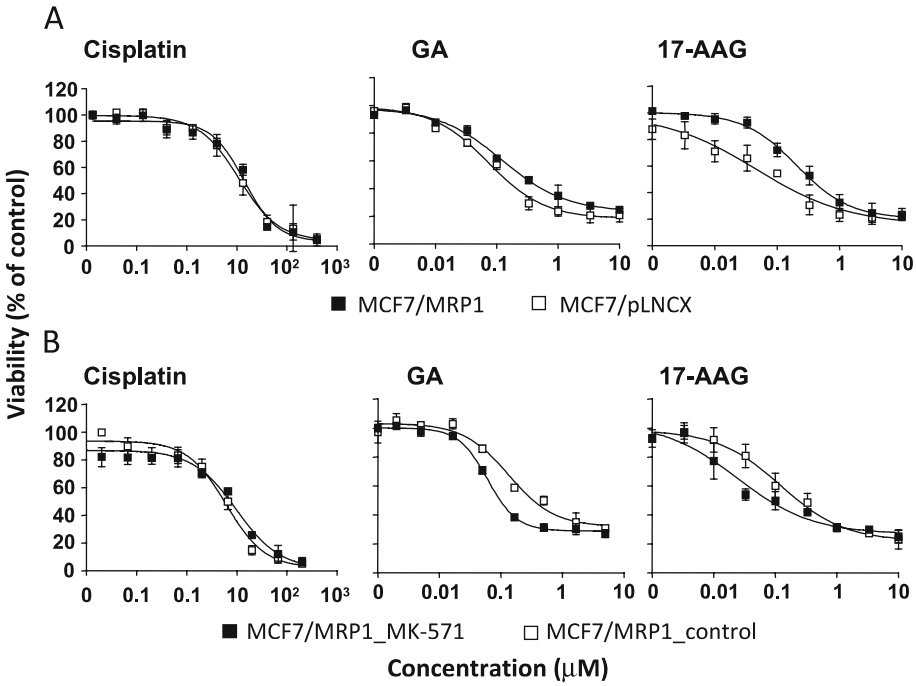

Fig. 4. Validation of MRP1-GA correlations in MRP1-transfected cell lines. **A** Growth inhibition curves are shown for MCF7/pLNCX (vector control) and MCF7/MRP1 cells in response to cisplatin (NSC 119875), GA (NSC 122750), and 17-AAG (NSC 330507). **B** Growth inhibition curves are shown for MCF7/MRP1 cells in the presence or absence of 50 μM MK-571. The cytotoxicity results are expressed as percentage survival of control cells without drug treatment (means + SD from three replicates).

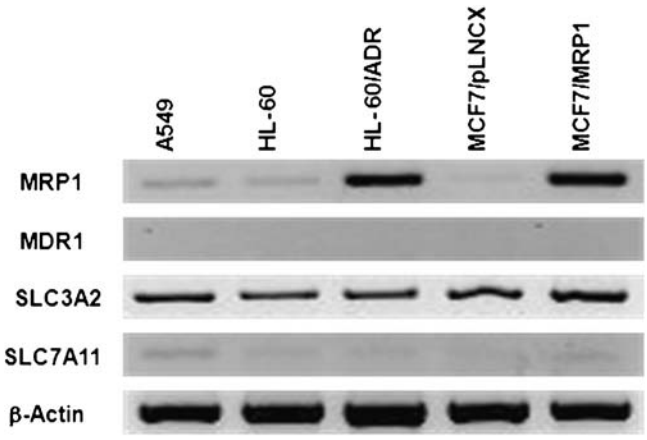

Fig. 5. Expression of MRP1, MDR1, SLC3A2, SLC7A11 and β-actin control was detected by RT-PCR and gel-electrophoresis analysis in A549, HL-60, HL-60/ADR, MCF7/pLNCX, and MCF7/MRP1 cells. The MRP1 showed a strong overexpression in HL-60/ADR and MCF7/MRP1 cells in comparison with HL-60 and MCF7/pLNCX cells. The MDR1 gene does not express in all the cells tested. SLC7A11 and SLC3A2 expression showed no difference between HL-60/ADR and MCF7/MRP1 cells and their parental cells.

