Taxane-based reversal agents modulate drug resistance mediated by P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein

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Abstract

Overexpression of ATP-binding cassette transport proteins, including P-glycoprotein (Pgp), multidrug resistance (MDR) protein (MRP-1), and breast cancer resistance protein (BCRP), is a well-characterized mechanism of MDR in tumor cells. Although the cytotoxic taxanes paclitaxel and docetaxel are substrates for Pgp-mediated efflux, the semisynthetic taxane analogue orataxel inhibits drug efflux mediated by Pgp as well as, as we recently demonstrated, MRP-1 and BCRP. Nevertheless, orataxel is not optimal for development as a clinical MDR modulator because of its cytotoxicity. We sought to identify noncytotoxic taxane-based broad-spectrum modulators from a library of noncytotoxic taxane-based reversal agents (tRAs) designed by eliminating the C-13 side chain of the taxane molecule, which inhibits microtubule depolymerization. Twenty tRAs, selected based on modulation of paclitaxel cytotoxicity in Pgp-overexpressing MDA435/LCC6^{mdr1} cells, were studied for modulation of retention and cytotoxicity of substrates of MRP-1 and BCRP as well as Pgp in established cell lines overexpressing each of these transporters. Four tRAs modulated MRP-1 and 17 modulated BCRP in addition to Pgp. The four broad-spectrum tRAs strongly modulated daunorubicin and mitoxantrone efflux and enhanced their cytotoxicity in cell lines overexpressing the three MRPs, decreasing IC_{50} values by as much as 97%. These tRAs, especially tRA 98006, have promise for development as clinical broad-spectrum MDR modulators and warrant more preclinical analysis to determine pharmacokinetic interactions and efficacy. (Mol Cancer Ther. 2003;2:1195–1205)

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Introduction

Despite advances in the field of cancer chemotherapy, drug resistance remains a major problem. Resistance may be intrinsic or may be induced by exposure to chemotherapeutic agents (1). Moreover, resistance may be to a specific chemotherapeutic agent or to chemically diverse agents; this latter form of resistance is called multidrug resistance (MDR). MDR frequently results from overexpression of cell membrane proteins belonging to the ATP-binding cassette (ABC) superfamily, which function as energy-dependent drug efflux pumps. These proteins include P-glycoprotein (Pgp), MDR protein (MRP-1), and breast cancer resistance protein (BCRP; 2).

Pgp, a 170-kDa membrane glycoprotein with 12 transmembrane spanning domains, is encoded by the mdr1 gene. Physiologically, Pgp functions as a xenobiotic pump in the intestine, liver, kidney, and placenta as well as in the blood-brain and blood-testes barriers. It effluxes neutral and cationic compounds in addition to a variety of chemotherapeutic agents, including anthracyclines, mitoxantrone, *Vinca* alkaloids, and taxanes (3, 4).

MRP-1, a 190-kDa protein with 17 transmembrane spanning domains encoded by the mrp1 gene, effluxes drugs that are either conjugated to or cotransported with glutathione; the N-terminal five transmembrane spanning domains are thought to be relevant in the glutathione requirement (5). Physiologically, MRP-1 is omnipresent throughout the body, with higher levels of expression in the adrenal gland, lung, heart, blood-brain barrier, and epithelial, muscle, and endocrine tissues. MRP-1 transports organic anions and leukotriene C-4 in addition to anthracyclines, mitoxantrone, and *Vinca* alkaloids (5).

BCRP (BCRP/MXR/ABCG2) was initially isolated from the MCF7 AdVp3000 breast cancer cell line, which demonstrated doxorubicin efflux in the absence of Pgp or MRP expression. BCRP, encoded by the mxr gene, is a 655-amino acid, 72-kDa protein with a N-terminal ATP-binding site and six transmembrane domains. It is a ''half-transporter'' likely to homodimerize or heterodimerize with a yet unidentified partner. Physiologically, BCRP is found in the placenta, intestine, breast, liver, and hematopoietic stem cells. BCRP may efflux mitoxantrone and anthracyclines as well as methotrexate and topoisomerase I inhibitors (6, 7); its substrate specificity depends on the amino acid sequence of the protein. BCRP protein with an arginine-to-threonine mutation at amino acid 482 (BCRP-T482) confers resistance

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to anthracyclines, whereas the wild-type protein (BCRP-R482) confers resistance to mitoxantrone but not to anthracyclines (8). This varying specificity suggests a role of amino acid 482 in drug binding.

Paclitaxel, isolated from the bark of the Pacific yew tree in the 1970s, is an antitumor drug that binds to β-tubulin and inhibits its depolymerization. Significant antitumor efficacy is seen in ovarian, breast, lung, head and neck, bladder, and esophageal cancers. Docetaxel is a more potent analogue of paclitaxel and is effective in breast, ovarian, lung, gastric, and prostate cancers. Both paclitaxel and docetaxel are substrates for Pgp- and MRP-1-mediated efflux, and their efficacy is thus compromised in cells that overexpress Pgp or MRP-1 (9).

The structure of paclitaxel has been altered in a variety of ways to create analogues that are both more potent and less susceptible to Pgp-mediated efflux (10). Our laboratory has identified orataxel (formerly IDN-5109, BAY 59-8862) as a potent paclitaxel analogue that modulates efflux mediated by Pgp (11), and we have recently demonstrated that orataxel also modulates efflux mediated by MRP-1 and BCRP (12). However, as cytotoxicity is not a desirable feature of a modulator, we sought to identify a noncytotoxic taxane analogue with activity as a modulator of all three of the ABC transporters that mediate MDR.

Noncytotoxic synthetic taxane-based reversal agents (tRAs) have the taxane baccatin backbone but lack the C-13 side chain of paclitaxel that binds β-tubulin and mediates cytotoxicity (13). More than 100 noncytotoxic tRAs have been synthesized to date, with diverse side chains; some of these tRAs are shown in Table 1 and Fig. 1. tRA 96023 was previously found to modulate Pgp, blocking efflux of doxorubicin in Pgp-overexpressing cell lines (11). We further demonstrated that tRA 96023 also modulates drug efflux mediated by BCRP but not by MRP-1 (12). In this study, we searched our library of synthetic tRAs to identify noncytotoxic modulators of Pgp, MRP-1, and BCRP that might be developed as broad-spectrum clinical MDR modulators.

