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Collateral Sensitivity of Multidrug-Resistant Cells to the Orphan Drug Tiopronin

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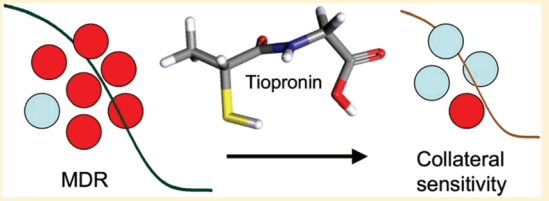
Supporting Information

ABSTRACT: A major challenge in the treatment of cancer is multidrug resistance (MDR) that develops during chemotherapy. Here we demonstrate that tiopronin (**1**), a thiol-substituted *N*-propanoylglycine derivative, was selectively toxic to a series of cell lines expressing the drug efflux pump P-glycoprotein (P-gp, ABCB1) and MRP1 (ABCC1). Treatment of MDR cells with **1** led to instability of the ABCB1 mRNA and consequently a reduction in P-gp protein, despite functional assays demonstrating that tiopronin does not interact with P-gp. Long-term exposure of P-gp-expressing cells to **1** sensitized them to doxorubicin and paclitaxel, both P-gp substrates. Treatment of MRP1-overexpressing cells with tiopronin led to a significant reduction in MRP1 protein. Synthesis and screening of analogues of tiopronin demonstrated that the thiol functional group was essential for collateral sensitivity while substitution of the amino acid backbone altered but did not destroy specificity, pointing to future development of targeted analogues.



INTRODUCTION

Chemotherapy is the front-line treatment option available for combating metastatic and hematological malignancies. However, multidrug resistance (MDR) conferred by the ATP binding cassette (ABC) family of efflux transporters, including ABCB1 (MDR1, P-glycoprotein, P-gp) and MRP1 (ABCC1), presents a significant clinical challenge to current cancer treatment, drug design, and drug development strategies.[1] The clinical significance of expression of the drug transporter BCRP (ABCG2) in cancer has yet to be fully determined, though it has been shown to efflux known chemotherapeutics. MDR associated ABC transporters reside predominately in the plasma membrane, where they actively prevent lipophilic drugs from entering the cell so that they do not accumulate and become cytotoxic. MDR can be intrinsic or acquired during chemotherapy through an increase in transporter expression and is known to negatively affect remission rates in patients suffering from leukemia, lymphoma, and a variety of solid tumors.[2]

Strategies employed to circumvent or resolve the reduced drug accumulation conferred by these promiscuous efflux transporters include the development of inhibitors of P-gp for adjuvant administration.[3] While a number have shown promise in vitro, they have not generally improved response to chemotherapy.[3] Therefore, alternative approaches are urgently required to improve the efficacy of chemotherapeutic agents.

One such approach is to exploit resistance by identifying drugs that target MDR cells over the nonresistant parental cells from which they were selected.[4] This phenomenon, termed "collateral sensitivity" (or MDR1-selectivity),[5] has been demonstrated using compounds such as NSC73306 (**11**)[6] and verapamil, which selectively kill P-gp-expressing cells and reduce P-gp expression at subtoxic concentrations.[7] This strategy holds promise for overcoming or preventing clinical MDR. Collateral sensitivity is assessed most readily in vitro by determining the cytotoxicity (IC_{50}) of a compound against a parental line relative to its MDR subline.[8]

In exploring strategies for targeting MDR, we unexpectedly found that the simple thiol-substituted *N*-propanoyl form of glycine called tiopronin (**1**, *N*-(3-mercaptopropanoyl)glycine, Figure 1) demonstrated collateral sensitivity. **1** is the condensation product of glycine and thiolactic acid and is an orphan drug that has been used over the past 30 years to treat a diverse range of pathophysiological conditions.[9] It can exert biological activity via a number of modalities: (i) in chelation therapy, it scavenges toxic metal ions;[10] (ii) in a related manner, **1** can protect against oto- and nephrotoxicity in patients receiving platinum chemotherapy;[11] (iii) in redox coupling ($2RSH \leftrightarrow RSSR + 2H^+ + 2e^-$), **1** reduces cystine disulfide bonds and dissolves cystine stones that develop in the kidneys, ureter, and bladder of patients suffering from cystinurea;[12,13] (iv) **1** potentially neutralizes reactive oxygen species (ROS) through its thiol, acting as an anti-inflammatory for rheumatoid arthritis patients;[14] (v) it

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serves as a radioprotectant;[15] (vi) it protects against the toxicity of enediynes such as neocarzinostatin (NCS);[16] (vii) it behaves as a neuroprotective agent against cerebral ischemia by scavenging the neurotoxic aldehyde 3-amino-propanal (currently in clinical trials).[17,18]

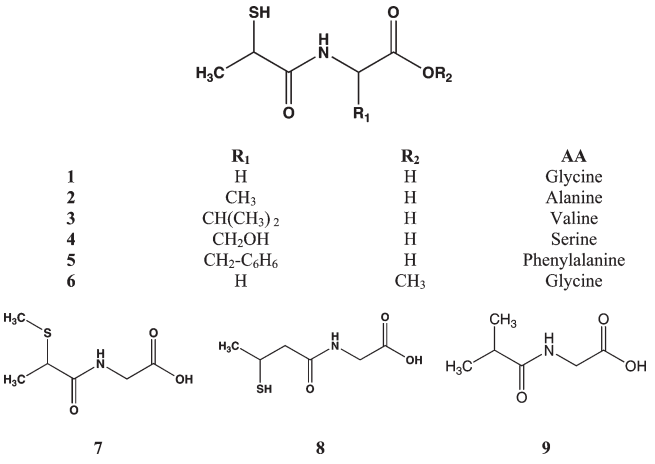

Figure 1. Chemical structures of tiopronin (**1**, [(2-mercaptopropanoyl)amino]acetic acid) and analogues. **1** is the condensation product of glycine and thiolactic acid, and a number of amino acid derivatives were generated (**2**−**6**). Structures of *S*-methyl tiopronin (**7**), *N*-(3-mercapto-2-methylpropanoyl)glycine (**8**), and (isobutyrylamino)acetic acid (**9**) are shown. All compounds were synthesized and tested as the racemate.

Here we demonstrate that (i) a series of human adenocarcinoma cell lines expressing the drug efflux pump P-gp and breast cancer cells expressing MRP1 are collaterally sensitive to **1** and (ii) the drug has a diverse range of effects on the cells at the level of mRNA and protein expression, though **1** is not a substrate of P-gp. Testing of analogues of **1** revealed that (i) removing, oxidizing, or methylating the thiol or (ii) altering the position of the thiol abrogated the activity of **1**. However, other structurally similar thiol-bearing compounds such as *N*-(3-mercapto-2-methylpropanoyl) glycine, D-penicillamine, and *N*-acetylcysteine did not demonstrate significant collateral sensitivity. We also describe the synthesis of a number of **1** analogues replacing Gly with Ser, Val, Ala, or Phe and their efficacy against P-gp-, MRP1-, and ABCG2-expressing cells.

RESULTS

The cytotoxicity of **1** (Figure 1) was assessed against parental KB-3-1 human adenocarcinoma cells and three MDR sublines expressing P-gp: KB-8-5, KB-8-5-11, and KB-V1 (Table 1).[19] The cell lines express increasing levels of ABCB1 mRNA and P-gp protein in the order KB-8-5 < KB-8-5-11 < KB-V1, resulting in greater resistance to toxic substrates and increased sensitivity to the collateral sensitivity agent **11**.[6] Collateral sensitivity (abbreviated as "RR" for resistance ratio) is calculated as the ratio of a compound's IC_{50} for parental cells divided by its IC_{50} for MDR cells. RR > 1 indicates that the compound kills MDR cells more effectively than parental cells, so-called collateral

Table 1. Determination of Cytotoxicity (IC_{50}, mM) of 1 and Collateral Sensitivity (RR) of Parental and MDR Sublines (Indented)[a]

cell line	resistance mechanism	selection	cytotoxicity (IC_{50}, mM)	RR	ref
KB-3-1			7.54 ± 0.20		19
KB-8-5	P-gp	colchicine-selected	5.68 ± 0.48	1.4	19
KB-8-5-11	P-gp	colchicine-selected	0.35 ± 0.09	33	19
KB-V1	P-gp	vinblastine-selected	0.15 ± 0.02	51	19
KB-A1	P-gp	adriamycin-selected	0.63 ± 0.10	14	30
KAS	pleiotropic	arsenite-selected	6.84 ± 1.27	1.2	31
CP20	pleiotropic	cisplatin-selected	9.61 ± 1.82	0.9	32
CHO			6.90 ± 1.30		33
C5	P-gp	colchicine-selected	8.27 ± 1.04	0.8	33
10001			17.2 ± 0.33		34
10193	β-tubulin mutant	colcemid-selected	10.11 ± 1.63	1.7	35
10576	α-tubulin mutant	paclitaxel-selected	4.84 ± 0.74	3.6	34
NIH 3T3			9.20 ± 2.12		36
G185	P-gp	*ABCB1*-transfected	4.00 ± 0.26	2.3	36
HeLa Tet-off			6.43 ± 4.45		23
HeLa MDR Tet-off	P-gp	*ABCB1*-transfected	5.14 ± 1.31	1.25	23
OVCAR8			5.99 ± 1.28		29
NCI/ADR-RES	P-gp	adriamycin-selected	9.05 ± 2.76	0.6	29
MCF-7			12.27 ± 2.34		37
VP-16	MRP1	etoposide-selected	0.29 ± 0.07	42.5	37
HEK-293 pc			0.15 ± 0.01		21
HEK-293 MRP1	MRP1	*ABCC1*-transfected	0.26 ± 0.04	0.6	21
H460			5.34 ± 0.24		38
TX50	P-gp	paclitaxel-selected	0.94 ± 0.25	5.7	28
MX20	ABCG2	mitoxantrone-selected	5.60 ± 0.32	0.95	38

[a] RR calculated as (IC_{50} parental)/(IC_{50} MDR cell line). The selection process and primary mechanism of MDR for each MDR cell line are shown.