RT-PCR. Fig. 6A shows that 48 h after transfection, Hs_ABCC1_3 HP (siRNA#3) was the most effective siRNA in down-regulating MRP1 mRNA expression in A549 cells, resulting in >80% reduction in MRP1 mRNA levels. Although the expression of MRP1 is much higher in HL-60/ADR cells than in the A549 cells, none of the four siRNA sets affected MRP1 expession in this cell line (data not shown). Therefore, we used siRNA#3 in the A549 cells for further experiments. To assess how siRNA-directed suppression of MRP1 affects drug sensitivity, we compared drug potency of siRNA-treated cells with that of mock-treated control cells. The sensitivity to GA and 17-AAG was significantly increased in MRP1 down-regulated cells. Fig. 6B illustrates the effects of siRNA on drug potency. Thus, these results obtained using siRNA are consistent with the results using MRP1 inhibitors.

DISCUSSION

The natural product geldanamycin (GA) and its synthetic analogues belong to a new class of anticancer agents inhibiting the molecular chaperone Hsp90. They demonstrate multiple downstream effects by simultaneous depletion of a number of

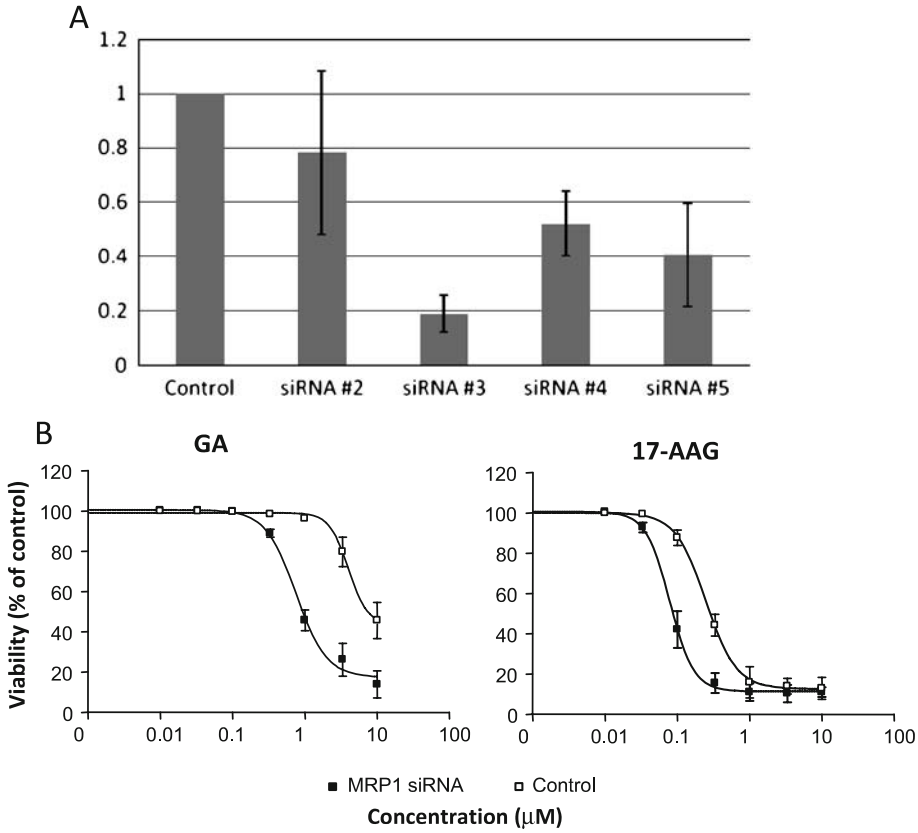

Fig. 6. Effects of siRNA against MRP1 gene on activity of GA and 17-AAG. **A** Quantitative RT-PCR analysis of MRP1 gene. The cells were transfected with four siRNA duplexes, Hs_ABCC1_2 HP (siRNA#2), Hs_ABCC1_3 HP (siRNA#3), Hs_ABCC1_4 HP (siRNA#4) and Hs_ABCC1_5 HP (SiRNA#5). After 48 h, relative MRP1 expression in A549 cells after transfection with different siRNA was determined by real-time RT-PCR. Expression levels were normalized to GAPDH levels. The MRP1 expression level in the control of A549 cells was set as 1. **B** Effects of siRNA transfection using siRNA#3 targeting MRP1 on drug potency in the cell line A549. Twenty-four hours after transfection, cells were exposed to GA or 17-AAG for 3 days and cell growth was measured using SRB assay.

oncogenic "client" proteins of Hsp90. This unique mechanism of action possibly impedes the development of resistance, an advantage over the agents inhibiting a single target. However, targeted therapy with GA and its analogues is also subject to general drug resistance mechanisms, such as, reduced drug accumulation due to abnormal membrane transport.