Materials and Methods

Cell Lines

The breast cancer cell line MDA435/LCC6 and its Pgp-overexpressing subline MDA435/LCC6^{mdr1}, transfected

Table 1. MRP-1 expression in cell lines

Cell line	Pgp	MRP-1	BCRP
HL60-wt	−	−	−
HL60-ADR	−	+	−
8226-wt	−	−	+
8226-Dox6	+	−	+
8226-MR20	−	−	+
MCF7/S	−	−	+
MCF7/R	+	−	+
MCF7/MRP1-10	−	+	+
MCF7/AdrVp	−	−	+[a]

[a]R482T mutation in BCRP.

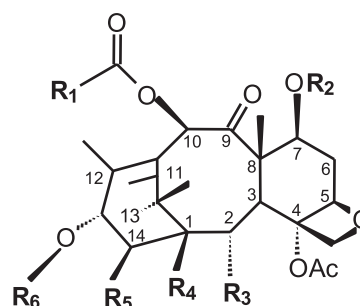

Figure 1. Backbone of tRAs.

with an engineered retrovirus to constitutively overexpress the mdr1 gene (gift from Dr. R. Clarke, Georgetown University Medical School, Washington, DC; 14), were used in the initial screening of tRAs for modulation of paclitaxel cytotoxicity. Wild-type MCF7 breast cancer (15), HL60 myeloid leukemia (16) and 8226 myeloma (17) cell lines, and resistant MCF7/R (drug selected, Pgp; 18), 8226/Dox6 (drug selected, Pgp and BCRP-R482; 17), HL60/ADR (drug selected, MRP-1; 16), MCF7/MRP1-10 (transfected, MRP-1; 19), 8226/MR20 (drug selected, BCRP-R482; 17), and MCF7 AdVp3000 (drug selected, mutant BCRP-T482; Table 1; 16) were used for subsequent study of Pgp, MRP-1, and BCRP modulation.

Suspension cell lines were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin (suspension cell lines; Life Technologies), and adherent cell lines in RPMI 1640 were supplemented with 5% heat-inactivated fetal bovine serum, 5% Nu-Serum, 10 mM HEPES, and 2 mM L-glutamine. All cell lines were incubated at 37°C in 5% CO_2 buffered air.

Drugs and Modulators

Paclitaxel, provided by Dr. I. Ojima, was solubilized in 100% DMSO to make a stock solution of 4 mM. Mitoxantrone (Sigma-Aldrich, St. Louis, MO) was solubilized in PBS to make a stock solution of 1.933 mM, doxorubicin (Sigma-Aldrich) was solubilized in distilled water to make a stock solution of 4 mM, and daunorubicin (Sigma-Aldrich) was solubilized in PBS to make a stock solution of 10 mM. The tRAs, synthesized as previously described (13), were solubilized in 100% DMSO to make stock solutions ranging from 1 to 10 mM.

Drug Efflux

To examine the efficacy of the tRAs as broad-spectrum modulators, retention of substrate drugs was studied in cell lines overexpressing Pgp, MRP-1, and BCRP. We have previously demonstrated that mitoxantrone is a substrate for all three of these proteins (17), whereas daunorubicin is known to be a substrate for Pgp, MRP-1, and BCRP-T482 but not for BCRP-R482 (8). Cells were incubated at 1 × 10^6/ml in RPMI 1640 with 3 μM mitoxantrone or 3 μM daunorubicin for 30 min at 37°C and washed with ice-cold PBS. An aliquot of cells was placed at 4°C for the analysis of uptake. The remaining cells were resuspended in RPMI

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Table 2. tRAs with activity against Pgp

tRA	R1	R2	R3	R4	R5	R6	%IC50 Reduction
95069	Me		OBz	OH	H	H	96
96023	Me		OBz	OH	H		88
97013	Me		OBz	See R5	(R5)O O(R4) O		91
97045	Me		OBz	OH	H		92
98005	Me		OBz	OH	H		91
98006	Me		OBz	OH	H		89
98007	Me		OBz	OH	H		89
98009	Me		OBz	OH	H		94
98010	Me		OBz	OH	H		91
99010	Me		OBz	OH	H		90
99011	Me		OBz	See R5	(R5)O O(R4) O		90
99018	Me		OBz	See R5	(R5)O O(R4) O		91
99019	Me		OBz	OH	H		83
99020	Me		OBz	OH	H		88
99021	Me		OBz	OH	(R5)O O(R4) O		92
99030	Me		OBz	OH	H		91
99031	Me		OBz	See R5	(R5)O O(R4) O		95
01015	Me			OH	H		91
01021	Me		OBz	OH	H		87
01069	Me		OBz	OH	H		86

Note: The structures are the tRAs selected for testing against other MDRs based on activity in modulating Pgp. The IC_{50} reduction represents the percent decrease in the IC_{50} of paclitaxel in the presence of 0.1 μM tRA in the MDA435/LCC6^{mdr1} cell line, which overexpresses Pgp.

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1640 with and without tRAs at a concentration of 10 μM, which is the highest concentration that can be delivered *in vivo* due to vehicle (Tween 80:ethanol [1:1] diluted in 0.9% NaCl Irrigation USP [Baxter Healthcare Corporation, Deerfield, IL]) toxicity. Mitoxantrone or daunorubicin efflux was allowed to occur at 37°C for 90 min in the presence and absence of each tRA. Cells were then washed with ice-cold PBS and placed on ice until analysis. Experiments were performed in triplicate, and the mean ± SE were calculated.

Flow Cytometry

Cellular mitoxantrone and daunorubicin content was analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped in standard fashion with an Argon laser for 488 nm excitation and 585/42 band-pass (FL2) or 670 long-pass (FL3) filters for emission collection as previously described (17, 20).