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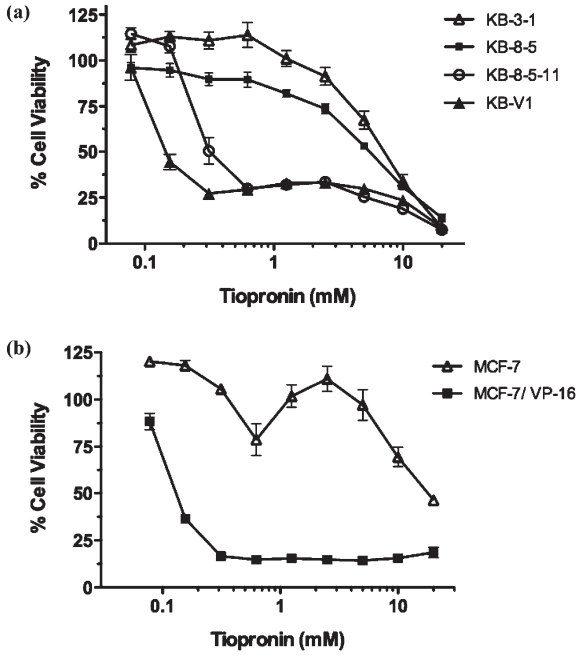

Figure 2. Collateral sensitivity of MDR cell lines to **1**. (a) Dose–response curves of **1** against the P-gp-expressing sublines KB-V1, KB-8-5-11, and KB-8-5 and their parental KB-3-1 human adenocarcinoma cell line treated with **1** for 72 h. P-gp expression is KB-V1 > KB-8-5-11 > KB-8-5, and P-gp expression corresponds with toxicity of **1**. (b) MRP1-expressing subline MCF-7/VP-16 and its parental line MCF-7. Data are the mean ± SD from three independent experiments.

sensitivity.[4] The cytotoxicity of **1** against parental KB-3-1 cells is relatively low (7.54 ± 0.2 mM), but **1** showed increasing cytotoxicity against the KB-8-5 (5.68 ± 0.48 mM, 1.4-fold collaterally sensitive, hereafter termed "selective"), KB-8-5-11 (0.349 ± 0.09 mM, 13-fold selective), and KB-V1 cells (0.147 ± 0.02 mM, 51-fold selective) (Table 1). Highly adriamycin-resistant KB-A1 cells expressing P-gp also showed sensitivity to **1** (0.633 ± 0.098 mM, 14-fold selective). Dose–response curves reveal a slight biphasic response of cells to **1**, with an initial strong response and a small population of cells surviving at higher concentrations before all cells are killed at ~10 mM (Figure 2a). This phenomenon is similar to the collateral sensitivity of P-gp-expressing Chinese hamster ovary (CHO) cells to verapamil.[20]

As a thiol, oxidation of **1** to its oxidized disulfide form (**10**, RSH → RSSR) is possible and either **10** or **1** may be the primary active species under physiological conditions. However, we determined that less than 10% of **1** was oxidized in aqueous solution after 5 days of incubation (Supporting Information Figure S1). Given that the oxidized, disulfide form of **1** (**10**) is expected to be reduced in the intracellular environment of the cell, it is unlikely that **10** is the form entering the cell or activated in the cell to yield the active form in vivo. Supporting this, oxidized **10** demonstrated reduced cytotoxicity and selectivity toward KB-V1 cells (Supporting Information Figure S2). To confirm that the collateral sensitivity was not elicited by the introduction of an initial pH gradient due to the carboxylic acid moiety of **1**, a pH gradient was introduced to cells by serial dilution of acetic acid, which did not elicit selectivity or cytotoxicity against KB cells (data not shown).

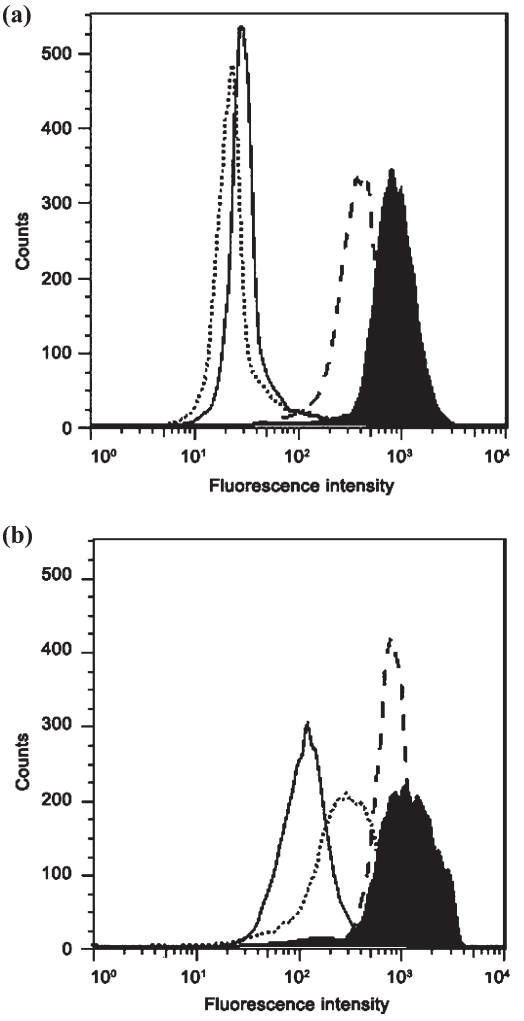

Figure 3. Effect of **1** on the transporter function of P-gp and MRP1. (a) **1** does not interfere with P-gp function. Cell lines were incubated with the P-gp substrate rhodamine 123 (4 μM) alone (solid line) or in the presence of **1** (20 mM, dotted line) or with the positive control P-gp inhibitor tariquidar (200 nM, dashed line) and compared with the fluorescence of the parental cell line KB-3-1 (black filled histogram). (b) **1** inhibits MRP1 function at high concentrations. Each cell line was incubated with the MRP1 substrate calcein-AM (0.25 μM) alone (solid line) or in the presence of **1** (20 mM, dotted line) or with the positive control MRP1 inhibitor MK-571 (50 μM, dashed line) and compared with the fluorescence of the parental cell line MCF-7 (black filled histogram).

The cytotoxicity of **1** against a series of MDR cells was determined to examine whether P-gp was necessary for mediating collateral sensitivity to **1** (Table 1). Cell lines with a range of MDR expression origins were chosen to assess activity. Relative to parental lines, **1** did not show strong selectivity toward other P-gp-expressing lines: the transfected murine cell line NIH 3T3 G185 (expressing human P-gp), adriamycin-selected NCI/ADR-RES human ovarian carcinoma cells, and colchicine-selected CHO C5 Chinese hamster ovary cells (Table 1). All showed less than 3-fold selectivity for the MDR subline, with a magnitude of cytotoxicity similar to that of parental KB cells. These data suggest that the expression of P-gp is not requisite for MDR cell sensitivity to **1**. As such, we examined MDR cell lines not expressing P-gp. The KB MDR sublines KAS (selected for resistance to arsenite)

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and CP20 (selected for resistance to cisplatin), neither of which express P-gp, did not show sensitivity to **1**. We also examined two MDR CHO cell lines (10193 and 10576) with tubulin mutations: while 10193 (colcemid-selected) did not show sensitivity, paclitaxel-selected 10576 cells showed modest collateral sensitivity (RR = 3.6) compared with the parental 10001 cell line.

A number of agents that demonstrate collateral sensitivity against MDR cells are substrates for P-gp.[4] Therefore, we examined whether **1** interacts directly with P-gp. We assessed this initially by testing whether **1** inhibited the efflux of the fluorescent substrate rhodamine 123 from P-gp-expressing KB-V1 cells (Figure 3a). At high concentrations, **1** (20 mM) had no effect on efflux, indicating that it did not act as either an inhibitor or a competitive substrate. As a positive control, we used the P-gp inhibitor tariquidar (XR9576), which was found to increase accumulation of rhodamine 123 in KB-V1 cells to levels similar to those in the parental KB-3-1 cells. Identical results were also found with the fluorescent P-gp substrate calcein-AM (Supporting Information Figure S3). We also found that **1** did not stimulate or suppress the activity of P-gp using a P-gp ATPase assay (Promega, WI, U.S.), confirming that it was not interacting as an inhibitor or substrate of P-gp (data not shown). When P-gp is inhibited, MDR cells lose sensitivity to the collateral sensitivity agent **11**, indicating that functional P-gp is required for selectivity. In contrast, inhibition of P-gp with the inhibitors cyclosporin A or tariquidar did not affect the activity of **1** against KB-V1 cells in an MTT cell toxicity assay (data not shown). These data collectively suggest that **1** does not interact directly with P-gp at its usual substrate or inhibition binding sites, and the protein does not mediate sensitivity to **1**.