This study initiated from a pharmacogenomics investigation, in which expression levels of several transporter genes obtained by microarray analysis were found significantly correlated with anticancer potency (i.e., *in-vitro* drug response) of GA analogues on 60 diverse human cancer cell lines (NCI-60) (10). The membrane efflux pumps ABCB1 (MDR1, or P-glycoprotein) and ABCC1 (MRP1), the cystine/glutamate transporter SLC7A11 (xCT, encoding the light chain of amino acid transport system x_c^-) and SLC3A2 (4F2hc, encoding the heavy chain of transport system x_c^-) displayed negative correlations with subset of GA analogues, suggesting a role in chemresistance. Subsequently, using cytotoxicity assay and transport analysis, all the GA analogues tested were confirmed to be not only substrates but also inhibitors of MDR1 (10). The SLC7A11 and SLC3A2 genes, encoding the heterodimeric amino acid transporter x_c^- , have been implicated in chemoresistance, by supplying cystine to the cell for glutathione synthesis (28). We experimentally confirmed that the x_c^- transporter confers resistance selectively to GA analogues with methoxy group or unsubstituted at C17 position, but not to analogues with amino group at the C17 position (29). Thus, using pharmacogenomics approach we have identified that, multiple transporters, either directly (by substrate-transporter interaction) or indirectly (by increasing glutathione levels), may contribute to tumors' sensitivity and resistance to GA analogues.

The current study focused on the interactions between MRP1 and GAs. As a member of the ABC transporter superfamily, MRP1 conveys chemoresistance to its substrates by decreased accumulation in target cells through active drug efflux (12). In NCI-60, its expression shows negative correlations with response to GAs. Thus, we hypothesized that GAs are MRP1 substrates, and that tumors cells that do not express MDR1 may develop resistance to GAs through MRP1 overexpression. The overlapping substrate profiles of MDR1 and MRP1 (12) support the conjecture that GAs may be substrates for both transporters. Combining pharmacogenomic and functional analysis in cell lines with low or high levels of MRP1 expression, our results support the above hypothesis. We examined the effects of suppressing MRP1 using known inhibitor MK-571, in MRP1 overexpressing cell lines, HL-60/ADR (obtained by doxorubicin selection) and MCF7/MRP1 (obtained by gene transduction). The resistant phenotype to GAs was significantly alleviated by MK-571. MK-571 also increased the intracellular accumulation of GA in HL-60/ADR cells. However, the potentiation by MK-571 in MCF7/MRP1 cells (Fig. 4B) were not as strong as that in HL-60/ADR cells (Fig. 3B), although the two cell lines showed similar expression levels of MRP1 (Fig. 5). One of the potential explanations is that in the drug selection procedure to obtain HL-60/ADR, the overexpression of MRP1 may be accompanied with other changes. Possible changes may happen in the levels of glutathione and/or glutathione S-transferases (GSTs), which commonly result in enhanced drug detoxification and resistance. The gene-transduced MCF7/MRP1 cells showed modest resistance to GA analogues probably because they have only MRP1 overexpression, but no other changes. Since MRP1 and GSTs or glutathione pathway have shown combined effects in drug resistance (12, 30, 31), we speculate that the activity of the MRP1 pump toward GA analogues could be made manifest by coexpression with GST gene family. However, the precise mechanism by which glutathione participates in MRP1-mediated efflux of drugs is currently unclear. Glutathione may form conjugates with GA analogues, which possibly renders them less cytotoxic, facilitates MRP-mediated transport out of the cells, and prevents binding to the target (Hsp90). Two recent reports showed that GA, 17-AAG, and 17-DMAG could react chemically (i.e., nonenzymatically) with glutathione (32, 33), although the conjugation proceeds rapidly with GA and less rapidly with 17-DMAG and 17-AAG. Further experiments should be done to examine the ability of GA analogues to target Hsp90 in the presence of glutathione and perform drug transport study in the presence or absence of glutathione. From the drug accumulation study shown in Fig. 2B, it is interesting to observe that after 1 h of incubation, the amount of GA remaining in the culture media of HL-60/ADR cells in the presence of MK-571 showed no significant difference from that in the absence of MK-571. However, in the cytotoxicity study the presence of MK-571 strongly sensitized the effects of GA after 3 or 4 days of drug exposure (Fig. 3). The inconsistency may result from the methodology we used: in the accumulation study, instead of measuring intracellular level of GA, we measured the drug remaining in the media. This method only detected the amount of drug removed from the medium, without considering drug transformations (i.e., metabolism) after the drug enters the cells. Therefore, even though the same amount of GA remains in the medium after 1 h, it is possible that the intracellular accumulation of active metabolites of GA show a difference in cells with different activity of MRP1. Further studies are needed to characterize the differences in the intracellular levels of GA metabolites.

