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Research Paper

Chemogenomic Analysis Identifies Geldanamycins as Substrates and Inhibitors of ABCB1

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Purpose. A prerequisite for geldanamycin (GA, NSC122750) to targeting heat shock protein 90 and inhibiting tumor growth is sufficient intracellular drug accumulation. We hypothesized that membrane transporters on tumor cells determine at least in part the response to GA analogues.
Materials and Methods. To facilitate a systematic study of chemosensitivity across a group of GA analogues with similar chemical structures, we correlated mRNA expression profiles of most known transporters with growth inhibitory potencies of compounds in 60 tumor cell lines (NCI-60). We subsequently validated the gene-drug correlations using cytotoxicity and transport assays.
Results. Geldanamycin analogues displayed a range of negative correlations coefficients with ABCB1 (MDR1, or P-glycoprotein) expression. Suppressing ABCB1 in multidrug resistant cells (NCI/ADR-RES and K562/DOX) and ABCB1-transfected cells (BC19) increased sensitivity to GA analogues, as expected for substrates. Moreover, ABCB1-mediated efflux of daunorubicin in K562/DOX cells could be blocked markedly by GA analogues in a dose-dependent fashion. The IC_{50} values (half-maximum inhibition of daunorubicin efflux) were 5.5, 7.3 and 12 μM for macbecin II (NSC330500), 17-AAG (NSC330507) and GA, respectively.
Conclusions. These observations demonstrate that GA analogues are substrates as well as inhibitors of ABCB1, suggesting that drug interactions between GA analogues and other agents that are ABCB1 substrates may occur via ABCB1 in normal or tumor cells.

KEY WORDS: ABCB1; chemogenomics; geldanamycin; membrane transporter and chemoresistance.

INTRODUCTION

Chemoresistance is a main obstacle to successful cancer chemotherapy. A systematic approach is needed to select drug candidates among a series of congeners that could avoid chemoresistance mechanisms. Correlation of gene expression in cancer cells with growth inhibitory drug potency represents a useful tool in the study of chemoresistance (1). Using cDNA microarrays, Scherf *et al.* (2) have correlated gene expression in a panel of 60 human tumor cell lines (NCI-60) to growth inhibitory potencies of anticancer drugs with known mechanism of action. High expression of a given gene in drug-sensitive cell lines yields a positive correlation whereas high expression in resistant cells gives negative correlations. For example, dihydropyrimidine dehydrogenase (DPYD) expression was negatively correlated with 5-FU activity, which is consistent with previous results that high DPYD levels—involved in the catabolism of pyrimidines and 5-FU—decrease exposure of cells to the active phosphorylated metabolites of 5-FU (2). As an extension of this study, we have previously used custom-designed microarrays to analyze gene expression, in the NCI-60 cell panel, of membrane transporters and channels, which govern cellular influx and efflux of ions, nutrients, and drugs. Correlating gene expression with the potencies of 119 standard anticancer drugs identified known drug-transporter interactions and suggested novel ones (3). In particular, correlation analysis of the ATP-binding cassette (ABC) transporter gene ABCB1 (or MDR1, encoding the multiple drug resistance protein 1 or P-glycoprotein) mRNA expression and drug potencies in the NCI-60 panel sensitively identified all known

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ABBREVIATIONS: NCI, The National Cancer Institute; DTP, Developmental Therapeutics Program; SiRNA, small interfering RNA; ABC, ATP-binding cassette; SRB, sulforhodamine B; GA, geldanamycin; Hsp90, heat shock protein 90.

ABCB1 substrates among the 119 drugs, and Baker's-antifol and a geldanamycin analogue as novel substrates. For specific transporter genes such as ABCB1, the gene-drug correlations enable one to search for additional likely substrates from a much larger database containing the potencies of >60,000 drugs against the NCI-60 cell panel (available from the NCI Developmental Therapeutics program (http://dtp.nci.nih.gov). For example, linking ABCB1 gene expression, cytotoxic potency, and chemical features of 7,466 compounds, we have identified novel substrates and inhibitors for ABCB1 among a group of ellipticine compounds (4). Ellipticine analogues showing negative correlations were found to be ABCB1 substrates, whereas those showing no or positive correlations appear to function as inhibitors.

In the present study, we focused on geldanamycin and its analogues because of their potent anticancer activity (5,6) and broad range of correlation coefficients with ABCB1 gene expression across the members of the compound class, similar to ellipticines. A prerequisite for reaching the drugs' intracellular target—heat shock protein 90 (Hsp90)—is sufficient intracellular drug accumulation, as the potency of GA analogues was found to be proportional to the intracellular drug concentrations (7). Therefore, GA analogues may also be subject to resistance mechanism mediated by ABCB1—decreased accumulation in cancer cells through active drug efflux. Negative correlations occur between ABCB1 gene expression and all the 25 GA analogues analyzed, with 12 compounds reaching statistical significance. Because ABCB1 substrates usually have poor or no inhibitory activity (8), our initial objective was to identify distinct groups of ABCB1 substrates and inhibitors among the 25 analogues. We employed cytotoxicity and transport assays, using ABCB1 inhibitor and siRNA-mediated ABCB1 down-regulation. Our results show that elevated expression of ABCB1 was associated with resistance to all the GA compounds tested. Unlike ellipticine analogues, which can be clearly defined as either ABCB1 substrate or inhibitor (4), GA analogues were shown to be not only substrates but also inhibitors of ABCB1. These results contribute to our understanding of the resistance mechanisms of geldanamycin-related drugs and the pharmacologic interactions between Hsp90 inhibitor compounds and ABC transporters.

MATERIALS AND METHODS

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 NCI-60. Array hybridization, data analysis and database were described in previous study (3). A second gene expression database, the Novartis microarray dataset, was also employed for a broad genome-wide screen. 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's Developmental Therapeutics Program (DTP) website (http://dtp.nci.nih.gov/mtargets/download.html).