To assess whether **1** could elicit collateral sensitivity in cells expressing other ABC transporters or alternatively suffer from resistance conferred by other transporters, **1** was tested against MRP1- and ABCG2-expressing cell lines (Table 1). MRP1-expressing MCF-7/VP-16 cells (IC_{50} = 0.29 ± 0.17 mM) selected for resistance to etoposide showed a 42.5-fold collateral sensitivity to **1** compared with parental MCF-7 cells (IC_{50} = 12.27 ± 2.34 mM) (Figure 2b, Table 1). MCF7 cells showed a biphasic response to **1** similar to that described above for KB cells. To assess whether this collateral sensitivity was mediated by MRP1, cytotoxicity of **1** was also assessed against HEK-293 human embryonic kidney cells transfected with *ABCC1* (IC_{50} = 0.26 ± 0.04 mM) and plasmid control (IC_{50} = 0.15 ± 0.01 mM), which showed approximately equivalent sensitivity (Table 1).[21] This indicated that MRP1 was not directly mediating collateral sensitivity, further supported by the observation that the MRP1 inhibitor MK-571 (3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]-phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl]-propionic acid) did not alter sensitivity of MRP1-expressing MCF-7/VP-16 cells (data not shown). Interestingly, while the HEK-293 cells were not collaterally sensitive, the pc line demonstrated an underlying sensitivity similar to that of P-gp- and MRP1-expressing lines.

Given that thiols and anionic compounds are common classes of MRP1 substrates,[22] we examined whether **1** interfered with MRP1 function. **1** (20 mM) partially inhibited the efflux of the MRP1 substrate calcein-AM, suggesting that **1** may be a substrate of MRP1 (Figure 3b). **1** did not show any selective activity (RR = 0.95) toward ABCG2-expressing H460 MX20 (IC_{50} = 5.6 ± 0.32 mM) cells compared with parental H460 cells (IC_{50} = 5.34 ± 0.24 mM).

As others have shown that agents inducing collateral sensitivity can down-regulate P-gp expression, we carried out qRT-PCR, Northern blotting, and Western blotting of P-gp. We used a stably transfected tetracycline-sensitive expression system (Tet-off) in order to separate the effects of **1** on the promoter regulation of ABCB1from a more direct effect on the stability of the *ABCB1* mRNA.[23] Following treatment of Tet-off cells with **1** (1 mM), RNA and protein were isolated and analyzed (Figure 4). Northern analysis using RNA prepared from cells treated for 24, 48, or 72 h demonstrated that there was a marked reduction in the amount of *ABCB1* message within 48 h but no further reduction at 72 h (Figure 4a). No specific *ABCB1* mRNA cleavage products were evident in the Northern blot, suggesting a mechanism of mRNA destabilization that affects the entire message rather than a specific and discrete cleavage site. By qRT-PCR, standardized using *PMCA4* mRNA, we determined that *ABCB1* transcript was reduced by nearly 30% at 24 h and 80% at 48 h (Figure 4b). *ABCB1* mRNA from KB-V1 cells was also down-regulated by treatment with **1** (data not shown).

We also tested the effect of **1** on P-gp protein expression levels by Western blotting. Tet-off cells were treated with 0.1, 1, or 10 mM **1** for 72 h prior to harvesting and Western blotting, and band scan densities were subsequently standardized against GAPDH. Corresponding to the reduced *ABCB1* mRNA levels, P-gp protein was reduced by 50%, 55%, and 65%, respectively, compared with control (untreated) cells (Figure 5c). Western blot analysis of MCF-7/VP16 cells treated with **1** (1 mM) also showed a substantial reduction in MRP1 protein (Figure 4d).

Given that **1** down-regulates MDR1, we assessed whether long-term treatment of KB-V1 cells with **1** (6 weeks, 5 mM) could resensitize them to conventional chemotherapeutics. The results shown in Figure 5 demonstrate that surviving KB-V1 cells treated with 5 mM **1** are 5.0- and 2.0-fold more sensitive to the P-gp substrates doxorubicin and paclitaxel, respectively. **1**-treated cells dosed with cisplatin (which is not a P-gp substrate) gave only a 1.5-fold sensitization compared with control nontreated KB-V1 cells. Measurement of cell surface P-gp expression demonstrated diminished P-gp expression of cells cultured in **1** that corresponded with the sensitization (Supporting Information Figure S4).

Given the properties of **1** against MDR cells, we sought to understand the molecular features of **1** essential for activity. We first examined *S*-methyl tiopronin (**7**, Figure 1, Table 2) in which the thiol functional group is replaced with a thioether. **7** showed no collateral sensitivity or cytotoxicity against KB-V1 cells (IC_{50} > 20 mM), suggesting that the thiol group of **1** is essential for its activity. Complete loss of activity was also found with (isobutyrylamino)acetic acid (**9**, Figure 1, Table 2), where the thiol group of **1** was replaced with a methyl group (IC_{50} > 20 mM).

Given that the thiol group is essential for activity of **1**, we examined whether collateral sensitivity observed in this study was simply the result of a general property of thiols or thiol reactivity. A number of thiol and thiol precursor compounds and disulfide compounds were tested against KB-3-1 and KB-V1 cells. The compounds stepronin, captopril, *N*-acetylcysteine, procysteine, racecadotril, D-penicillamine, thiorphan, Cys-Gly, cystamine, cystine, and glutathione (GSH) do not confer significant collateral sensitivity (Table 2). Interference of intracellular GSH levels by **1** is unlikely to account for the strong collateral sensitivity of KB-V1 cells because treatment with buthionine sulfoximine, a strong inhibitor of GSH synthesis, has no significant collateral sensitivity

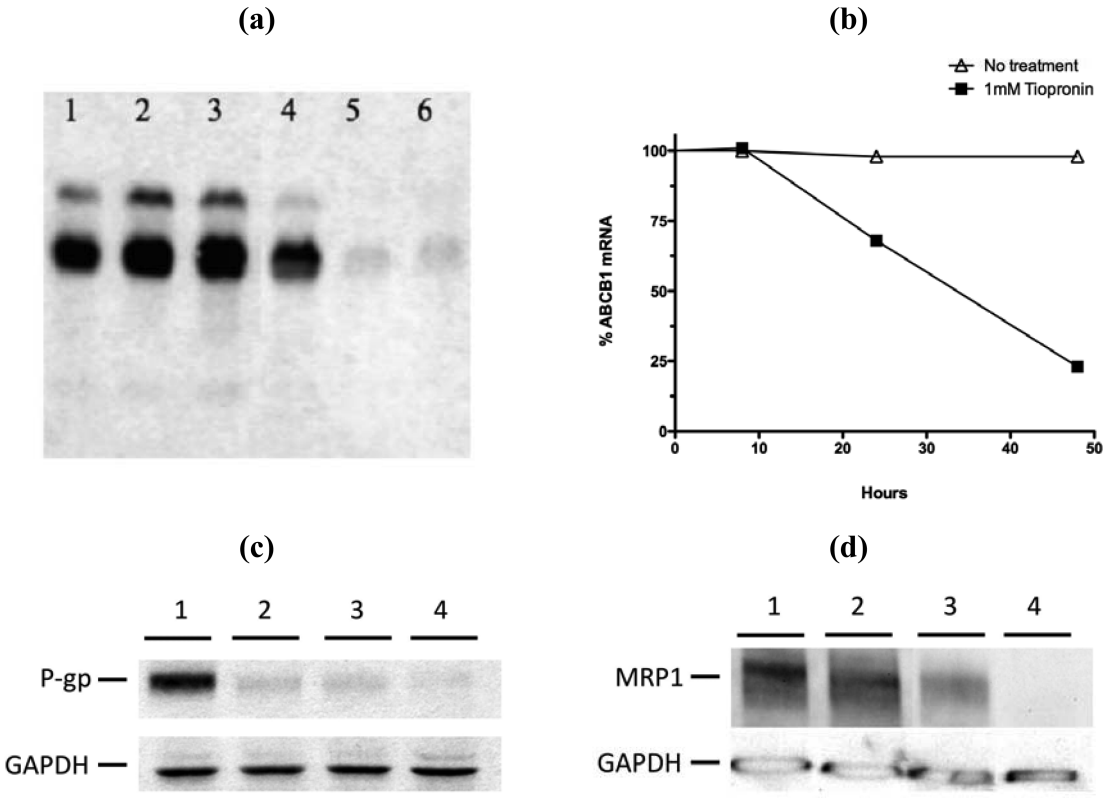

Figure 4. **1** significantly down-regulates *ABCB1* mRNA. (a) Northern blotting analysis of HeLa MDR Tet-off cells grown in the absence (lanes 1−3) or presence of 1 mM **1** (lanes 4−6) for 24 h (lanes 1 and 4), 48 h (lanes 2 and 5), or 72 h (lanes 3 and 6), prior to RNA extraction and Northern blotting with an ABCB1 cDNA biotin labeled probe. (b) Quantitative analysis of the ABCB1 mRNA extracted from HeLa MDR Tet-off cells cultured for 8, 24, or 48 h in the absence (line with triangles) or presence of 1 mM **1** (line with filled squares) prior to RNA extraction and Taqman Q-RT-PCR analysis. (c) Western blot of protein extracts from HeLa Tet-off MDR (P-gp expressing) cells treated with 0 mM **1** (lane 1), 0.1 mM (lane 2), 1 mM (lane 3), or 10 mM **1** (lane 4) for 24 h prior to harvesting and Western blot analysis with the anti-P-gp antibody C219. GAPDH control was used to calibrate loading. (d) **1** strongly down-regulates the amount of MRP1 in MCF7/VP-16 cells. Cells were treated with 0 mM (lane 1), 0.1 mM (lane 2), 1 mM (lane 3), or 10 mM (lane 4) **1** for 72 h prior to harvesting, Western blotting and analysis of the ABCC1/MRP1 protein levels with the primary antibody QCRL and a loading control anti-GAPDH antibody.