For several GA analogues (e.g., 17-AAG) and doxorubicin, the Pearson correlation coefficients were not significant (Fig. 1), while the experimental results demonstrated that they are MRP1 substrates as well. The false negative prediction could result from the interference of other genes. These may include the presence of cofactors such as MDR1, SLC7A11 or GSTs, which may express discordantly in the 60 cells and mask the contribution of less potent transporters of the same drugs. The false negative correlation of 17-AAG and other drugs with the expression of MRP1 is probably a consequence of the positive correlation of the expression of MRP1 with SLC7A11 (r=0.36) and NAD(P)H: quinone oxidoreductase 1 (NQO1) (r=0.31). MRP1, SLC7A11 and NQO1 genes are under regulation by NF-E2-related factor-2 (NRF2), a transcription factor that binds to antioxidant response element (ARE) sequences located in the gene promoters (34–36). For 17-AAG, expression of the metabolizing enzyme NQO1 was found important for tumor cell sensitivity (37). Thus, drugs showing positive correlation with NQO1, such as 17-AAG (r=0.2) are therefore less likely to have strong negative correlation with MRP1 expression.

In conclusion, GA analogues should be added to the extensive list of drugs that can be affected by the multidrug resistance mechanism mediated by MRP1. In many tumor cells,

the amount of MRP1 expression is significantly elevated above that of normal cells. This active efflux renders cells resistant and requires elevated dosages of chemotherapy, which in turn results in increased toxicity in normal cells. Therefore, expression of MRP1, together with MDR1, SLC7A11 and SLC3A2, could serve as the predictor for resistance to GA analogues. In clinical terms, documentation of MRP1 overexpression might preclude prescription of GA-related agents.