Flow Cytometry Data Analysis

Data were analyzed with WinList software (Verity Software House, Topsham, ME). Distribution histograms of mean fluorescence intensity following efflux in the presence and absence of modulator were compared by the Kolmogorov-Smirnov statistic (21), expressed as a *D*-value, which ranges from 0 (identical histograms) to 1.0 (no overlap in histograms). *D*-values ≥ 0.2 indicate a significant separation of histograms.

Cytotoxicity

To study cytotoxicity in suspension cell lines, cells were cultured in 24-well plates at 5×10^4 cells/well or in 48-well plates at 2.5×10^4 cells/well. Drugs were diluted in RPMI 1640 from frozen stock solutions to achieve the desired concentrations. Final DMSO concentrations were <0.1%. Cells were incubated with drugs at concentrations spanning a 5–6-log range, with and without modulators, for 96 h. Cells in each well were counted with a Coulter counter (Coulter Electronics, Fullerton, CA) as previously described (22). Experiments were performed at least in triplicate, with duplicates within each experiment.

To study cytotoxicity in the adherent cell lines, cells were seeded at 600–2000 cells/well (varying by cell line) in 96-well plates and incubated for 18–24 h at 37°C to allow for attachment. Drugs were diluted in RPMI 1640 + 2% HEPES from frozen stock solutions to arrive at the desired concentrations. Final DMSO concentrations were <0.1%. Cells were incubated with drugs at concentrations spanning a 5–6-log range, with and without modulators, for 72 h, analyzed for growth inhibition, and quantified with the sulforhodamine B dye-based assay as previously described (23). Experiments were performed at least in quintuplicate.

Cytotoxicity Data Analysis

IC_{50} values, or drug concentrations required to inhibit control growth by 50%, were determined using the Datalog and Gplate Microsoft FORTRAN software program developed by Dr. W. Greco at Roswell Park Cancer Institute (11). Briefly, data were fitted using the Sigmoid-Emax concentration-effect model (24) with nonlinear regression, weighed by the reciprocal of the square of the predicted response. The software uses the Marquardt (25) algorithm as adapted by Nash (26) for the nonlinear regression. Modulators were assessed for cell growth inhibition at various concentrations.

The IC_{50} values of mitoxantrone in the absence and presence of each tRA at 0.1, 1, and 10 μM were compared in

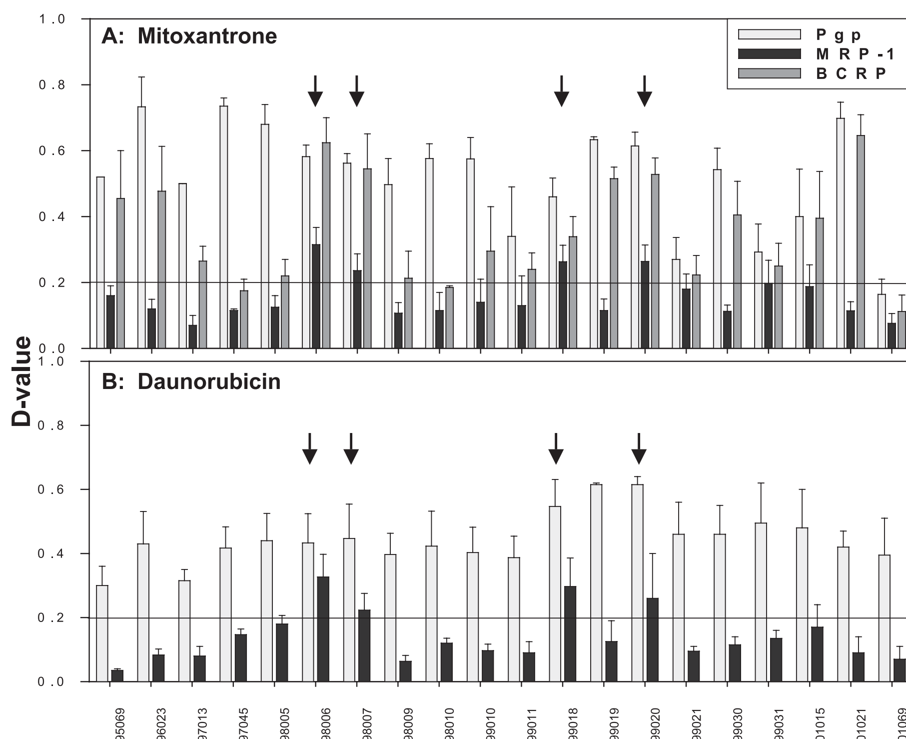

Figure 2. Effect of tRAs on drug efflux in resistant cell lines. Results are expressed as *D*-values comparing efflux of mitoxantrone (**A**) and daunorubicin (**B**) in the presence and absence of tRAs. Screening of tRAs for modulation of mitoxantrone efflux mediated by MRP-1 (HL60/ADR) and BCRP (8226/MR20) is demonstrated; Pgp (8226/Dox6) is also shown (**A**). Modulation of daunorubicin efflux mediated by MRP-1 (HL60/ADR) as well as Pgp (8226/Dox6) is also shown (**B**). *Arrows,* the four tRAs that are broad-spectrum modulators: 98006, 98007, 99018, and 99020.

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each cell line by calculating the resistance-modifying factor (RMF) as (IC_{50} drug) / (IC_{50} drug + modulator). RMF > 1 indicates enhanced drug sensitivity in the presence of tRA, RMF = 1 indicates no effect, and RMF < 1 indicates an antagonistic effect. Statistical error was calculated for the RMFs, and enhancement of drug sensitivity was defined as (RMF − SE) > 1; the greater the RMF magnitude, the more significant the effect.