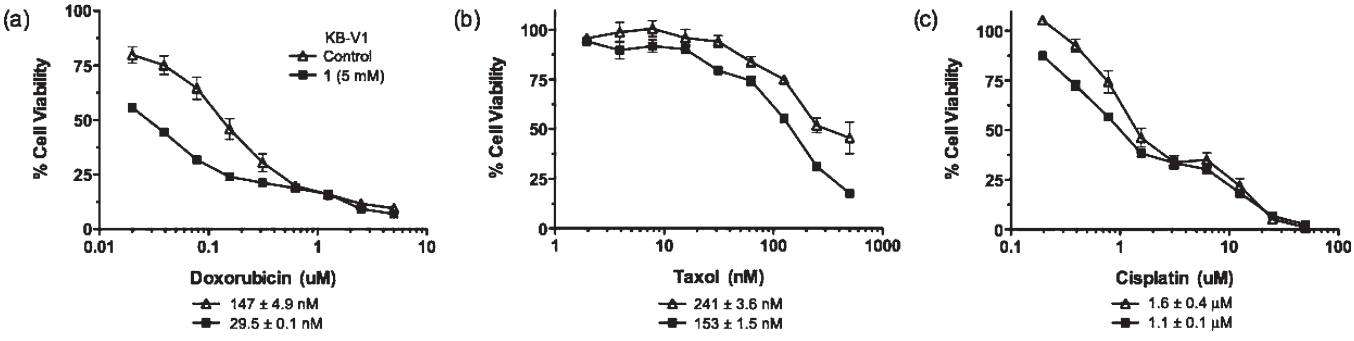

Figure 5. Long-term culture of KB-V1 cells with **1** leads to the partial reversal of the MDR phenotype and consequent resensitization of the cells to chemotherapeutics. Dose−response curves of KB-V1 cells that have previously been selected for growth in and cultured in the absence (line with triangles) or presence (line with filled squares) 5 mM **1** for 6 weeks prior to the cell viability assay with the chemotherapeutics: (a) doxorubicin, (b) paclitaxel, or (c) cisplatin. The IC_{50} values for each drug are shown below each graph.

activity either alone or in combination with **1** (data not shown). Of note is *N*-(3-mercapto-2-methylpropanoyl)glycine (**8**) which is also inactive (IC_{50} > 20 mM) against both KB-3-1 and KB-V1 cell lines despite only differing from **1** by the incorporation of a $-CH_2-$ spacer to extend the thiol group away from the amide bond (Figure 1). Neither of the two constituent components of **1**, glycine and thiolactic acid (RR = 1.05), are selective. Furthermore, agents that react with thiols or disulfides such as dithiothreitol, β-mercaptoethanol, *N*-ethylmaleimide, and DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) were not particularly selective (Table 2).

Having established that the thiol group is essential for the activity of **1**, we synthesized a number of analogues retaining the

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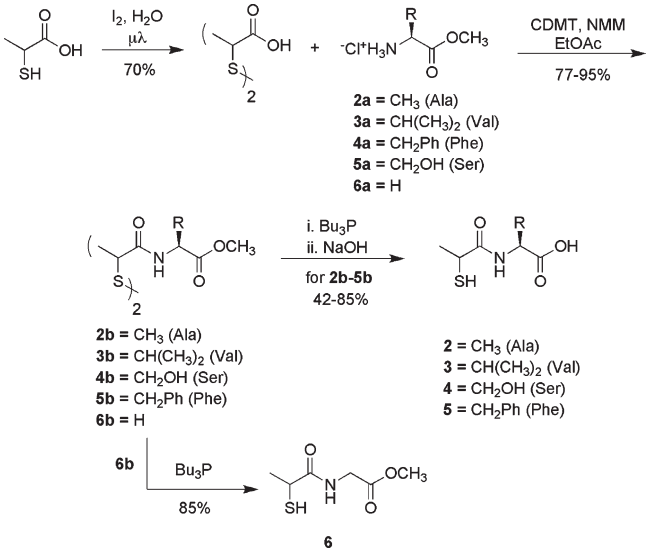
Table 2. Cytotoxicity of 1 and Analogues, and Sulfur Containing Compounds Against Parental KB-3-1 Human Adenocarcinoma Cells and the P-gp-Expressing Subline KB-V1 (IC_{50}, mM)[a]

	cytotoxicity (IC_{50}, mM)		
	KB-3-1	KB-V1	RR
1	7.54 ± 0.2	0.147 ± 0.02	51
7	>20	>20	
8	11.78 ± 0.18	9.24 ± 0.23	1.28
9	>20	>20	
glutathione	>20	>20	
thiolactic acid	3.34 ± 0.21	3.5 ± 0.4	1.05
glycine	>50	>50	
thiol compounds			
stepronin	0.86 ± 0.12	0.89 ± 0.04	0.97
captropril	15.67 ± 0.82	13.07 ± 0.51	1.2
N-acetylcysteine	29.9 ± 1.16	28.8 ± 2.11	1.04
procysteine	28 ± 3.24	29.52 ± 3.13	0.95
racecadotril	2.93 ± 0.47	1.27 ± 0.33	2.30
D-penicillamine	4.04 ± 0.37	6.93 ± 0.11	0.58
thiorphan	4.87 ± 0.32	2.84 ± 0.16	1.71
disulfide compounds			
Cys-Gly	9.42 ± 0.43	4.23 ± 0.26	2.23
cystamine	0.79 ± 0.07	1.55 ± 0.09	0.51
cystine	3.12 ± 0.49	2.14 ± 0.23	1.45
disulfide/thiol reactive			
dithiothreitol	2.37 ± 0.24	1.89 ± 0.26	1.25
N-ethyl maleimide	0.030 ± 0.0004	0.016 ± 0.0003	1.97

[a] Selectivity (RR) is calculated as the ratio of a compound's IC_{50} against parental cells divided by its IC_{50} against resistant cells. IC_{50} values are the mean ± SD from three independent experiments. Structures of the thiol compounds in this table are shown in Supporting Information Figure S6.

Scheme 1



racemic *N*-2-mercaptopropanoyl moiety but replacing the glycine backbone of **1** in order to determine whether structural variation was allowed at the amino acid side chain position (Figure 1). Analogues containing alanine (**2**), valine (**3**), serine (**4**), and phenylalanine (**5**) were prepared. The methyl ester derivative of **1** (**6**) was also synthesized to confirm that the acidity of **1** was not responsible for activity of **1** and to ascertain whether the overall negative charge of **1** was critical to activity. The synthesis of analogues **2**−**6** is shown in Scheme 1. Thiolactic acid was oxidized to 2,2′-dithiobispropionic acid in 70% yield with iodine under microwave irradiation. The bisacid was then coupled with a series of amino acid methyl ester hydrochlorides (**2a**−**6a**) via chlorodimethoxytriazine (CDMT) activation of 2,2′-dithiobispropionic acid to cleanly afford bisamide disulfides **2b**−**6b** in 77−95% yield. Disulfide reduction mediated by tributylphosphine (Bu_3P) and methyl ester saponification with 2 M sodium hydroxide (NaOH) of **2b**−**5b** was carried out in a one-pot operation to afford the target analogues **2**−**5** in 42−85% yield. Tributylphosphine mediated disulfide reduction of **6b** afforded **6**, the methyl ester analogue **1**, in 85% yield.

All analogues were tested against P-gp-expressing (KB-V1), MRP1-expressing (MCF-7/VP-16), and ABCG2-expressing (H460 MX20) cells and their respective parental cell lines (KB-3-1, MCF-7, and H460). Analogues **2**−**5** demonstrated collateral sensitivity toward P-gp- and MRP1-expressing cells (Table 3). While all analogues were active (RR > 1), there was a large variation in both the IC_{50} values and the fold-selectivity between different drug-resistant cell line pairs. Ser-tiopronin (**4**) was the most selective analogue against KB-V1 cells (RR = 74.7) but showed minimal selectivity for the MRP1-expressing VP-16 cells (RR = 1.7). ABCG2-expressing cells were not collaterally sensitive to **1** (RR = 0.95) or its analogues **2**, **3**, and **5** (RR = 1.2, 1.6, 1.56, respectively) (Table 3). Methylating the **1** carboxyl group to produce the nonacidic ester analogue **6** resulted in a significantly more cytotoxic compound (IC_{50} = 0.51 mM against KB-3-1 versus 7.54 mM for **1**) perhaps because of greater cell entry of the neutral species. Selectivity was reduced against both KB-V1 and VP-16 cells compared with **1**, and it failed to elicit collateral sensitivity (RR = 0.90) of ABCG2-expressing cells.