Compound Potency Database for NCI-60

The September 2003 release of the National Cancer Institute (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 non-confidential screening results and chemical structural data from the DTP. 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 (9,10). The data provide GI_{50} values for each compound-cell line pair (GI_{50}, the concentration causing 50% growth inhibition).

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 (4). Correlation analysis was performed for expression profiles measured with 712 oligonucleotide and 12,626 U95Av2 oligoprobe data sets against the potency of GA analogs. Unadjusted p values were obtained using Efron's bootstrap resampling method (11), with 10,000 bootstrap samples for each gene-drug comparison. Alternatively, 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. 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. K562/DOX cell line (a gift from J.P. Marie, INSERM, E9912, University of Paris, France) was obtained by *in vitro* passaging of K562 in progressively increasing doses of daunorubicin (12), cultured as the other cell lines, and retreated with 0.1 μM doxorubincin once a month. BC19 cells were kindly provided by Dr. Kenneth Cowan at Eppley Cancer Center of University of Nebraska Medical Center. Other cell lines were obtained from Division of Cancer Treatment and Diagnosis at NCI.

Cytotoxicity Assay

Growth inhibitory potency was tested using a proliferation assay with sulforhodamine B (SRB), a protein-binding reagent (Sigma), described before (3,13). Cells growing in suspension were assessed with the MTS assay (14). For SRB assay, 3,000–4,000 cells/well, for MTS assay, 5,000–10,000 cells/well were seeded in 96-well plates and incubated for 24 h. Before exposure to test compounds, cells were treated individually with CsA 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 significance.

siRNA-mediated Downregulation of Gene Expression

siRNA duplexes for ABCB1 were chemically synthesized by QIAGEN Inc. (Valencia, California) and described in previous report (3). Chemically synthesized mock siRNA (fluorescein-labeled, non-silencing) was also purchased from QIAGEN. Transfection was performed with HiPerFect Transfection Reagent (QIAGEN) using 5 nM siRNA. The effect of down-regulation was detected by real-time RT-PCR and described previously (3,15). For RNA extraction for real-time RT-PCR, cells were harvested 48 h after transfection. To measure cytotoxic drug potency, 24 h after 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 compounds for 4 days before cytotoxicity assay.

FACS Analysis—FITC-GA Efflux Assay

1×10^6 Cells were pre-incubated for 5 min at 37°C in RPMI 1640 medium, with or without 5 μM CsA. 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, CA). Acquisition of events was stopped at 12,000 counts.

FACS Analysis—Daunorubicin (DNR) Accumulation Assay

The capacity of a compound to inhibit ABCB1-mediated efflux from K562/DOX cells, was measured by flow cytometry (16). The cells were centrifuged (50 g for 5 min) at room temperature and resuspended in RPMI 1640 medium with 10% FBS (37°C) at a concentration of 6,000,000 cells/ml. Aliquots (50 μl) of the cell suspension were transferred to tubes containing 1.95 ml of the incubation media containing 5 μM DNR. The cells were incubated in the presence of absence of test drugs in RPMI 1640 medium with 10% FBS with DNR for 50 min at 37°C. After centrifugation (50 g for 5 min at 4°C) and removal of supernatant, cold PBS was added to each tube, and cell suspension was transferred to FACS tubes, which were placed on ice (less than 1 h) until analysis.

Flow cytometry was performed with a Becton-Dickinson FACS Calibur (San Jose, California) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 585/42 nm for DNR). Analysis was based on acquisition of data from 10,000 cells. Log fluorescence intensities of individual cells were recorded as histograms. The median fluorescence intensity of 10,000 cells was used for analysis. CsA (10 μM affording maximal inhibition of MDR1-mediated efflux of DNR in K562/DOX) served as a positive control and for normalization of the results across experiments. The % inhibition of fluorescent dye efflux was calculated as follows:

$$Relative\ fluorescence = \frac{Fluorescence\ median\ of\ a\ sample}{Fluorescence\ median\ of\ 10\ \mu M\ CsA} \times 100$$

RESULTS

Correlation of Transporter Gene Expression With Growth Inhibitory Potency of GA Analogues

The mRNA expression was measured in the NCI-60 cell panel with a customized microarray containing oligonucleotide probes targeting the majority of transporter genes presently known to be relevant to drug transport (3). To identify genes potentially involved in chemosensitivity to GA analogues, we performed correlation analysis between gene expression profiles and growth inhibitory potency of six representative GA analogues across the NCI-60. This yielded Pearson correlation coefficients (*r*) for each gene-drug pair. The statistical significance of the correlation for each gene-drug pair was assessed by computing unadjusted bootstrap *p* values (11). Genes that exhibited significant correlations for at least one of the six compounds ($|r|>0.35$ and $p<0.05$) were selected. This was a relatively low-stringent criterion. To avoid potential false positives, we selected genes which were previously implicated in chemosensitivity. As a result, five transporter genes were extracted (Table I): membrane efflux pumps *ABCB1* (*MDR1*, or P-glycoprotein) and *ABCC1* (*MRP1*), the cystine/glutamate transporter *SLC7A11* (*xCT*, encoding the light chain of transport system x_c^-) and *SLC3A2* (*4F2hc*, encoding the heavy chain of transport system x_c^-) displayed negative correlations with GA analogues, suggesting a role in chemoresistance; the nucleoside transporter *SLC29A1* (*ENT1*, encoding equilibrative nucleoside transporter 1) demonstrated positive correlations. In addition, three non-transporter genes showed significant correlations with at least one GA analogues (Table I): *ATP1B1* (encoding the beta 1 subunit of Na, K-ATPase), *EGFR* (epidermal growth factor receptor) and *ADAM9* (encoding one member of the family of transmembrane disintegrin-containing metalloproteinases). To further validate the results of correlation analysis, we queried a genome-wide gene expression database of the NCI-60, the Novartis microarray dataset obtained by the Affymetrix U95Av2 array, and calculated the correlation coefficients between potency of the six analogues and expression of the selected eight genes. The results from two microarray types were highly consistent (data not shown).