Results

Screening for Pgp Modulation

Synthetic tRAs (n = 101) were screened for Pgp modulation by treating Pgp-transfected MDA435/LCC6^{mdr1} breast carcinoma cells with a range of paclitaxel doses in the presence and absence of 0.1 μM tRA. When cytotoxicity was examined in the presence of higher concentrations of tRAs, a large percentage of the tRAs was efficacious at lowering the paclitaxel IC_{50}. Thus, a tRA concentration of 0.1 μM was chosen to identify the most effective Pgp modulators. The IC_{50} of paclitaxel alone in the MDA435/LCC6^{mdr1} cells was 221 ± 6.6 nM, representing over 100-fold resistance to the agent as compared with the parental cell line MDA435/LCC6. At a concentration of 0.1 μM, 49 of 101 tRAs produced a >50% decrease in the IC_{50} of paclitaxel and 26 tRAs produced a >75% decrease (data not shown). Importantly, the tRAs were also screened in the parental nontransfected cell line in parallel and neither tRA cytotoxicity nor enhancement of paclitaxel cytotoxicity was noted. The 20 tRAs, which produced the greatest decreases in the IC_{50} of paclitaxel (Table 2), were selected for testing for MDR modulation in cell lines overexpressing MRP-1 and BCRP as well as Pgp.

Modulation of Drug Efflux in Cells Lines Overexpressing Pgp, MRP-1, and BCRP

The 20 tRAs selected based on modulation of paclitaxel cytotoxicity in MDA435/LCC6^{mdr1} cells were tested for modulation of mitoxantrone efflux in resistant cell lines overexpressing Pgp, MRP-1, and BCRP. The tRAs were initially tested at 10 μM, the maximum concentration that could be delivered *in vivo* due to vehicle toxicity (see ''Materials and Methods''). The tRAs did not have inherent fluorescence and did not modulate mitoxantrone in the non-MDR-overexpressing HL60/wt cell line (data not shown). Following uptake of mitoxantrone, 8226/Dox6, HL60/ADR, and 8226/MR20 cells, which overexpress Pgp, MRP-1, and BCRP-R482, respectively, effluxed 40–50% of their intracellular mitoxantrone content during 90 min incubation in medium alone. With the exception of tRA 01069, all of the tRAs studied modulated efflux of mitoxantrone in Pgp-overexpressing 8226/Dox6 cells, with D-values of at least 0.2 for the comparison of efflux in the presence and absence of tRA, consistent with the initial criterion for the selection of these tRAs. Four tRAs modulated mitoxantrone efflux in HL60/ADR cells and 17 modulated mitoxantrone efflux in 8226/MR20 cells; the tRAs that modulated efflux in 8226/MR20 cells included those that modulated efflux in HL60/ADR cells (Fig. 2A). Thus, four tRAs were identified as having broad-spectrum activity, modulating mitoxantrone efflux in resistant cell lines overexpressing each of the three MDR-associated drug efflux pumps (Fig. 2A).

The tRAs were also tested for modulation of daunorubicin efflux in 8226/Dox6 and HL60/ADR cells. Because daunorubicin is not a substrate for BCRP-R482, modulation of daunorubicin efflux was not studied in 8226/MR20 cells but was examined in MCF7 AdVp3000 cells, which overexpress BCRP-T482 (see below). The results were concordant with those for mitoxantrone; all 20 agents modulated daunorubicin efflux in 8226/Dox6 cells, and the four tRAs that modulated mitoxantrone efflux in HL60/ADR cells also modulated daunorubicin efflux (Fig. 2B).

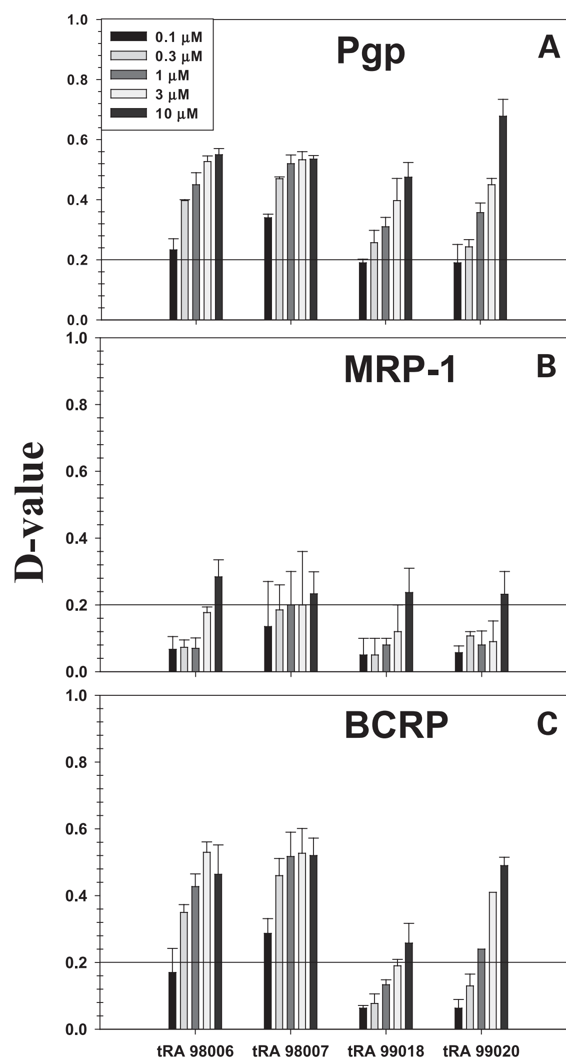

Figure 3. Dose-effect of broad-spectrum tRAs on mitoxantrone efflux in MDR-expressing suspension cell lines. Mitoxantrone efflux was studied by flow cytometry in the presence and absence of tRAs 98006, 98007, 99018, and 99020 at varying concentrations. Efflux in the presence and absence of each tRA was compared by the Kolmogorov-Smirnov statistic, expressed as a D-value. The tRAs modulated efflux in 8226/Dox6 cells at concentrations as low as 100 nM (**A**), in HL60/ADR cells only at 10 μM (**B**), and in 8226/MR20 cells at concentrations as low as 0.1 μM (**C**).