DISCUSSION AND CONCLUSIONS

We have shown here that while not all MDR cell lines are collaterally sensitive to **1**, its cytotoxic activity is independent of P-gp or MRP1 drug efflux transporters and may therefore be targeting a more general feature of MDR cells that has been acquired during drug selection. The collateral sensitivity shown by MDR cell lines to **1** does not require expression of either P-gp or MRP1 but does seem to require alterations associated with the development of MDR. This is in contrast to **11**, a thiosemicarbazone compound that requires expression and functionality of P-gp (transfected or selected) for its selective activity.[6] Cell lines such as KB-A1, 10576, 10193, and H460/TX50 that have been selected for resistance to a number of agents (adriamycin, paclitaxel, colcemid, and paclitaxel, respectively) were sensitized to **1**.

Thiols are known to elicit toxicity through redox cycling and generation of reactive oxygen species (ROS), and we recently hypothesized that the collateral sensitivity of MDR cells may be caused by ROS.[24] By testing **1** analogues (Figure 1, Table 1) and thiol-bearing compounds (Table 2), we determined that the thiol group is necessary for **1**'s activity, but a thiol functional group is not generally sufficient for collateral sensitivity. In fact, **1** is exquisitely sensitive to structural variation around the thiol functional group: S-methylation (**7**), replacement of the thiol

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Table 3. Cytotoxicity (IC_{50} mM) of Analogues 1−6 against Parental and Drug-Resistant Cell Line Pairs: KB-3-1 Human Adenocarcinoma Cells and the P-gp-Expressing Subline KB-V1, MCF-7 Human Breast Cancer Cells and the MRP1-Expressing Subline MCF-7/VP-16, and the Human Large Cell Lung Cancer Cell Line H460 and its ABCG2-Expressing Subline H460/MX20[a]

	cytotoxicity (IC_{50}, mM)								
	P-gp			MRP1			ABCG2		
	KB-3-1	KB-V1	RR	MCF-7	VP-16	RR	H460	MX20	RR
1	7.54 ± 0.2	0.15 ± 0.02	51.0	12.27 ± 2.34	0.29 ± 0.17	42.5	5.34 ± 0.24	5.60 ± 0.32	0.95
2	18.25 ± 2.61	0.49 ± 0.02	37.1	20.2 ± 2.58	0.51 ± 0.05	39.5	18.38 ± 0.57	15.38 ± 1.1	1.20
3	12.77 ± 3.84	0.36 ± 0.10	36.0	14.23 ± 3.22	0.55 ± 0.03	26.0	6.33 ± 0.54	3.95 ± 0.26	1.60
4	17.85 ± 0.25	0.24 ± 0.03	74.7	1.30 ± 0.32	0.76 ± 0.45	1.7	>20	>20	
5	4.96 ± 1.26	0.33 ± 0.05	15.3	2.92 ± 3.13	0.36 ± 0.10	8.0	11.32 ± 1.58	7.24 ± 0.0.13	1.56
6	0.51 ± 0.26	0.08 ± 0.02	6.2	0.22 ± 0.14	0.14 ± 0.01	1.6	0.23 ± 0.03	0.25 ± 0.03	0.90

[a] IC_{50} values are the mean ± SD from three independent experiments. Compounds are tiopronin (**1**), Ala-tiopronin (**2**), Val-tiopronin (**3**), Ser-tiopronin (**4**), Phe-tiopronin (**5**), and tiopronin methyl ester (**6**).

with a methyl group (**9**), or even insertion of a $-CH_2-$ spacer to extend the thiol group away from the amide bond (**8**) all resulted in compounds incapable of eliciting collateral sensitivity. In agreement with the importance of the thiol (RSH), oxidation of **1** (Supporting Information Figure S2) to yield the disulfide **10** led to a marked reduction in selectivity toward KB-V1 cells. **1** is known to be cell permeable,[25,26] and intracellular activity (at millimolar concentrations) has been reported for a number of modalities.[16,17,27]

In contrast, structural variation of **1** by substitution of the glycine portion of the molecule (Figure 1) for other amino acids retained selectivity, and in the case of **4** (replacement of $-H$ with $-CH_2OH$) selectivity increased (RR = 74.7) for KB-V1 cells. Interestingly, conversion of the carboxyl group in **1** to a methyl ester (**6**) considerably increased absolute cytotoxicity (perhaps because of increased hydrophobicity and hence cell penetration) but reduced its selectivity for MDR cells. This understanding points the way to further development of analogues with increased potency, replacing glycine in **1** for natural and non-natural amino acids and peptides for targeted delivery and improved efficacy. The H460/MX20 cell line expressing ABCG2 was not particularly sensitive to **1** or its analogues (**2**−**6**), which may indicate that cells resistant to mitoxantrone are not sensitized to **1**.

The HeLa MDR Tet-off cells, transfected with *ABCB1*, were not significantly more (RR = 1.25) sensitive to **1** than the control HeLa Tet-off cells. However, **1** does reduce *ABCB1* mRNA and consequently P-gp protein expression, both in MDR cells that show collateral sensitivity to **1** (KB-V1) and cells that do not (Hela MDR Tet-off), suggesting that the reduction of the *ABCB1* mRNA by **1** is not a promoter-specific interaction. We do not yet know whether **1** interacts directly with the mRNA as part of a protein complex or initiates an unknown downstream cellular mechanism (currently the subject of investigation). Reduction in transporter activity can resensitize cells to P-gp substrate chemotherapeutics such as doxorubicin and paclitaxel (Figure 5). Treatment of cells with 0.1 mM **1** or more results in a reduction of mRNA and consequently P-gp protein, potentially explaining the resensitization of MDR cells to chemotherapy by **1** but not the collateral sensitivity. **1** also reduces the amount of MRP1 protein in MCF7/VP16 cells.

1 is an orphan drug used for many years to treat patients with such diverse conditions as cystinuria, rheumatoid arthritis, and metal poisoning. While the absolute cytotoxicity of **1** (high μM against MDR cell lines) is relatively low, there is a notable lack of serious side effects experienced by patients during treatment for cystinuria with **1**, despite daily drug dosing regimens on the order of hundreds of milligrams to gram quantities per patient per day. There are a number of specific clinical examples where the local administration of drug at very high concentrations to treat MDR malignancies is possible, such as the lumen of the bladder or the peritoneal cavity. From the perspective of potential clinical utilization, **1** may offer the dual advantage of targeting the MDR cell phenotype (collateral sensitivity) at the same time as reducing the amount of drug transporter in cells. For example, by treatment of cancer cells with **1** prior to chemotherapy, the amount of P-gp or MRP1 may be reduced and thereby improve response to subsequent chemotherapy. Alternatively, co-treatment could suppress the appearance of the MDR phenotype.

Work is currently underway to understand the mechanisms behind the selectivity of **1**, and further medicinal chemistry and xenograft experiments are planned to optimize targeting of **1** to MDR cancer cells.

EXPERIMENTAL SECTION

Chemicals. Tiopronin (**1**), D-penicillamine, captopril, thiorphan, *N*-acetylcysteine (NAC), buthionine sulfoximine (BSO), cysteine−glycine dipeptide, cystamine, cystine, DTT, *N*-ethylmaleimide, procysteine, glutathione, glycine, and thiolactic acid were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). *S*-Methyl tiopronin, racecadotril and *N*-(3-mercapto-2-methylpropanoyl)glycine were purchased from Toronto Research Chemicals (Toronto, Canada). The tiopronin prodrug stepronin was purchased from Sequoia Research Products (Pangbourne, U.K.), and (isobutyrylamino)acetic acid was from Oakwood Products (West Columbia, SC, U.S.).

Synthesis. The amino acid methyl ester hydrochlorides were obtained from Bachem (Torrance, CA) and used as received unless otherwise noted. Reactions were magnetically stirred under an argon atmosphere and monitored by thin layer chromatography (TLC) with 0.25 mm Sigma-Aldrich precoated aluminum-backed silica gel plates with fluorescent indicator. TLC visualization was achieved using 254 or 360 nm UV lamp detection and/or staining with cerium molybdate (Hannesian's stain), phosphomolybdic acid (PMA), or potassium permanganate. Flash column chromatography was performed on an Ana-Logix IntelliFlash 280 system, using Biotage SNAP cartridges and SNAP Samplet cartridges with KP-Silica 60 μm. Analytical HPLC analyses were performed on an Agilent 1200 series instrument equipped with multiwavelength detectors using a

Zorbax StableBond C-18 column (4.6 mm × 50 mm, 3.5 μm) with a flow rate of 0.5 or 1.0 mL/min. Solvent A was 0.05% trifluoroacetic acid (TFA) in water (H_2O). Solvent B was 0.05% TFA in acetonitrile (ACN), and a linear gradient of 5% B to 95% B over 10 min was used. ESI or APCI mass spectrometry (MS) was performed on an LC/MSD TrapXCl Agilent Technologies instrument or on a 6130 quadrupole LC/MS Agilent Technologies instrument equipped with a diode array detector. In each case, purity of compounds was confirmed to be ≥95% by the analytical HPLC trace displaying only a single peak during analysis. Microwave irradiation was carried out in a CEM Discover synthesizer with 150 W max power. ^{1}H and ^{13}C NMR spectra were recorded on a Varian spectrometer operating at 400 and 100 MHz, respectively. Chemical shifts are reported relative to chloroform (δ 7.26), dichloromethane (δ 5.32), or deuterium oxide (δ 4.79) for ^{1}H NMR and chloroform (δ 77.0) or dichloromethane (δ 54.0) for ^{13}C NMR.