Table I. Genes Whose Expression Levels Correlated With Potency of GA Analogues

Gene	Alias	GA Analogs (NSC #)					
		122750 GA	330500 Macbecin II	330507 17-AAG	707545 17-DMAG	658514 –	661581 –
Transporter							
ABCB1	*MDR1*	−0.19	−0.49*	−0.31*	−0.49*	−0.16	−0.16
ABCC1	*MRP1*	−0.53**	−0.39*	−0.22	−0.36*	−0.41**	−0.48**
SLC7A11	*XCT*	−0.52**	−0.09	0.02	−0.02	−0.48**	−0.46**
SLC3A2	*4F2hc*	−0.40**	−0.10	−0.14	−0.14	−0.28*	−0.35*
SLC29A1	*ENT1*	0.36*	0.30*	0.12	0.17	0.37*	0.30*
Non-transporter							
ADAM9	*MDC9*	−0.50**	−0.49**	−0.29*	−0.29*	−0.59**	−0.53**
EGFR	*ERBB1*	−0.34*	−0.48**	−0.30*	−0.27*	−0.35**	−0.40**
ATP1B1	–	−0.49**	−0.33*	−0.16	−0.1	−0.50**	−0.49**

The columns labeled NSC numbers and corresponding chemical names of compound contain Pearson correlation coefficients between compound potency and gene expression. Genes with compound-gene correlation coefficients > 0.35 or < −0.35 for at least one compound were selected and shown. The correlations were computed by gene expression data obtained from a DNA microarray data base (3). *: P value<0.05; **: P value<0.001.

One limitation of this chemogenomic approach is that the relationships established between compounds and genes are merely correlative, not causal. Therefore, the hypotheses generated have to be experimentally validated (1). We subsequently tested the correlations between the gene expression and growth inhibitory potencies of GA analogues and the roles of these genes in chemoresistance. The present study focuses on the role of ABCB1 serving as the chemoresistance factor for GA analogues. Since ABCB1 confers multidrug resistance by active efflux of drugs out of cells, the potency patterns of its substrates are expected to be negatively correlated with the level of ABCB1 expression. Negative correlations indicative of chemoresistance occur between ABCB1 gene expression and all six GAs, with 3/6 compounds reaching statistical significance (Table I). As small differences in chemical structure may determine whether a compound is ABCB1 substrate or inhibitor, we systematically studied the compound class containing multiple GA analogues that has been tested against the NCI-60 drug screening panel and their interactions with ABCB1.

Identification of the Geldanamycin Compound Class

Searching through ~43,000 compounds in the September 2003 release of the NCI antitumor drug screening database, we identified 25 GA analogues according to the common chemical substructures, the benzoquinone ansamycin moiety (Table II). All 25 analogues showed negative ABCB1-correlations (Fig. 1a), and the mean correlation coefficient is −0.27, suggesting that the majority of these compounds are substrates. The correlation coefficients for 12 analogues showed statistical significance (P<0.05) (Fig. 1a). 17-DMAG (NSC 704545) showed the lowest correlation coefficient, −0.49 (Fig. 1a, b). The parent compound GA (NSC122750) showed negative, but not statistically significant correlation (Fig. 1b). NSC 255108 showed no correlation (r=0.05) (Fig. 1b).

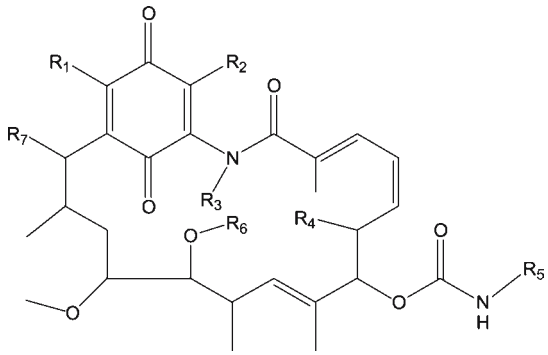
If there is a connection between resistance to the GA analogues and ABCB1 expression levels, we would expect to detect a difference in compound potency between cell lines over- and under-expressing this gene. To corroborate GA-ABCB1 correlations, we calculated the difference in potency between NCI/ADR-RES cells, expressing relatively high levels of ABCB1, and OVCAR-8 cells, expressing low levels of ABCB1 while otherwise being similar in overall gene expression pattern to NCI/ADR-RES (2). On average the 25 GA analogues are approximately 19 times less potent against NCI/ADR-RES than OVCAR-8. 17-DMAG (NSC 707545) and macbecin II (NSC 330500) are ~1,000 times less potent against NCI/ADR-RES than OVCAR-8, supporting the hypothesis that they are ABCB1 substrates.

Evidence That High ABCB1 Expression Confers Resistance to GA Analogues

Available for experimental investigations were parent compound GA (NSC 122750), analogues 17-AAG (NSC 330507), 17-DMAG (NSC 707545), macbecin II (NSC 330500), NSC 658514, and 661581. Also included in our experimental investigations are two novel derivatives of 17-AAG, 17-(2-(pyrrolidin-1-yl)ethyl)amino-17-demethoxygeldanamycin (17-AEP-GA) and 17-(dimethylaminopropylamino)-17-demethoxygeldanamycin (17-DMAP-GA) (not shown in Table II); growth inhibitory activity data for these two compounds are not available in the NCI-60 database (17).