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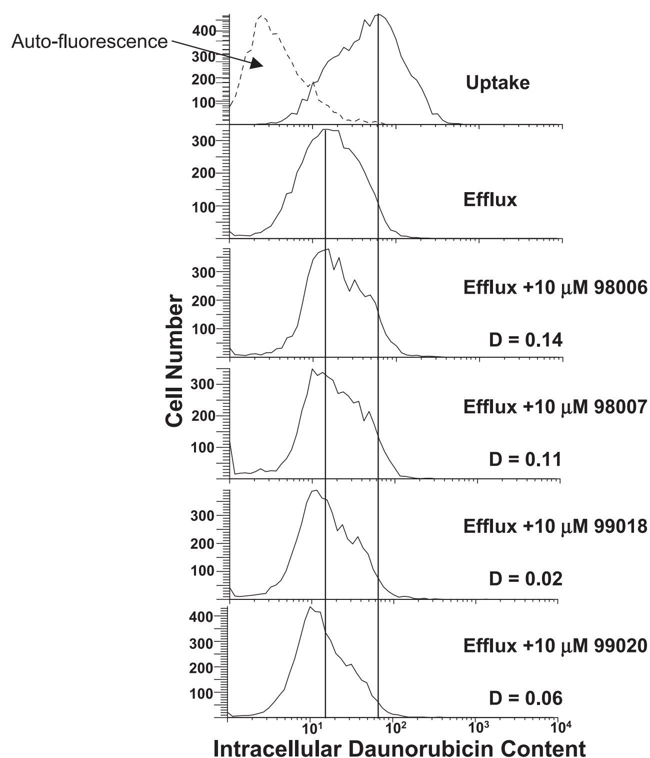

Figure 4. Effect of tRAs on daunorubicin efflux in MCF7 AdVp3000 cells. Daunorubicin efflux was studied by flow cytometry in the presence and absence of tRAs 98006, 98007, 99018, and 99020 at 10 μM. Intracellular daunorubicin content was measured as log-scale fluorescence intensity (FL2). Efflux in the presence and absence of each tRA was compared by the Kolmogorov-Smirnov statistic, expressed as a *D*-value. None of the tRAs modulated efflux mediated by BCRP-T482.

tRAs 98006, 98007, 99018, and 99020, the four tRAs that were identified as modulators of efflux mediated by Pgp, MRP-1, and BCRP-R482, were further studied for concentration-dependent effects on mitoxantrone efflux. Each of the tRAs modulated Pgp-mediated efflux in a concentration-dependent manner, starting at concentrations as low as 0.1–0.3 μM (Fig. 3A), with *D*-values of 0.23–0.68. Each of the tRAs modulated efflux mediated by MRP-1 at a concentration of 10 μM, with *D*-values ranging from 0.23 to 0.28, but not at lower concentrations (Fig. 3B). The tRAs also modulated efflux mediated by BCRP-R482 (Fig. 3C) in a concentration-dependent manner: tRA 98006 modulated efflux at 0.3 μM and higher, with *D*-values of 0.35–0.46; tRA 98007 modulated efflux at concentrations of 0.1 μM and higher, with *D*-values of 0.29–0.52; tRA 99018 modulated efflux only at 10 μM, with a *D*-value of 0.26; and tRA 99020 modulated efflux at concentrations of 1 μM and higher, with *D*-values of 0.24–0.49.

To test the ability of the broad-spectrum tRAs to modulate efflux mediated by BCRP-T482, we studied drug uptake and efflux in MCF7 AdVp3000 breast cancer cells. Concentration-dependent tRA modulation of both mitoxantrone and daunorubicin efflux was studied under the same experimental conditions as described above, with tRA concentrations ranging from 0.1 to 10 μM. At concentrations up to 10 μM, the tRAs were ineffective modulators of efflux of mitoxantrone (data not shown) and daunorubicin (Fig. 4) mediated by BCRP-T482 in MCF7 AdVp3000 cells.

Enhancement of Cytotoxicity

Each of the four tRAs was studied for effects on mitoxantrone cytotoxicity at a range of concentrations (0.1, 1, and 10 μM). Controls of tRA alone within each experiment confirmed that each tRA was noncytotoxic at all concentrations tested for modulation. Cytotoxicity experiments were also performed separately for each tRA, and toxicity was negligible at doses up to 30 μM, as shown for tRA 98006 in Fig. 5. Moreover, toxicity at high concentrations is attributable to the solvent DMSO. All four tRAs produced a concentration-dependent decrease of the IC_{50} of mitoxantrone in 8226/Dox6 cells, seen as a cytotoxicity curve shift to the left in Fig. 6A, with RMFs of 1–13 (Table 3). Modulation of MRP-1-mediated resistance to mitoxantrone

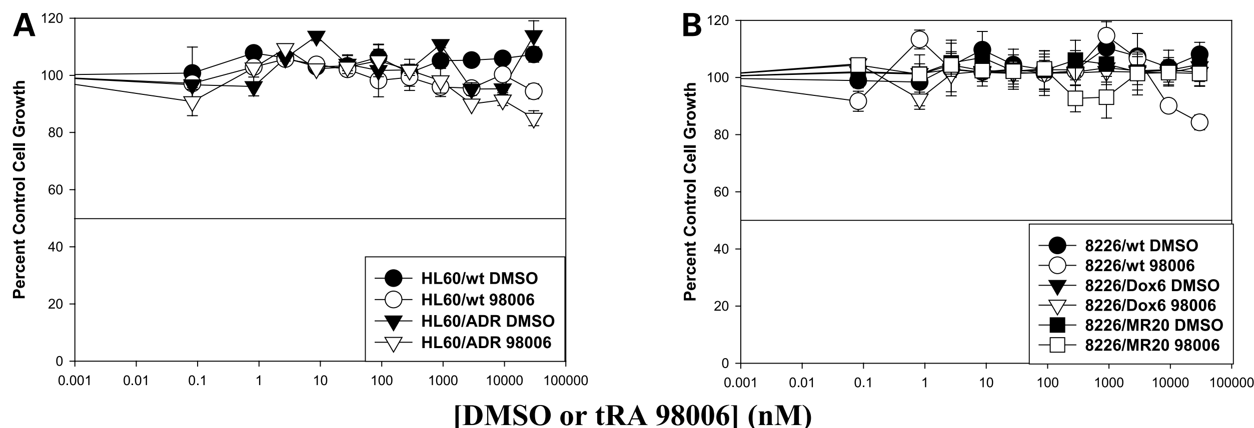

Figure 5. Toxicity of tRA 98006 and DMSO on myeloma/leukemia cell lines. Results are percent of control growth *versus* log-scale drug concentration. HL60/wt (**A**), HL60/ADR (**A**), 8226/wt (**B**), 8226/Dox6 (**B**), and 8226/MR20 (**B**) cells were treated with tRA 98006 (*open symbols*) or comparable concentrations of DMSO (*closed symbols*) for 96 h. *Horizontal line,* 50% control cell growth; experimental values are representative of at least triplicate experiments.