2,2′-Dithiobispropionic Acid. Iodine (5.72 g, 22.5 mmol) was added in portions to a solution of racemic thiolactic acid (2.39 g, 22.5 mmol) in water (H_2O) (12 mL). The resulting reaction mixture was heated to 100 °C under microwave irradiation for 30 min, after which TLC [1:1 hexane (Hex)/ethyl acetate (EtOAc)] showed reaction completion. The reaction was quenched by addition of a saturated aqueous solution of sodium thiosulfate ($Na_2S_2O_3$) and the mixture extracted with EtOAc twice. The combined organic layers were washed twice with $Na_2S_2O_3$ and once with brine, dried over magnesium sulfate ($MgSO_4$), and concentrated. The crude mixture was purified by recrystallization from toluene, affording **2** (1.61 g, 70% yield) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.66 (bs, 2H), 3.57 (m, 2H), 1.50 (d, J = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.8, 47.5, 46.8, 16.6. MS (m/z) = 210.1 $(M + 1)^+$.

General Procedure for the Coupling of Bisacid Disulfide ***2*** *with Amino Acid Methyl Ester Hydrochlorides* ***2a*****−*****6a***. *Synthesis of Compounds* ***2b*****−*****6b***. A mixture of the bisacid disulfide 2,2′-dithiobispropionic acid (1 equiv), the amino acid methyl ester hydrochloride **2a**−**6a** (2.05 equiv), and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) (2.05 equiv) in EtOAc was cooled to 0 °C. To this cooled mixture was added slowly a solution of *N*-methylmorpholine (NMM) (5 equiv) in EtOAc. The resulting reaction mixture was allowed to stir at 0 °C for 5 min and at room temperature for 1−2 h while being monitored by TLC.

Workup A. Reaction mixture was diluted EtOAc and H_2O. The phases were separated, and the organic layer was washed twice with 1 M HCl, once with brine, dried over $MgSO_4$, and concentrated. Crude product was purified by flash column chromatography to afford the bisamide disulfide compound.

Workup B. Reaction mixture was filtered, and the insoluble salts were rinsed with EtOAc. The filtrate was concentrated and the residue purified by flash column chromatography to afford the bisamide disulfide compound.

Bisamide Disulfide 2b. The following reagents and conditions were used: 2,2′-dithiobispropionic acid (400 mg, 1.90 mmol), alanine derivative **2a** (544 mg, 3.90 mmol), CDMT (685 mg, 3.90 mmol) in EtOAc (15 mL); NMM (962 mg, 9.51 mmol) in EtOAc (10 mL); TLC (1:1 Hex/EtOAc), 2 h. Workup A and flash column chromatography using silica gel and 75% EtOAc in hexanes afforded **2b** (714 mg, 95% yield) as a syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.16 (t, J = 9.6 Hz, 1H), 6.80 (bs, 1H), 4.65−4.58 (m, 2H), 3.76 (s, 6H), 3.63−3.52 (m, 2H), 1.54−1.41 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.3, 173.5, 173.2, 171.6, 171.5, 52.2, 49.6, 48.7, 48.5, 48.2, 18.2, 18.1, 18.0, 17.9, 17.2, 16.7. MS m/z = 381.1 $[M + H]^+$.

Bisamide Disulfide 3b. The following reagents and conditions were used: 2,2′-dithiobispropionic acid (400 mg, 1.90 mmol), valine derivative **3a** (654 mg, 3.90 mmol), CDMT (685 mg, 3.90 mmol) in EtOAc (15 mL); NMM (962 mg, 9.51 mmol) in EtOAc (10 mL); TLC (1:1 Hex/EtOAc), 2 h. Workup A and flash column chromatography using silica gel and 40% EtOAc in hexanes afforded **3b** (803 mg, 94% yield) as a syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.03 (t, J = 10.4 Hz, 1H), 6.93 (bs, 1H), 4.57−4.51 (m, 2H), 3.75 (s, 3H), 3.73 (s, 3H), 3.67−3.55 (m, 2H), 2.24−2.14 (m, 2H), 1.52−1.42 (m, 6H), 1.00−0.92 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.9, 172.6, 172.4, 172.2, 171.8(x2), 171.5, 57.6, 52.2, 49.2, 48.8, 31.1, 30.9, 19.0 (×2), 17.9 (×2), 17.8, 17.2. MS m/z = 437.2 $[M + H]^+$.

Bisamide Disulfide 4b. The following reagents and conditions were used: 2,2′-dithiobispropionic acid (350 mg, 1.66 mmol), serine derivative **4a** (531 mg, 3.41 mmol), CDMT (599 mg, 3.41 mmol) in EtOAc (15 mL); NMM (842 mg, 8.32 mmol) in EtOAc (10 mL); TLC (EtOAc), 1 h. Workup B and flash column chromatography using silica gel and EtOAc afforded **4b** (501 mg, 73% yield) as a waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43−7.34 (m, 2H), 4.74−4.63 (m, 2H), 3.87−3.81 (m, 4H), 3.80 (s, 3H), 3.78 (s, 3H), 3.72−3.67 (m, 2H), 1.46−1.41 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.4, 172.3, 171.8, 170.9(x2), 63.3, 62.5, 62.4, 56.1, 55.1 (×2), 52.8, 17.4, 16.7, 16.4. MS m/z = 437.3 $[M + Na]^+$.

Bisamide Disulfide 5b. The following reagents and conditions were used: 2,2′-dithiobispropionic acid (400 mg, 1.90 mmol), phenylalanine derivative **5a** (841 mg, 3.90 mmol), CDMT (685 mg, 3.90 mmol) in EtOAc (15 mL); NMM (962 mg, 9.51 mmol) in EtOAc (10 mL); TLC (EtOAc), 1 h. Workup A and flash column chromatography using silica gel and 35% EtOAc in hexanes afforded **5b** (945 mg, 94% yield) as a syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.31−7.12 (m, 10H), 6.98 (bs, 1H), 6.64 (bs, 1H), 4.93−4.82 (m, 2H), 3.74−3.70 (m, 6H), 3.56−3.43 (m, 2H), 3.19−2.99 (m, 4H), 1.41−1.37 (m, 3H), 1.33−1.23 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.9, 171.6, 171.4, 129.3, 129.2, 128.5 (×2), 127.1, 53.6, 53.4, 52.4, 50.1, 49.4, 48.8, 47.7, 37.9, 37.7 (×2), 37.6, 17.3, 16.7 (×2). MS m/z = 533.1 $[M + H]^+$.

Bisamide Disulfide 6b. The following reagents and conditions were used: 2,2′-dithiobispropionic acid (300 mg, 1.43 mmol), glycine derivative **6a** (367 mg, 2.92 mmol), CDMT (513 mg, 2.92 mmol) in EtOAc (10 mL); NMM (722 mg, 7.13 mmol) in EtOAc (10 mL); TLC (3:1 EtOAc/Hex), 2 h. Workup B and flash column chromatography using silica gel and 75% EtOAc in hexanes afforded **6b** (385 mg, 77% yield) as a syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.16 (bs, 2H), 4.23 (dd, J = 18 Hz, 6.0 Hz, 1H), 4.10 (d, J = 5.6 Hz, 1H), 3.77 (s, 3H), 3.76 (s, 3H), 3.67 (q, J = 7.2 Hz, 1H), 3.61 (q, J = 7.2 Hz, 1H), 1.49 (d, J = 7.2 Hz, 3H), 1.45 (d, J = 7.2 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.4, 172.2, 171.0 (×2), 52.5, 49.6, 47.8, 41.4, 17.2, 16.7. MS m/z = 353.1 $[M + H]^+$.

Synthesis of Analogues 2−5. A solution of the racemic bisamide disulfide compounds **2b**−**5b** (1 equiv) in 20% H_2O in tetrahydrofuran (THF) (v/v) was thoroughly degassed by bubbling argon through the solution for 5−10 min. Tributylphosphine (Bu_3P) (3.5 equiv) was added slowly and the resulting reaction mixture allowed to stir at room temperature for 5 min and monitored by TLC. The reaction mixture was diluted with ethanol (EtOH), 2 M sodium hydroxide (NaOH) added, and stirring continued for 1 h.

Workup A. The reaction mixture was diluted with EtOH and concentrated under reduced pressure. The residue was taken up in H_2O, extracted twice with EtOAc, and the organic layers were discarded. The aqueous layer was acidified with 1 M HCl and extracted twice with EtOAc. The combined organic layers were dried over $MgSO_4$ and concentrated to afford the **1** analogue.

Workup B. The reaction mixture was diluted with EtOH and concentrated under reduced pressure. The residue was taken up in H_2O, extracted twice with EtOAc, and the organic layers were discarded. The aqueous layer was acidified with 1 M HCl and concentrated under reduced pressure. The solid residue was extracted with EtOH, and the insoluble salts were filtered. The filtrate was concentrated to afford the **1** analogue.