To test whether high expression of ABCB1 confers resistance to the GA analogues, we used cytotoxicity assays on NCI/ADR-RES, K562 and K562/DOX cell lines. NCI/ADR-RES cells express the highest levels of ABCB1 in the NCI-60 panel and show chemoresistance to ABCB1 substrates (3). K562/DOX cells were obtained by passaging K562 cells in progressively increasing doses of doxorubicin resulting in doxorubicin resistance due to ABCB1 overexpression (12). K562 cells lack significant ABCB1 expression. These cell lines were treated with increasing concentrations of compounds, with and without the presence of 5 uM cyclosporin A (CsA), a known inhibitor of ABCB1. Alternatively, NCI/ADR-RES cells were treated with ABCB1-specific siRNA (3), using non-silencing siRNA as the negative control. Shown in Fig. 2a, CsA sensitized the NCI/ADR-

Table II. Chemical Structures of 25 GA Analogues



NSC	Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7
122750	GA	OMe	H	H	OMe	H	H	H
210753		OMe	C=N-t-Bu	H	OMe	H	H	H
255104		OH	H	H	OMe	H	H	H
255109	17-AG	NH2	H	H	OMe	H	H	H
255111		OMe	C=N-$C_5H_{10}N$*	H	OMe	H	H	H
305978	herbimycin	H	H	H	OMe	H	Me	OMe
320877		NCH_2CH_2Cl	H	H	OMe	H	H	H
330499	macbecin I	H	H	H	Me	H	Me	OMe
330507	17-AAG	$NCH_2CH{=}CH_2$	H	H	OMe	H	H	H
330512		OMe	H	Me	OMe	Me	H	H
658514		OMe	H	H	OMe	H	$C(=O)(CH_2)_3NH_2$	H
661580		OMe	H	H	OMe	H	$C{=}(O)CH_2NH_2$	H
661581		OMe	H	H	OMe	H	$C(=O)(CH_2)_2NH_2$	H
682300		N-Pr	H	H	OMe	H	$C(=O)(CH_2)_3NH_2$	H
683201		$NCH_2CH{=}CH_2$	H	H	OMe	H	$C(=O)(CH_2)_3NH_2$	H
683659		$NCH_2CH_2NMe_2$	H	H	OMe	H	H	H
683661		$NCH_2CH{=}CH_2$	H	H	OMe	H	$C{=}(O)CH_2NH_2$	H
683662		$NCH_2CH{=}CH_2$	H	H	OMe	H	$C{=}(O)CH_2NMe_2$	H
683663		$NCH_2CH{=}CH_2$	H	H	OMe	H	$C(=O)(CH_2)_2NH_2$	H
683664		$NCH_2CH{=}CH_2$	H	H	OMe	H	$C(=O)(CH_2)_2NMe_2$	H
683666		$NCH_2CH{=}CH_2$	H	H	OMe	H	$C(=O)(CH_2)_4NH_2$	H
697886		$NCH_2CH{=}CH_2$	H	H	OMe	H	$C(=O)(CH_2)_3NMe_2$	H
707545[a]	17-DMAG	$NCH_2CH_2NMe_2$	H	H	OMe	H	H	H
330500[b]	macbecin II	H	H	H	Me	H	Me	OMe
255108[b]		OMe	H	H	OMe	H	H	H

The compounds available for experimental validation are italic.
*$C_5H_{10}N$ = 1-piperidinyl; [a] HCl salt of NSC 683659
[b] NSC 330500 is not a benzoquinone, but the hydroquinone form (18, 21 dihydroquinone) of NSC 330499; NSC 255108 is hydrogeldanamycin 18,21-diacetate.

RES cells to GA and 17-AAG. siRNA also increased sensitivity to GA and 17-AAG in NCI/ADR-RES cells but was only partially effective, presumably because transient transfection of siRNA could not fully suppress ABCB1 expression (Fig. 2b). ABCB1 (−) K562 cells were more sensitive to GA and 17-AAG than ABCB1 (+) K562/DOX cells (Fig. 2c). In the presence of CsA, K562/DOX cells was strongly sensitized, but the potency of K562 cells did not change appreciably. The same experiments were done for all other available GA analogues showing that the resistance in NCI/ADR-RES and K562/DOX cells was reversible by CsA in each case (Table III). K562/DOX and NCI/ADR-RES cells are drug-selected cells and therefore may overexpress other drug-resistance genes that may affect the sensitivity to GAs. We performed the same experiments using the breast tumor cells MCF7 and its ABCB1-transfected variant BC19 cells. Compared to MCF7 cells, BC19 cells showed a 9-fold higher ABCB1 protein level and >13-fold higher mRNA expression (18). MCF7 cells were more sensitive to GA and 17-AAG than BC19 cells (Fig. 2d). In the presence of 2.5 μM CsA, BC19 cells were strongly sensitized, but the potency against MCF7 cells did not change appreciably. To rule out the role of other genes in these cell lines, we performed real-time RT-PCR analysis for ABCB1, ABCC1, SLC7A11 and SLC3A2 genes. As can be seen from the Supplementary Figure, the expression levels of ABCB1 in K562/DOX and NCI/ADR-RES were higher than other cells tested to a great extent. In contrast, the expression levels of ABCC1, SLC7A11 and SLC3A2 in K562/DOX and NCI/ADR-RES cells were low. These results suggest that ABCB1 may mediate the efflux of GA analogues, leading to decreased accumulation of compounds in the ABCB1 overexpressing cells.