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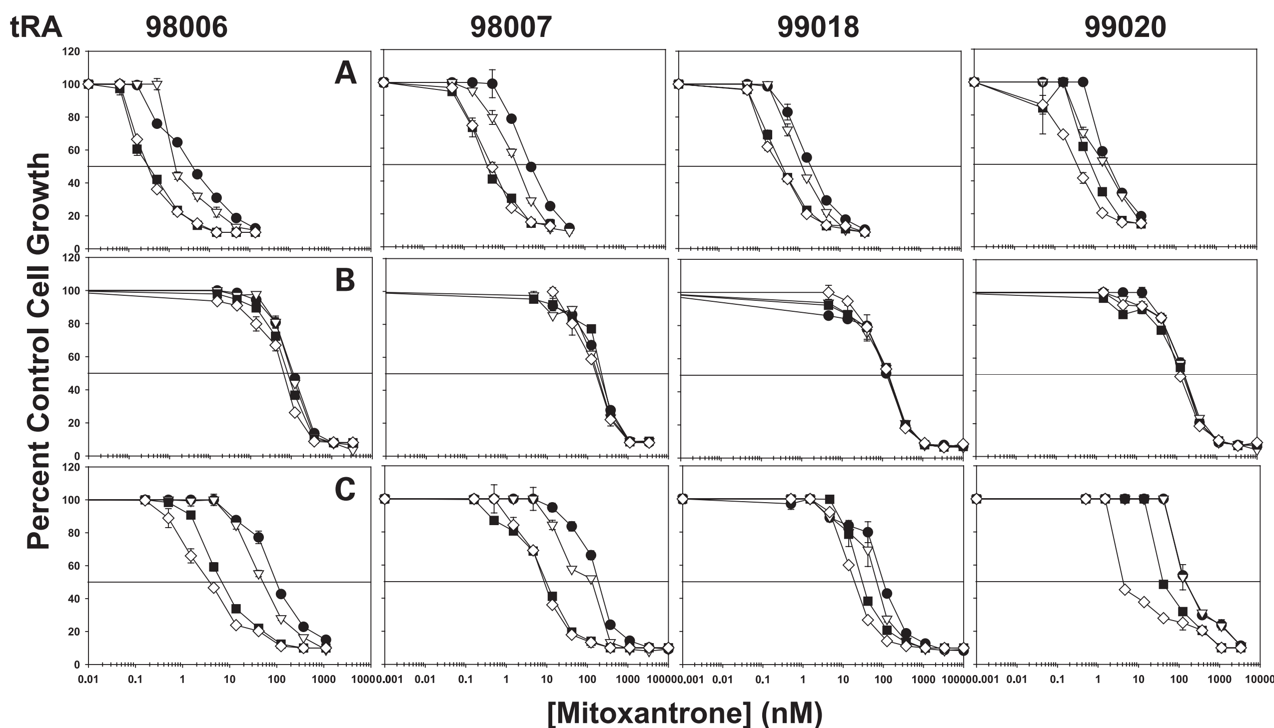

Figure 6. Effect of tRAs on mitoxantrone cytotoxicity in resistant cell lines. Results are percent of control growth *versus* log-scale drug concentration. 8226/Dox6 (**A**), HL60/ADR (**B**), and 8226/MR20 (**C**) cells were treated with mitoxantrone for 96 h in the absence (●) and presence of tRAs at 0.1 μM (▽), 1 μM (■), and 10 μM (◇). *Horizontal line,* 50% control cell growth; experimental values are representative of at least triplicate experiments.

in HL60/ADR cells was observed with tRAs 98006, 98007, and 99020 but not with tRA 99018 (Fig. 6B); the degree of modulation was less than that observed for Pgp, with a maximum RMF of 2.2 (Table 3). Finally, all four tRAs modulated mitoxantrone resistance in 8226/MR20 cells with overexpression of BCRP-R482 (Fig. 6C), and the degree of modulation was greater than that observed for Pgp and MRP-1, with RMFs of 1–62 (Table 3).

tRA 98006 was identified as the lead broad-spectrum modulator based on its degree of modulation of MRP-1 and BCRP-R482 in addition to Pgp. This agent was further studied by measuring its effects on anthracycline cytotoxicity in the MCF7 breast cancer cell line model. Wild-type MCF7 cells, MCF7/R cells (which overexpress Pgp), MCF7/MRP1-10 cells (which overexpress MRP-1), and MCF7 AdVp3000 cells (which overexpress BCRP-T482) were treated with doxorubicin, daunorubicin, and mitoxantrone with and without tRA 98006 at 0.1, 1, and 10 μM (Fig. 7 and Table 4). A minimal degree of modulation was seen in wild-type MCF7 cells (Fig. 7A), likely due to their low-level BCRP expression (8). tRA 98006 modulated resistance to all three drugs in MCF7/R cells at concentrations as low as 0.1 μM (Fig. 7B); RMFs ranged between 2.5 and 20 (Table 4). tRA 98006 also markedly enhanced cytotoxicity of all three drugs