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REFERENCE

1. L. Whitesell, E. G. Mimnaugh, B. De Costa, C. E. Myers, and L. M. Neckers. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. U. S. A*. **91**:8324–8328 (1994) doi:10.1073/pnas.91.18.8324.
2. V. Smith, E. A. Sausville, R. F. Camalier, H. H. Fiebig, and A. M. Burger. Comparison of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17DMAG) and 17-allylamino-17-demethoxygeldanamycin (17AAG) *in vitro*: effects on Hsp90 and client proteins in melanoma models. *Cancer Chemother. Pharmacol*. **56**:126–137 (2005) doi:10.1007/s00280-004-0947-2.
3. P. Workman. Altered states: selectively drugging the Hsp90 cancer chaperone. *Trends Mol. Med*. **10**:47–51 (2004) doi:10.1016/j.molmed.2003.12.005.
4. L. Neckers. Development of small molecule Hsp90 inhibitors: utilizing both forward and reverse chemical genomics for drug identification. *Curr. Med. Chem*. **10**:733–739 (2003) doi:10.2174/0929867033457818.
5. R. K. Ramanathan, D. L. Trump, J. L. Eiseman, C. P. Belani, S. S. Agarwala, E. G. Zuhowski, J. Lan, D. M. Potter, S. P. Ivy, S. Ramalingam, A. M. Brufsky, M. K. Wong, S. Tutchko, and M. J. Egorin. Phase I pharmacokinetic-pharmacodynamic study of 17-(allylamino)-17-demethoxygeldanamycin (17AAG, NSC 330507), a novel inhibitor of heat shock protein 90, in patients with refractory advanced cancers. *Clin. Cancer. Res*. **11**:3385–3391 (2005) doi:10.1158/1078-0432.CCR-04-2322.
6. A. Maloney, P. A. Clarke, and P. Workman. Genes and proteins governing the cellular sensitivity to HSP90 inhibitors: a mechanistic perspective. *Curr. Cancer Drug. Targets*. **3**:331–341 (2003) doi:10.2174/1568009033481822.
7. Y. Huang, S. Penchala, A. N. Pham, and J. Wang. Genetic variations and gene expression of transporters in drug disposition and response. *Expert. Opin. Drug. Metab. Toxicol*. **4**:237–254 (2008) doi:10.1517/17425255.4.3.237.
8. Y. Huang, P. Anderle, K. J. Bussey, C. Barbacioru, U. Shankavaram, Z. Dai, W. C. Reinhold, A. Papp, J. N. Weinstein, and W. Sadee. Membrane transporters and channels: role of the transportome in cancer chemosensitivity and chemoresistance. *Cancer Res*. **64**:4294–4301 (2004) doi:10.1158/0008-5472.CAN-03-3884.
9. Y. Huang, and W. Sadee. Membrane transporters and channels in chemoresistance and sensitivity of tumor cells. *Cancer Lett*. **239**:168–182 (2006) doi:10.1016/j.canlet.2005.07.032.
10. Y. Huang, P. E. Blower, R. Liu, Z. Dai, A. N. Pham, H. Moon, J. Fang, and W. Sadee. Chemogenomic analysis identifies geldanamycins as substrates and inhibitors of ABCB1. *Pharm. Res. (2007)*. **24**:1702–1712 (2007).
11. M. M. Gottesman, T. Fojo, and S. E. Bates. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat. Rev. Cancer.* **2**:48–58 (2002) doi:10.1038/nrc706.
12. G. D. Kruh, and M. G. Belinsky. The MRP family of drug efflux pumps. *Oncogene*. **22**:7537–7552 (2003) Medline doi:10.1038/sj.onc.1206953.
13. Z. S. Chen, T. Furukawa, T. Sumizawa, K. Ono, K. Ueda, K. Seto, and S. I. Akiyama. ATP-dependent efflux of CPT-11 and SN-38 by the multidrug resistance protein (MRP) and its inhibition by PAK-104P. *Mol. Pharmacol*. **55**:921–928 (1999).
14. E. M. Leslie, R. G. Deeley, and S. P. Cole. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol*. **204**:216–237 (2005) Medline doi:10.1016/j.taap.2004.10.012.
15. A. Monks, D. A. Scudiero, G. S. Johnson, K. D. Paull, and E. A. Sausville. The NCI anti-cancer drug screen: a smart screen to identify effectors of novel targets. *Anticancer. Drug Des*. **12**:533–541 (1997).
16. J. N. Weinstein, T. G. Myers, P. M. O'Connor, S. H. Friend, A. J. Fornace Jr., K. W. Kohn, T. Fojo, S. E. Bates, L. V. Rubinstein, N. L. Anderson, J. K. Buolamwini, W. W. van Osdol, A. P. Monks, D. A. Scudiero, E. A. Sausville, D. W. Zaharevitz, B. Bunow, V. N. Viswanadhan, G. S. Johnson, R. E. Wittes, and K. D. Paull. An information-intensive approach to the molecular pharmacology of cancer. *Science*. **275**:343–349 (1997) doi:10.1126/science.275.5298.343.
17. Y. Huang, P. E. Blower, C. Yang, C. Barbacioru, Z. Dai, Y. Zhang, J. J. Xiao, K. K. Chan, and W. Sadee. Correlating gene expression with chemical scaffolds of cytotoxic agents: ellipticines as substrates and inhibitors of MDR1. *Pharmacogenomics J*. **5**:112–125 (2005) doi:10.1038/sj.tpj.6500297.
18. B. Efron, and R. Tibshirani. *An introduction to the bootstrap*. Chapman Hall, New York, 1993.
19. P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst*. **82**:1107–1112 (1990) doi:10.1093/jnci/82.13.1107.
20. S. P. Cole, G. Bhardwaj, J. H. Gerlach, J. E. Mackie, C. E. Grant, K. C. Almquist, A. J. Stewart, E. U. Kurz, A. M. Duncan, and R. G. Deeley. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science*. **258**:1650–1654 (1992) doi:10.1126/science.1360704.
21. C. F. Manohar, J. A. Bray, H. R. Salwen, J. Madafiglio, A. Cheng, C. Flemming, G. M. Marshall, M. D. Norris, M. Haber, and S. L. Cohn. MYCN-mediated regulation of the MRP1 promoter in human neuroblastoma. *Oncogene*. **23**:753–762 (2004) doi:10.1038/sj.onc.1207151.
22. Y. Huang. Pharmacogenetics/genomics of membrane transporters in cancer chemotherapy. *Cancer Metastasis. Rev*. **26**:183–201 (2007) doi:10.1007/s10555-007-9050-6.
23. S. Gollapudi, and S. Gupta. Lack of reversal of daunorubicin resistance in HL60/AR cells by cyclosporin A. *Anticancer. Res*. **12**:2127–2132 (1992).
24. K. Bhalla, A. Hindenburg, R. N. Taub, and S. Grant. Isolation and characterization of an anthracycline-resistant human leukemic cell line. *Cancer Res*. **45**:3657–3662 (1985).
25. D. Marquardt, and M. S. Center. Drug transport mechanisms in HL60 cells isolated for resistance to adriamycin: evidence for nuclear drug accumulation and redistribution in resistant cells. *Cancer Res*. **52**:3157–3163 (1992).
26. L. Llauger-Bufi, S. J. Felts, H. Huezo, N. Rosen, and G. Chiosis. Synthesis of novel fluorescent probes for the molecular chaperone Hsp90. *Bioorg. Med. Chem. Lett*. **13**:3975–3978 (2003) doi:10.1016/j.bmcl.2003.08.065.
27. C. S. Morrow, C. Peklak-Scott, B. Bishwokarma, T. E. Kute, P. K. Smitherman, and A. J. Townsend. Multidrug resistance protein 1 (MRP1, ABCC1) mediates resistance to mitoxantrone via glutathione-dependent drug efflux. *Mol. Pharmacol*. **69**:1499–1505 (2006) doi:10.1124/mol.105.017988.
28. Y. Huang, Z. Dai, C. Barbacioru, and W. Sadee. Cystine-glutamate transporter SLC7A11 in cancer chemosensitivity and chemoresistance. *Cancer Res*. **65**:7446–7454 (2005) doi:10.1158/0008-5472.CAN-04-4267.
29. R. Liu, P. E. Blower, A. N. Pham, J. Fang, Z. Dai, C. Wise, B. Green, C. H. Teitel, B. Ning, W. Ling, B. D. Lyn-Cook, F. F. Kadlubar, W. Sadee, and Y. Huang. Cystine-glutamate transporter SLC7A11 mediates resistance to geldanamycin but not to 17-(allylamino)-17-demethoxygeldanamycin. *Mol. Pharmacol*. **72**:1637–1646 (2007) doi:10.1124/mol.107.039644.