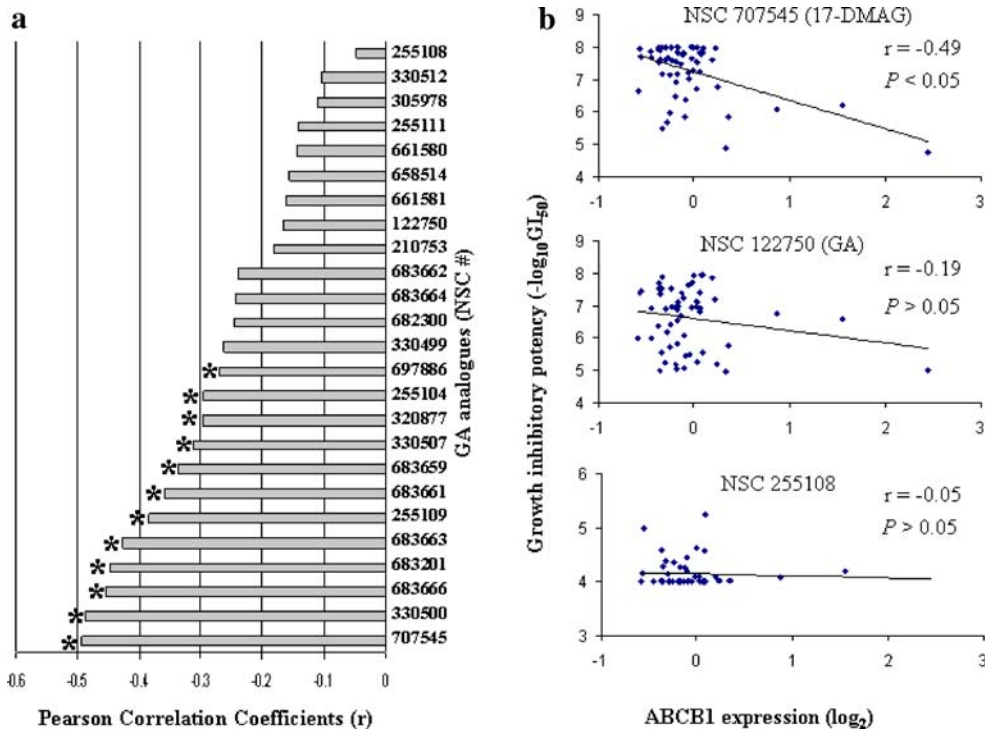


Fig. 1. Relationship between drug potency and ABCB1 expression in the NCI-60. **a**, Plot of sorted Pearson correlation coefficients (r) between ABCB1 gene expression and cytotoxic potencies of 25 GA analogues. *: *P* value<0.05; **b**, Scatter plot showing the correlation (r) of ABCB1 expression with sensitivity of the 60 cells to NSC 707545 (17-DMAG), NSC 122750 (GA) and NSC 255108.

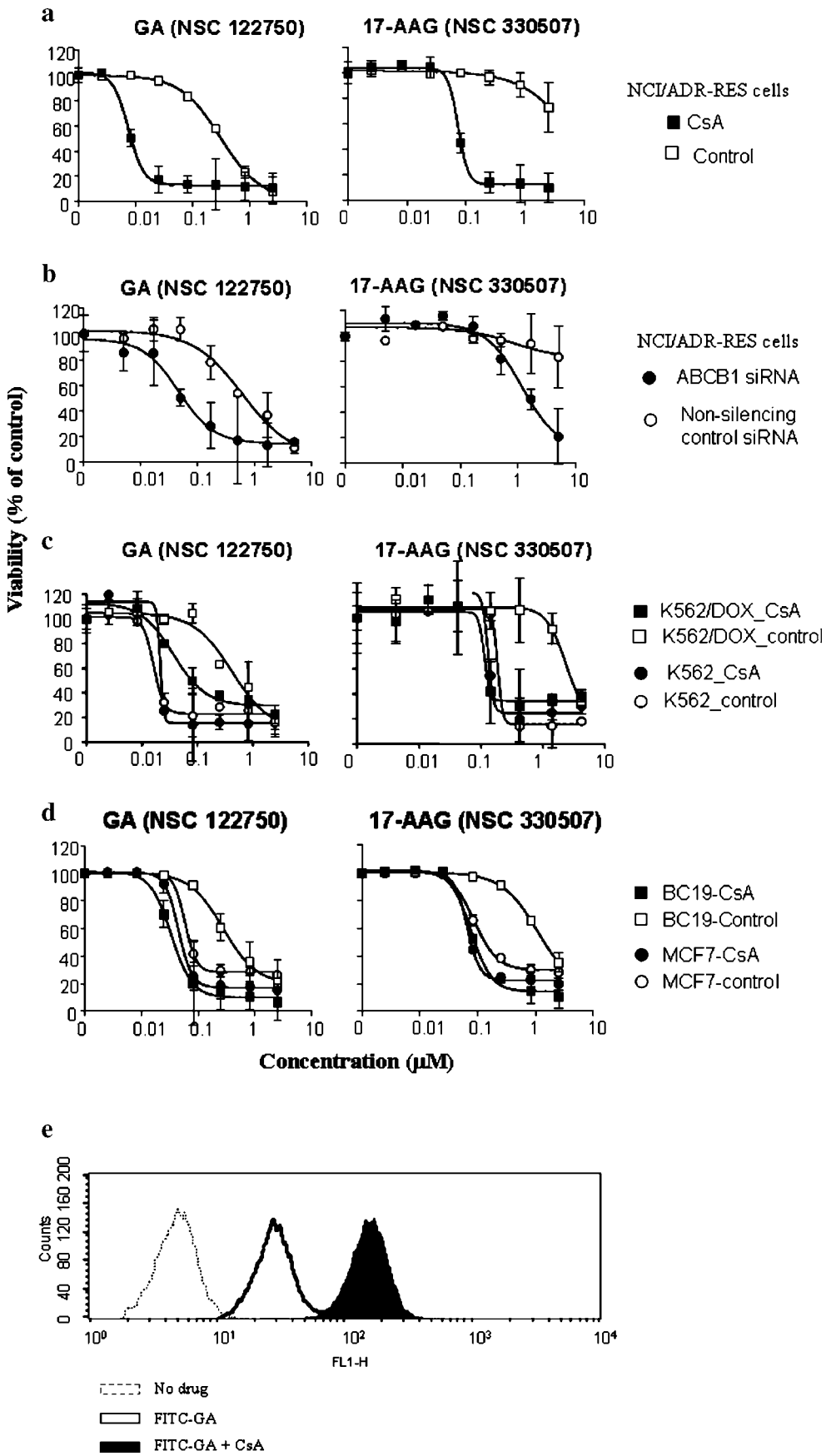
The availability of a fluorescent derivative of GA, FITC-GA, allowed us to further investigate the role of ABCB1 on drug transport and efflux. Following incubation with FITC-GA for 10 min at 37°C, K562/DOX cells contained less of the fluorescent compound than did the K562 cells. The decreased accumulation in K562/DOX cells was reversible by addition of 5 μM CsA (which had no effect on the K562 cells), further supporting the notion that GA is an ABCB1 substrate (Fig. 2e).