Table 3. RMFs of broad-spectrum tRAs on mitoxantrone cytotoxicity in resistant cell lines

	tRA 98006			tRA 98007			tRA 99018			tRA 99020		
	0.1 μM	1 μM	10 μM	0.1 μM	1 μM	10 μM	0.1 μM	1 μM	10 μM	0.1 μM	1 μM	10 μM
8226/Dox6	3.11 ± 1.29	13.8 ± 3.2	13.9 ± 4.2	2.78 ± 0.3	11.8 ± 2.7	14.5 ± 2.1	1.53 ± 0.6	6.05 ± 0.9	7.2 ± 1.8	1.12 ± 0.4	2.7 ± 0.17	9.8 ± 3.6
HL60/ADR	1.09 ± 0.14	1.78 ± 0.11	2.2 ± 0.17	1.16 ± 0.09	1.21 ± 0.12	1.4 ± 0.2	1.05 ± 0.06	1.25 ± 0.26	1.11 ± 0.11	0.97 ± 0.04	1.34 ± 0.18	1.61 ± 0.37
8226/MR20	2.04 ± 0.6	22.7 ± 5.9	19 ± 5.3	2.83 ± 0.9	30 ± 5.7	31.8 ± 1.4	1.59 ± 0.06	9.78 ± 6.8	5.2 ± 0.9	1.25 ± 0.04	15.9 ± 5.3	50.7 ± 15.2

Note: 8226/Dox6, HL60/ADR, and 8226/MR20 cells were treated with mitoxantrone for 96 h in the absence and presence of tRAs 98006, 98007, 99018, or 99020 at 0.1, 1, and 10 μM. Results are expressed as RMFs calculated as (IC_{50} mitoxantrone) / (IC_{50} mitoxantrone + tRA). Experimental values are representative of at least triplicate experiments over which the SE was calculated.

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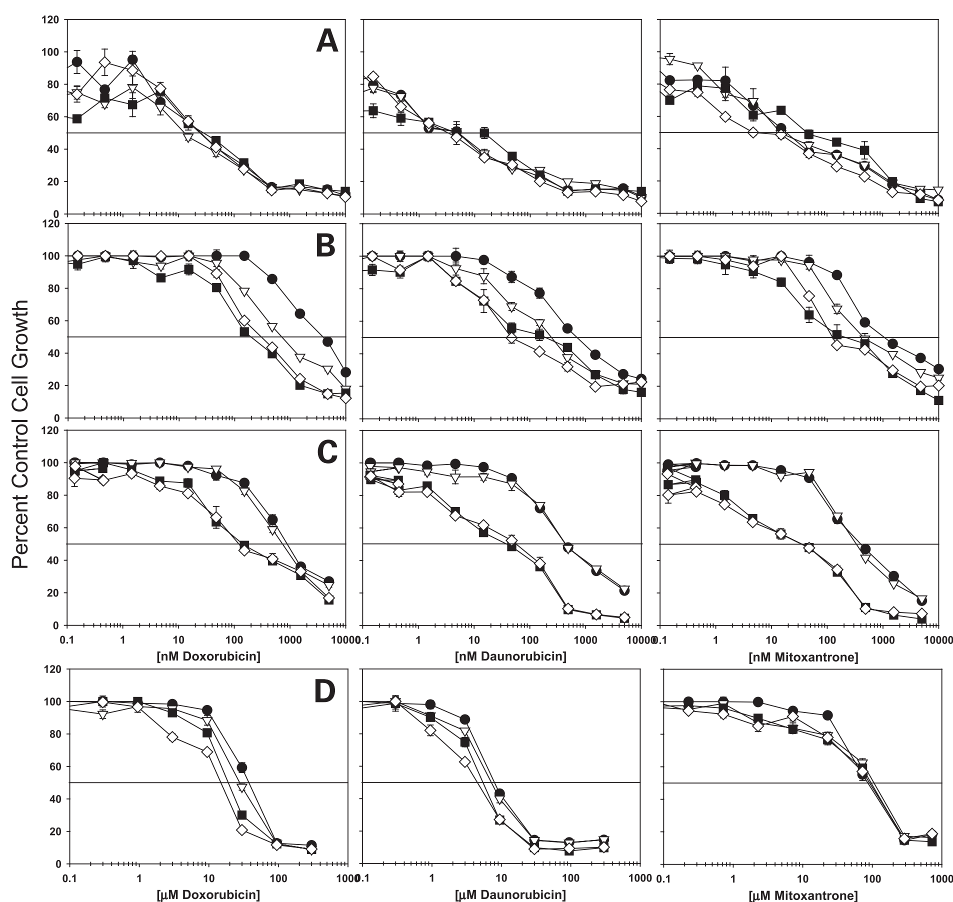

Figure 7. Effect of tRA 98006 on cytotoxicity in MCF7 cells. Results are percent of control growth *versus* log-scale drug concentration. MCF7/S (**A**), MCF7/R (**B**), MCF7/MRP1-10 (**C**), and MCF7 AdVp3000 (**D**) cells were treated with doxorubicin, daunorubicin, or mitoxantrone for 72 h in the absence (●) or presence of tRA 98006 at 0.1 µM (▽), 1 µM (■), and 10 µM (◇). *Horizontal line,* 50% control cell growth; experimental values are representative of at least triplicate experiments.

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in MCF7/MRP1-10 cells at concentrations of 1 and 10 µM (Fig. 7C); RMFs ranged from 1 to 24 (Table 4). tRA 98006 did not modulate mitoxantrone cytotoxicity in MCF7 AdVp3000 cells and only modulated doxorubicin and daunorubicin cytotoxicity at a concentration of 10 µM (Fig. 7D), with correlating RMFs up to 3 (Table 4).

Discussion

In this study, we identified four noncytotoxic synthetic tRAs that modulate efflux and cytotoxicity of substrate drugs in multidrug resistant cell lines overexpressing Pgp, MRP-1, and BCRP-R482. The most effective of the four modulators was tRA 98006.