30. P. Depeille, P. Cuq, S. Mary, I. Passagne, A. Evrard, D. Cupissol, and L. Vian. Glutathione S-transferase M1 and multidrug resistance protein 1 act in synergy to protect melanoma cells from vincristine effects. *Mol. Pharmacol.* **65**:897–905 (2004) doi:10.1124/mol.65.4.897.
31. P. Depeille, P. Cuq, I. Passagne, A. Evrard, and L. Vian. Combined effects of GSTP1 and MRP1 in melanoma drug resistance. *Br. J. Cancer.* **93**:216–223 (2005) doi:10.1038/sj.bjc.6602681.
32. R. L. Cysyk, R. J. Parker, J. J. Barchi Jr, P. S. Steeg, N. R. Hartman, and J. M. Strong. Reaction of Geldanamycin and C17-Substituted Analogues with Glutathione: Product Identifications and Pharmacological Implications. *Chem. Res. Toxicol.* **19**:376–381 (2006) doi:10.1021/tx050237e.
33. W. Lang, G. W. Caldwell, J. Li, G. C. Leo, W. J. Jones, and J. A. Masucci. Biotransformation of geldanamycin and 17-allylamino-17-demethoxygeldanamycin by human liver microsomes: reductive *versus* oxidative metabolism and implications. *Drug Metab. Dispos.* **35**:21–29 (2007) doi:10.1124/dmd.106.009639.
34. P. Moi, K. Chan, I. Asunis, A. Cao, and Y. W. Kan. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. U. S. A.* **91**:9926–9930 (1994) doi:10.1073/pnas.91.21.9926.
35. A. Y. Shih, D. A. Johnson, G. Wong, A. D. Kraft, L. Jiang, H. Erb, J. A. Johnson, and T. H. Murphy. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J. Neurosci.* **23**:3394–3406 (2003) Medline.
36. H. Sasaki, H. Sato, K. Kuriyama-Matsumura, K. Sato, K. Maebara, H. Wang, M. Tamba, K. Itoh, M. Yamamoto, and S. Bannai. Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *J. Biol. Chem.* **277**:44765–44771 (2002) doi:10.1074/jbc.M208704200.
37. L. R. Kelland, S. Y. Sharp, P. M. Rogers, T. G. Myers, and P. Workman. DT-Diaphorase expression and tumor cell sensitivity to 17-allylamino, 17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. *J. Natl. Cancer Inst.* **91**:1940–1949 (1999) doi:10.1093/jnci/91.22.1940.