Inhibition of ABCB1-mediated Efflux by GA Analogues

We next tested the GA analogs for their ability to inhibit ABCB1-mediated efflux from multi-drug resistant cells K562/DOX, using daunorubicin (DNR) as fluorescent substrates (16). The level of DNR fluorescence in K562/DOX cells without drugs was significantly lower than that of K562 cells (data not shown). Inhibition of efflux increases intracellular fluorescence intensity from DNR, which can be detected using FACS analysis of at least 10,000 cells. CsA was used as positive control at 10 μM, which maximally inactivated MDR1 efflux. We tested three analogs, GA, 17-AAG and macbecin II. All of them inhibited ABCB1-mediated efflux of DNR in a dose-dependent fashion (0.1–100 μM). They strongly enhanced the accumulation of DNR in K562/DOX cells. A representive FACS histograph is depicted in Fig. 3a. A quantitative estimation of the inhibitory effects are depicted in Fig. 3b–d. Macbecin II (NSC 330500) yielded the highest inhibition (127.5% relative to 10 μM CsA). 17-AAG (NSC 330500) achieved 84.3% inhibition of DNR efflux relative to 10 μM CsA. The IC_{50} values of the test compounds (half-maximum inhibition of marker efflux) that were determined from inhibition curves at different inhibitor concentrations, were 5.5, 7.3 and 12 μM for macbecin II, 17-AAG and GA, respectively, which do not appear to be related to their cytotoxicity IC_{50}. These results indicate that these three GA analogues are inhibitors of ABCB1.

DISCUSSION

Circumventing drug resistance is important to successful cancer therapy. Similar to traditional chemotherapeutic agents, emerging molecularly targeted drugs such as imatinib/Gleevec, gefitinib/Iressa and geldanamycin analogues are also subject to mechanisms of resistance (19). While chemoresistance of tumor cells are frequently associated with decreased intracellular accumulation of anticancer drugs or abnormal expression of membrane transporters, comprehensive analysis of transporter-drug relationships is lacking. The NCI-60 databases containing data of mRNA gene expression profiles of transporters as well as drug potencies enabled a systematic search for transporter-drug relationships and have been used to identify genes encoding transporters and non-transporters showing significant correlations with growth inhibitory potencies of anticancer drugs (3,20). In this study we applied a chemogenomics approach, in which we analyzed the correlations between mRNA expression profiles of human transporter genes and patterns of growth inhibitory potency of a group of GAs with similar chemical structures in the NCI-60 panel. We demonstrated the power of this approach to identifying and validating ABC transporter gene ABCB1 conveying chemoresistance to these compounds. Exploiting available information on drug structure, drug activity and gene expression, this approach can be applied to any compound class and gene set for elucidating novel


a
GA (NSC 122750)
17-AAG (NSC 330507)
NCI/ADR-RES cells
CsA
Control
b
GA (NSC 122750)
17-AAG (NSC 330507)
NCI/ADR-RES cells
ABCB1 siRNA
Non-silencing control siRNA
c
GA (NSC 122750)
17-AAG (NSC 330507)
K562/DOX_CsA
K562/DOX_control
K562_CsA
K562_control
d
GA (NSC 122750)
17-AAG (NSC 330507)
BC19-CsA
BC19-Control
MCF7-CsA
MCF7-control
Viability (% of control)
Concentration (μM)
e
Counts
FL1-H
No drug
FITC-GA
FITC-GA + CsA

◀ **Fig. 2.** Validation of ABCB1-GA correlations by inhibiting ABCB1 function with cyclosporin A (CsA) or suppressing mRNA levels of ABCB1 with gene specific siRNA. **a**, Growth inhibition curves are shown for NCI/ADR-RES cells in response to GA and 17-AAG, with or without treatment with CsA (5 μM). **b**, Growth inhibition curves are shown for NCI/ADR-RES cells in response to GA and 17-AAG, with or without treatment with ABCB1-siRNA or non-silencing control siRNA. **c**, Growth inhibition curves for K562/DOX and K562 cells in response to GA and 17-AAG, with or without treatment with CsA. **d**, Growth inhibition curves for MCF7 and BC19 cells in response to GA and 17-AAG, with or without treatment with CsA (2.5 μM). The cytotoxicity results are expressed as percentage survival of control cells with no drug treatment (means+SD from three replicates). **e**, Accumulation of the fluorescent compound FITC-GA in ABCB1-overexpressing K562/DOX cells. Representative histograms show the fluorescence of cells after incubation with 5 μM FITC-GA for 10 min at 37°C in the presence or absence of 5 μM CsA.

mechanisms of drug resistance and for optimizing selection of drug candidates.