The amino acid at position 482 of the BCRP protein is known to determine substrate specificity (8); based on the evidence presented here, the amino acid at position 482 of the BCRP protein also determined modulator efficacy. The tRAs modulated drug efflux and resistance mediated by BCRP-R482 but not by BCRP-T482. Although BCRP-T482 is found in MCF7 AdVp3000 cells, in which BCRP-mediated resistance was initially described (6), it has not been demonstrated in clinical samples. BCRP-R482 has been found to be present in all cases of acute myeloid leukemia (AML; 27) and acute lymphoblastic leukemia (28) studied to date; BCRP mutations have not been found. Thus, based on data to date, the tRAs should be

Table 4. Effect of tRA 98006 on anthracycline cytotoxicity in MCF7 cells

	Doxorubicin			Daunorubicin			Mitoxantrone		
	0.1 µM	1 µM	10 µM	0.1 µM	1 µM	10 µM	0.1 µM	1 µM	10 µM
MCF7/S	2.76 ± 0.7	1.3 ± 0.2	1.3 ± 0.07	1.6 ± 0.14	2.1 ± 0.6	2.1 ± 1.1	1.8 ± 0.3	3.1 ± 1.5	2.5 ± 0.5
MCF7/R	4.62 ± 0.7	15 ± 3	20.1 ± 1	5.2 ± 0.9	15 ± 5.7	13.1 ± 0.6	3.6 ± 0.7	6 ± 0.8	19.74 ± 2.6
MCF7/MRP1-10	1.6 ± 0.3	4.9 ± 0.07	4.61 ± 0.2	1.5 ± 0.4	4.7 ± 0.2	16.99 ± 0.1	3.7 ± 1.7	19.8 ± 0.6	24.02 ± 0.8
MCF7 AdVp3000	1.04 ± 0.06	1.6 ± 0.3	2.98 ± 0.16	1.2 ± 0.18	1.3 ± 0.2	1.67 ± 0.4	0.9 ± 0.08	0.97 ± 0.09	0.96 ± 0.17

Note: MCF7/S, MCF7/R, MCF7/MRP1-10, and MCF7 AdVp3000 cells were treated with doxorubicin, daunorubicin, or mitoxantrone for 72 h in the absence and presence of tRA 98006 at 0.1, 1, and 10 µM. Results are expressed as RMFs. Experimental values are representative of at least triplicate experiments over which the SE was calculated.

effective broad-spectrum modulators in the acute leukemias, modulating Pgp, MRP-1, and BCRP-R482.

The relevance of the three MDRs has been thoroughly studied in AML due to the ease of obtaining tumor cells for study and the ability to correlate MDR expression with treatment response, which is heterogeneous. Pgp, MRP-1, and BCRP are all known to be expressed in AML cells. Pgp has been shown to have clinical relevance in AML (29–32), and the relevance of MRP expression has also been demonstrated (31–33). Moreover, the relevance of BCRP has been suggested in studies performed to date (34–37). Coexpression of multiple MDRs in AML cells (37, 38) provides a strong rationale for studying broad-spectrum modulators in the treatment of this disease.

Most MDR modulation clinical trials to date have focused on inhibiting Pgp using modulators such as cyclosporin A (CsA) and PSC-833. The results thus far have been disappointing for the most part (39–41). Lack of efficacy in most clinical trials may be due in part to coexpression of multiple MRPs in many cases. The only positive clinical trials in AML have been with CsA (42, 43), an immunomodulator that has been shown to modulate MRP-1 in addition to Pgp and may partially modulate BCRP (44). The CsA trial demonstrated a significant reduction in the frequency of resistance to induction therapy in the population of patients who received modulator ($P = 0.0077$) as well as an increase in relapse-free (at 2 years) and overall survival ($P = 0.031$ and 0.046, respectively; 43). The efficacy of CsA may be due in part to its activity against multiple MRPs, suggesting that identification of clinically applicable broad-spectrum MDR modulators may be a promising avenue of investigation.

Broad-spectrum modulators have potential advantages and disadvantages. As noted, the ability to block more than one efflux pump would be useful in the treatment of malignancies, such as AML, in which multiple pumps are expressed. Agents capable of blocking the largest number of clinically relevant MDR pumps may have the greatest potential for success in patients. Moreover, regimens using a broad-spectrum modulator from the onset of treatment could mitigate induction of expression of multiple MDR pumps (6, 45). An additional advantage may be increased absorption following oral administration, as multiple MDRs are expressed in the mucosal cells of the gastrointestinal tract (46), or increased penetration of drugs into pharmacological sanctuaries such as the brain (47, 48).

Potential concerns about the use of broad-spectrum modulators include possible increased toxicity, as efflux from such ''protected'' areas (*i.e.*, brain and testes) would also be blocked. Moreover, detoxifying organs, including the liver and the kidney, also express MDRs (49), and modulation of these proteins might also alter the pharmacokinetics (PK) of chemotherapeutic agents.

The potential for PK interaction is a large concern and is one of the biggest obstacles in MDR modulation. As mentioned, MDRs are expressed in many tissues normally and these proteins may be modulated. Thus, increased amounts of drug will enter peripheral organs, and the function of the main detoxifying organs (liver and kidneys) will be altered. In clinical trials carried out thus far, doses of chemotherapeutic agents have had to be decreased by up to 60% in the modulation arm, as increases in the drug's area under curve and half-life, accompanied by decreased drug clearance, have been noted with CsA and PSC-833 (45). New third-generation modulators, including both specific and broad-spectrum agents, have been found to have minimal to no PK interactions when combined with doxorubicin or paclitaxel (50–52); PK interactions remain an important aspect of the tRAs to be studied.

Additionally, as chemotherapeutic agents are not the only substrates for MDR pumps, patients receiving modulation therapy must be diligently surveyed for other drugs that they may be taking to treat comorbid conditions. Antiarrythmic agents, antihypertensive agents, hormones, and antihistamines (46) are also Pgp substrates and are contraindicated in the setting of modulation therapy. These potential problems need to be explored for each agent as part of preclinical and subsequent clinical testing.

The most effective broad-spectrum modulator identified in this study was tRA 98006. Preclinical testing will continue with this agent, with the eventual goal of clinical testing in AML and other malignancies.

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