Ala-tiopronin (2). The following reagents and conditions were used: bisamide disulfide **2b** (700 mg, 1.84 mmol), 20% H_2O in THF

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(v/v, 10 mL), Bu_3P (1.30 g, 6.44 mmol); TLC (1:1 Hex/EtOAc), 5 min; EtOH (4 mL), 2 M NaOH (5 mL), 1 h. Workup A was used to afford Ala-tiopronin analogue **2** (271 mg, 42% yield) as a fluffy solid. ^{1}H NMR (400 MHz, D_2O): δ 4.35 (q, J = 7.2 Hz, 1H), 3.67−3.58 (m, 1H), 1.49−1.42 (m, 6H). ^{13}C NMR (100 MHz, D_2O): δ 176.4 (×2), 48.8, 36.2, 20.4, 15.9. MS m/z = 178.1 $[M + H]^+$.

Val-tiopronin (3). The following reagents and conditions were used: bisamide disulfide **3b** (800 mg, 1.83 mmol), 20% H_2O in THF (v/v, 10 mL), Bu_3P (1.30 g, 6.44 mmol); TLC (1:1 Hex/EtOAc), 5 min; EtOH (5 mL), 2 M NaOH (5 mL), 1 h. Workup A was used to afford Val-tiopronin analogue **3** (577 mg, 77% yield) as a white solid. ^{1}H NMR (400 MHz, CD_2Cl_2): δ 6.86 (d, J = 8.0 Hz, 1H), 4.45 (td, J = 8.4, 4.8 Hz, 1H), 3.52−3.47 (m, 1H), 2.26−2.20 (m, 1H), 2.18−2.14 (m, 1H), 1.52 (d, J = 7.2 Hz, 3H), 0.97−0.94 (m, 6H). ^{13}C NMR (100 MHz, CD_2Cl_2): δ 174.4, 173.4, 57.3, 37.9, 30.8, 21.8, 18.8, 17.3. MS m/z = 206.1 $[M + H]^+$.

Ser-tiopronin (4). The following reagents and conditions were used: bisamide disulfide **4b** (500 mg, 1.21 mmol), 20% H_2O in THF (v/v, 8 mL), Bu_3P (858 mg, 4.24 mmol); TLC (EtOAc), 5 min; EtOH (3 mL), 2 M NaOH (3 mL), 1 h. Workup B was used to afford Ser-tiopronin analogue **4** (305 mg, 65% yield) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 4.58−4.52 (m, 1H), 4.04−3.97 (m, 1H), 3.96−3.91 (m, 1H), 3.72−3.67 (m, 1H), 1.51−1.49 (m, 3H). ^{13}C NMR (100 MHz, D_2O): δ 176.7, 173.3, 60.8, 54.9, 36.4, 20.5. MS m/z = 194.1 $[M + H]^+$.

Phe-tiopronin (5). The following reagents and conditions were used: bisamide disulfide **5b** (460 mg, 0.863 mmol), 20% H_2O in THF (v/v, 6 mL), Bu_3P (611 mg, 3.02 mmol); TLC (1:1 Hex/EtOAc), 10 min; EtOH (2 mL), 2 M NaOH (2 mL), 1 h. Workup A was used to afford Phe-tiopronin analogue **5** (371 mg, 85% yield) as a thick syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (bs, 1H), 7.31−7.23 (m, 3H), 7.18−7.14 (m, 2H), 6.93−6.86 (m, 1H), 4.86−4.79 (m, 1H), 3.43−3.39 (m, 1H), 3.26 (ddd, J = 14.4, 5.6, 2.8 Hz, 1H), 3.12 (dd, J = 14.0, 6.4 Hz, 1H), 1.96 (d, J = 8.0 Hz, 1H), 1.46 (d, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.6, 173.3, 135.5, 129.4, 128.6, 127.3 (×2), 53.3, 37.9, 37.2, 21.8 (×2). MS m/z = 254.1 $[M + H]^+$.

Tiopronin-Me Ester (6). A solution of bisamide disulfide **6b** (380 mg, 1.08 mmol) in 20% H_2O in THF (v/v, 10 mL) was thoroughly degassed by bubbling argon through the solution for 10 min. Bu_3P (763 mg, 3.77 mmol) was added slowly and the resulting reaction mixture stirred for 10 min under argon. TLC (EtOAc) showed complete reduction. Reaction mixture was concentrated under reduced pressure, and the residue was subjected to flash column chromatography using silica gel and 1:1 Hex/EtOAc to afford the tiopronin-Me ester analogue **6** (332 mg, 85% yield) as a thick syrup. ^{1}H NMR (400 MHz, D_2O): δ 4.05 (s, 1H), 3.79 (s, 3H), 3.68 (q, J = 7.2 Hz, 1H), 1.51 (d, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, D_2O): δ 177.2, 171.9, 52.7, 41.3, 36.4, 20.6. MS m/z = 178.1 $[M + H]^+$.

Cell Lines. The cell lines used were the following: the human cervical epithelial adenocarcinoma cell line KB-3-1 (a HeLa derivative) and its P-gp-expressing MDR sublines KB-A1, KB-V1, KB-8-5-11, and KB-8-5; the human breast cancer cell line MCF-7 and its MRP1-expressing MDR subline MCF-7/VP16; the human lung carcinoma cell line H460 and its ABCG2-expressing MDR subline H460/MX20 and P-gp-expressing variant H460/TX50; NIH-3T3 murine fibroblast cells and its mutant human P-gp-expressing variant NIH-3T3 G185; OVCAR8 human ovarian carcinoma cells and its P-gp-expressing variant NCI/ADR-RES. All cell lines were grown at 37 °C in 5% CO_2 and cultured as follows. The KB, NIH-3T3, and MCF-7 lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 5 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin, all obtained from Life Technologies (Carlsbad, CA, U.S.). The H460 lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium from Life Technologies (Carlsbad, CA, U.S.) and supplemented as described above. Resistant cell lines were additionally cultured in the following cytotoxic drugs to maintain transporter expression: KB-8-5, colchicine (10 ng/mL); KB-8-5-11, colchicine (100 ng/mL); KB-V1, vinblastine (1 μg/mL); KB-A1, adriamycin (1 μg/mL); NIH-3T3 G185, colchicine (60 ng/mL); H460/TX50, paclitaxel (50 ng/mL); MCF-7/VP16, etoposide (4 μM); H460/MX20, mitoxantrone (20 nM).[19,28] The parental Clontech Hela "Tet-off" and the HeLa "MDR Tet-off" (American Type Culture Collection, Manassas, VA, U.S.) derived cell lines were grown in high glucose (DMEM) medium supplemented with 10% tetracycline free fetal bovine serum and 5 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin from Life Technologies (Carlsbad, CA, U.S.). The medium of the HeLa MDR Tet-off cell line was additionally supplemented with colchicine (20 ng/mL) to maintain P-gp expression.

MTT Cytotoxicity Assay. Cytotoxicity was measured with a colorimetric viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Molecular Probes, Eugene, OR) as previously described.[29] Cells (5000 cells per well of a 96-well plate) were allowed to attach for 24 h. Stock solutions of compounds (3 M) were prepared in H_2O and then 2-fold serially diluted in medium to give a range of final tissue culture concentrations of 20 mM to 78 μM. After 72 h, cell viability was examined. Cytotoxicity (IC_{50}) was defined as the drug concentration that reduced cell viability to 50% of the untreated control. Resistance ratios (RR) are also reported for each cell line pair, determined by dividing the IC_{50} of the parental cell line by that of the transporter-expressing cell line. RR > 1 indicates that the MDR cell population is collaterally sensitive to the tested drug, while RR < 1 indicates that the MDR cells are resistant to the drug relative to the parental cell line.[4]

ABCB1 and ABCC1 mRNA Analysis. 1×10^6 MDR Tet-off cells were seeded for 24 h before treating (in the absence of doxycycline) either with or without **1** (1 mM) for 8, 24, or 48 h. Cells were then trypsinized, rinsed with PBS three times, and the total RNA was purified using an RNeasy mini kit according to the manufacturer's instructions (Qiagen, Germantown, MD, U.S.). First strand cDNA was prepared from 1 μg of total RNA using the high capacity cDNA reverse transcription kit (Applied Biosciences, Foster City, CA, U.S.) followed by PCR analysis using either ABCB1 (Hs00184491_m1) or the *PMCA4* control (Hs00608058_m1) Taqman probe sets from TaqMan Universal PCR Master Mix and loaded on an ABI Prism 7900 HT sequence detection system according to the manufacturer's instructions (Applied Biosciences, Foster City, CA, U.S.). The percentage of ABCB1 mRNA remaining was calculated from the PCR crossing point threshold (Ct) values and the total amount of RNA adjusted using the RNA control.

Northern Blotting. The pTM1 plasmid containing the ABCB1 cDNA was digested with XhoI and NcoI restriction enzymes, and following agarose gel electrophoresis, the bands were purified using a QIAquick gel extraction kit (Qiagen, Germantown, MD, U.S.). The cDNA was biotinylated using the Brightstar psoralen-biotin kit (Ambion, Austin, TX, U.S.). An amount of 2 μg of total RNA from MDR Tet-off cells was resolved on a nondenaturing 1% TAE agarose gel. Equal loading of RNA in the gel was confirmed by ethidium bromide staining showing 18S and 28S rRNA bands (Supporting Information Figure S5). Northern blots were performed using NorthernMax membranes and detection was performed with BrightStar BioDetect according to the manufacturer's instructions (Ambion, Austin, TX, U.S.).