Several genes and proteins have been associated with sensitivity to GA analogues, including expression levels or mutation status of key Hsp90 client proteins such as ERBB2, BRAF, Bcr-Abl and AKT (21,22) as well as levels of Hsp90 family members such as Hsp70 (23,24). For 17-AAG, the metabolizing enzyme NQO1 is important for sensitivity (25). Thus, like commonly used anticancer agents, multiple factors contribute to the cellular sensitivity and resistance to GA analogues. Sufficient intracellular drug accumulation is a prerequisite for GAs to targeting Hsp90 (7). Membrane transporters play a key role in chemosensitivity, affecting the entry of anti-cancer drugs into cells and extrusion of drugs from them (26). We hypothesized that membrane transporters determine at least in part the differential sensitivity to GA analogues. Our results showed that expression levels of several transporter and non-transporter genes significantly correlated with growth inhibitory potencies of GA and its analogues. Although all identified genes will need to be investigated for their pharmacological functions in drug resistance, we first focused on the interactions between ABCB1 and these compounds because: (1) ABCB1 shows a wide range of correlations with chemoresistance to GAs; (2) ABCB1 expression conveys chemoresistance to its substrates by decreased accumulation in target cells through active drug efflux; (27,28) (3) numerous compounds of dissimilar structures can serve as ABCB1 substrates and/or inhibitors; (4) use of ABCB1-specific inhibitors, such as cyclosprin A and verapamil, can suppress ABCB1-related resistance, but clinical use of these inhibitors remains problematic (29); (5) the interaction of compound with ABCB1 also influence pharmacokinetic drug interactions (30). Therefore, designing or searching for compounds to avoid ABCB1-mediated chemoresistance is desirable.

In our previous study, a class of ellipticine analogues displayed a range of correlation coefficients with ABCB1 gene expression (4). Ellipticines with negative correlations were shown to be ABCB1 substrates, whereas those with neural or positive correlations served as ABCB1 inhibitors, which escape ABCB1-mediated chemoresistance. The question was whether GA analogues interact with ABCB1 in a similar fashion. Compounds in the GA class share common chemical substructure—the benzoquinone ansamycin moiety, and are functionally related—targeting Hsp90. The correlation coefficients with ABCB1 expression range from −0.05 to −0.49. Combining chemogenomic data and functional assays in cancer cell lines with low or high levels of ABCB1, our results suggested that GA analogues tested could be substrates as well as inhibitors of ABCB1. Pearson correlation coefficients were rather small between ABCB1 expression and several GA analogues, e.g., GA (NSC 122750) and NSC 255108. Nevertheless, the experimental results demonstrate that they are ABCB1 substrates as well. These false negative observations could be due to the interference of other genes expressed in NCI-60. Several cell lines, e.g., NCI-H322M and A549 cells are relatively resistant to GA (NSC122750), but do not have a high expression level of ABCB1. The resistance mechanism for GA (NSC 122750) in these cell lines may involve other genes and/or pathways independent of ABCB1 transport function. Therefore, gene-drug relationships gleaned from gene-drug correlations across the NCI-60 can produce false negative result. However, if one focuses on a class of compounds, significant negative correlations within

Table III. Effects of Inhibition of ABCB1 Activity on Cytotoxicity of GA Analogues in NCI/ADR-RES and K562/DOX Cells

NSC #	Name	IC50 (μM)			
		NCI/ADR-RES		K562/DOX	
		(−) CsA	(+)CsA	(−)CsA	(+)CsA
122750	GA	0.84±0.06	0.02±0.01* (45)	0.33±0.02	0.01±0.00* (32)
330500	Macbecin II	5.7±0.92	1.0±0.01* (5.7)	2.4±0.14	1.2±0.22* (2.1)
330507	17-AAG	4.1±0.32	0.08±0.01* (54)	>2.5	0.23±0.01* (>11)
707545	17-DMAG	>5.0	0.47±0.18* (>12)	>5.0	0.69±0.17* (>7.5)
–	17-AEP-GA	>4.3	0.40±0.19* (>13)	>4.3	0.23±0.05* (>19)
–	17-DMAP-GA	>4.3	2.3±1.0* (>2.1)	>4.3	1.3±0.12* (>3.3)
658514		>25	5.3±0.11* (>4.7)	9.9±0.24	2.0±0.37* (5.1)
661581		8.9±2.2	0.66±0.06* (13)	2.2±0.14	0.43±0.10* (5.4)

To validate ABCB1-GA correlations, effects of inhibiting ABCB1 activity on cytotoxicity of GAs were determined in ABCB1-overexpressing NCI/ADR-RES and K562/DOX cells. 5 μM CsA was used to suppress ABCB1 transport activity. The IC_{50} values are the concentration (μM) that produced 50% inhibition of cell growth compared to controls, obtained from the SRB (for NCI/ADR-RES) or MTS (for K562/DOX) assays. Numbers in the parentheses represent fold-reversal, which is the IC_{50} for the compounds in control cells divided by that in CsA-treated cells ($IC_{50}/[IC_{50}]_{CsA}$). Results represent mean+SD of at least three experiments. *: $p<0.05$ versus controls without adding CsA.

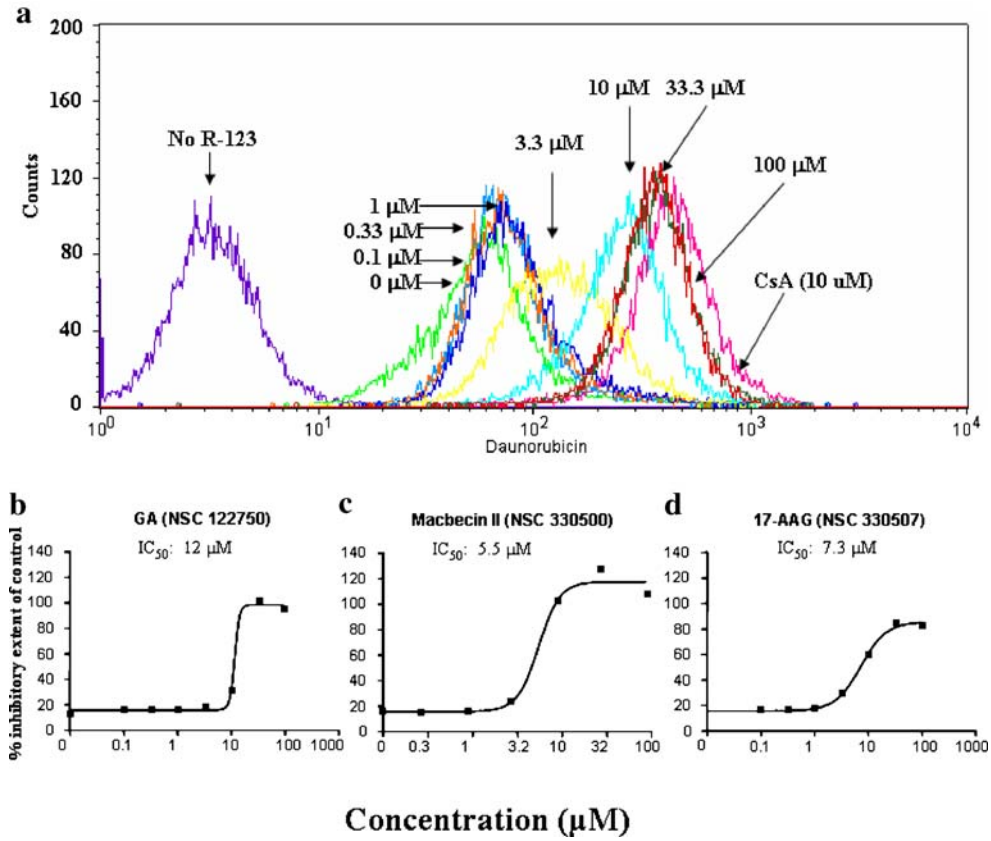


Fig. 3. Effects of selected GA compounds on intracellular accumulation of DNR in K562/DOX cells. **a**. Effect of 17-AAG (NSC 330507) (0–100 μM) on intracellular accumulation of Daunorubicin in K562/DOX cells. Histograms represent the cell counter numbers versus fluorescent intensity expressed as log relative fluorescence. The median of fluorescent intensity for No R-123, 0, 0.1, 0.33, 1, 3.3, 10, 33.3, 100 μM 17-AAG, 10 μM CsA are 12.86, 56.74, 70.41, 71.69, 78.44, 127.49, 254.83, 361.9, 355.45, 429.35, respectively. **b**, **c**, **d**, Graphs showing fluorescent intensity in the presence of different concentration of GA analogs relative to that observed in the presence of the control, 10 μM CsA (%).

this class could be indicative of possible interactions for all members, which requires experimental investigation.

We analyzed the effects of suppressing ABCB1 gene using the known inhibitor cyclosporin A and siRNA down-regulation of ABCB1 level, in ABCB1 overexpressing cell lines, K562/DOX and NCI/ADR-RES. Both lines were markedly resistant to GA, 17-AAG and other analogues. The resistant phenotype could be significantly alleviated by cyclosporin A. Cyclosporin A also increased the intracellular accumulation of FITC-GA in K562/DOX cells. GA analogues are substrates of cytochrome P-450, CYP3A4 (31). It has been reported that many substrate of CYP3A4 are also substrates of ABCB1 (32). Therefore, we confirm that GA analogues should be added to the extensive list of drugs that can be affected by the multidrug resistance mechanism mediated by ABCB1. Many anticancer agents are actively removed from tumors by ABCB1, and in many tumor cells, the amount of ABCB1 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 ABCB1 could serve as predictor for resistance to GA analogues. In clinical terms, documentation of ABCB1 overexpression might preclude prescription of GA-related agents. We cannot exclude the possibility that other genes also contribute to the resistance to this group of compounds, which is subject of a separate investigation. In other type of cancer cells, other genes, e.g., ABCC1 (MRP1) also plays a role to confer the resistance to GA (unpublished results).

GA analogues were also examined for ability to inhibit ABCB1-mediated efflux. In K562/DOX cells, GA, 17-AAG and macbecin II significantly inhibited daunorubicin transport. These data indicate that GA analogues are not only substrates but also inhibitors of ABCB1. The extent of the inhibitory effects of GA and macbecin II is higher than that of cyclosporin A—the most potent ABCB1 inhibitor presently known. Known ABCB1 inhibitors can be divided into two groups: those that are transported themselves and those that are not (8,33). GA and its analogues probably belong to the former. While the majority of ABCB1 substrates have poor ABCB1 inhibitory activity, imatinib/Gleevec was also shown to serve as ABCB1 substrate and inhibitor (34). Treatment with GA analogues would be expected to be synergistic with co-administered agents that are substrates of ABCB1. The IC_{50} values (half-maximum inahibition of daunorubicin efflux) were 5.5, 7.3 and 12 μM for macbecin II (NSC330500), 17-AAG (NSC330507) and GA, respectively. These concentrations are clinically achievable (35). Furthermore, GA analogues may change the pharmacokinetics and

pharmacodynamics of ABCB1 substrate drugs, such as doxorubicin and vincristine, because ABCB1 is present in high levels in the small intestine, liver, kidney and brain. Therefore, ABCB1-mediated drug-drug interaction with other ABCB1 substrate drugs needs to be considered. However, the accumulation assay was conducted in the presence of 10% fetal bovine serum. We cannot exclude the possibility that geldanamycin binds to protein in the culture media and thereby affect the unbound free concentration of DNR.

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