Western Blot Analysis. The expression of ABC transporters in each cell line was visualized by Western blot analysis. Protein samples were prepared and run on a gel as described by Brimacombe et al.[29] In brief, lysed cells were incubated in sodium dodecyl sulfate (5×) buffer, loaded onto a 3−8% NuPAGE Novex Tris-acetate gel (Invitrogen Corp., Carlsbad, CA, U.S.), and transferred to nitrocellulose membranes. Dry blots were blocked in 20% milk for 30 min at 21 °C. Blots were then probed for expression of P-gp, MRP1, and ABCG2 protein using the three

primary antibodies C219 (1:10,000), QCLR (1:5,000), and BXP-21 (1:10,000), respectively, for 60 min at 21 °C, washed 3 × 10 min, immunoprobed with the secondary antibody ImmunoPure goat anti-mouse IgG peroxidase-conjugated (GAMP, 1:10000, Pierce Biotechnology, Rockford, IL, U.S.) for 60 min at 21 °C, and washed again. Each blot was immunoprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Ambion, Austin, TX, U.S.) as a loading control.

Resensitization Assay. KB-V1 cells were grown in **1** at 0.1 mM and then 1 mM and finally 5 mM over 6 weeks. In parallel, control KB-V1 cells were grown for the same period of time in the absence of selecting agent (vinblastine). Cells were then tested for rensensitization to doxorubicin, paclitaxel, or cisplatin using a standard MTT cell viability assay.

Inhibition of Transporter Function. Since transporter substrates (at high concentrations) can inhibit ABC transporter function as competitive substrates, we determined whether **1** could inhibit the function of P-gp. We measured inhibitory activity by the uptake of rhodamine 123 as described previously.[29] For each condition, 2×10^5 cells were suspended in 1 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal bovine serum. The cells were first pretreated with a P-gp inhibitor (positive control, 200 nM tariquidar), **1** (20 mM), or medium (negative control) for 10 min in a 37 °C water bath. Cells were isolated by centrifugation, resuspended in 1 mL of IMDM, and incubated with rhodamine 123 (4 μM). Cells were then incubated in the dark for 45 min in a 37 °C water bath, centrifuged, resuspended in 300 μL of 0.1% bovine serum albumin in 1× PBS, and kept on ice until analysis. For each cell treatment fluorescence intensity (cellular uptake of fluorescent substrate) was recorded for a total of 10 000 cells using a FACS Calibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, U.S.). FACS data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, U.S.).

■ ASSOCIATED CONTENT

Supporting Information. Assessment of **10** (Figures S1 and S2), calcein-AM P-gp efflux assay supporting the Rh123 data presented in Figure 3a (Figure S3), evidence for down-regulation of P-gp in long-term culture with **1** (Figure S4), ethidium bromide loading control for Figure 5a (Figure S5), and structures of compounds described in Table 2 (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ABC, ATP-binding cassette; GSH, glutathione; MDR, multidrug resistance; RR, resistance ratio

■ REFERENCES

(1) Gottesman, M. M.; Fojo, T.; Bates, S. E. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat. Rev. Cancer* **2002**, *2*, 48–58.

(2) Gottesman, M. M. Mechanisms of cancer drug resistance. *Annu. Rev. Med.* **2002**, *53*, 615–627.

(3) McHugh, K.; Callaghan, R. Clinical Trials on MDR Reversal Agents. In *Multidrug Resistance: Biological and Pharmaceutical Advances in Antitumour Treatment*; Colabufo, N. A., Ed.; Research Signpost: Kerala, India, 2008; pp 321−353.

(4) Hall, M. D.; Handley, M. D.; Gottesman, M. M. Is resistance useless? Multidrug resistance and collateral sensitivity. *Trends Pharmacol. Sci.* **2009**, *30*, 546–556.

(5) Szybalski, W.; Bryson, V. Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of *Escherichia coli* to fifteen antibiotics. *J. Bacteriol.* **1952**, *64*, 489–499.

(6) Ludwig, J. A.; Szakacs, G.; Martin, S. E.; Chu, B. F.; Cardarelli, C.; Sauna, Z. E.; Caplen, N. J.; Fales, H. M.; Ambudkar, S. V.; Weinstein, J. N.; Gottesman, M. M. Selective toxicity of NSC73306 in MDR1-positive cells as a new strategy to circumvent multidrug resistance in cancer. *Cancer Res.* **2006**, *66*, 4808–4815.

(7) Muller, C.; Bailly, J. D.; Goubin, F.; Laredo, J.; Jaffrezou, J. P.; Bordier, C.; Laurent, G. Verapamil decreases P-glycoprotein expression in multidrug-resistant human leukemic cell lines. *Int. J. Cancer* **1994**, *56*, 749–754.

(8) Hall, M. D.; Salam, N. K.; Hellawell, J. L.; Fales, H. M.; Kensler, C. B.; Ludwig, J. A.; Szakacs, G.; Hibbs, D. E.; Gottesman, M. M. Synthesis, activity, and pharmacophore development for isatin-beta-thiosemicarbazones with selective activity toward multidrug-resistant cells. *J. Med. Chem.* **2009**, *52*, 3191–3204.

(9) de Angelis, L. Tiopronin. *Drugs Today* **1978**, *14*, 37–40.

(10) Fujimoto, T.; Fuyuta, M.; Kiyofuji, E.; Hirata, S. Prevention by tiopronin (2-mercaptopropionyl glycine) of methylmercuric chloride-induced teratogenic and fetotoxic effects in mice. *Teratology* **1979**, *20*, 297–301.

(11) Fetoni, A. R.; Quaranta, N.; Marchese, R.; Cadoni, G.; Paludetti, G.; Sergi, B. The protective role of tiopronin in cisplatin ototoxicity in Wistar rats. *Int. J. Audiol.* **2004**, *43*, 465–470.

(12) Chow, G. K.; Streem, S. B. Medical treatment of cystinuria: results of contemporary clinical practice. *J. Urol.* **1996**, *156*, 1576–1578.

(13) Remien, A.; Kallistratos, G.; Burchardt, P. Treatment of cystinuria with thiola (alpha-mercaptopropionyl glycine). *Eur. Urol.* **1975**, *1*, 227–228.

(14) Amor, B.; Mery, C.; de Gery, A. Tiopronin (*N*-[2-mercaptopropionyl] glycin) in rheumatoid arthritis. *Arthritis Rheum.* **1982**, *25*, 698–703.

(15) Devi, P. U. Protection of mouse intestine against gamma irradiation by 2-mercaptopriopionylglycine (MPG). *J. Radiat. Res. (Tokyo)* **1977**, *18*, 160–163.

(16) Oda, T.; Yamamoto, H.; Miki, T.; Maeda, H. Differential neutralizing effect of tiopronin on the toxicity of neocarzinostatin and SMANCS: a new rescue cancer chemotherapy. *Jpn. J. Cancer Res.* **1989**, *80*, 394–399.

(17) Ivanova, S.; Batliwalla, F.; Mocco, J.; Kiss, S.; Huang, J.; Mack, W.; Coon, A.; Eaton, J. W.; Al-Abed, Y.; Gregersen, P. K.; Shohami, E.; Connolly, E. S., Jr.; Tracey, K. J. Neuroprotection in cerebral ischemia by neutralization of 3-aminopropanal. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5579–5584.

(18) Kim, G. H.; Kellner, C. P.; Hickman, Z. L.; Zacharia, B. E.; Starke, R. M.; Hwang, B. Y.; Ducruet, A. F.; Fernandez, L.; Mayer, S. A.; Tracey, K. J.; Connolly, E. S., Jr. A phase I clinical trial of tiopronin, a putative neuroprotective agent, in aneurysmal subarachnoid hemorrhage. *Neurosurgery* **2010**, *67*, 182−185; discussion 186.

(19) Shen, D. W.; Cardarelli, C.; Hwang, J.; Cornwell, M.; Richert, N.; Ishii, S.; Pastan, I.; Gottesman, M. M. Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show

changes in expression of specific proteins. *J. Biol. Chem.* **1986**, *261*, 7762–7770.

(20) Warr, J. R.; Brewer, F.; Anderson, M.; Fergusson, J. Verapamil hypersensitivity of vincristine resistant Chinese hamster ovary cell lines. *Cell Biol. Int. Rep.* **1986**, *10*, 389–399.

(21) Robey, R. W.; Lin, B.; Qiu, J.; Chan, L. L.; Bates, S. E. Rapid detection of ABC transporter interaction: potential utility in pharmacology. *J. Pharmacol. Toxicol. Methods* **2011**, *63*, 217–222.

(22) Cole, S. P.; Deeley, R. G. Transport of glutathione and glutathione conjugates by MRP1. *Trends Pharmacol. Sci.* **2006**, *27*, 438–446.

(23) Aleman, C.; Annereau, J. P.; Liang, X. J.; Cardarelli, C. O.; Taylor, B.; Yin, J. J.; Aszalos, A.; Gottesman, M. M. P-Glycoprotein, expressed in multidrug resistant cells, is not responsible for alterations in membrane fluidity or membrane potential. *Cancer Res.* **2003**, *63*, 3084–3091.

(24) Munday, R. Toxicity of thiols and disulphides: involvement of free-radical species. *Free Radical Biol. Med.* **1989**, *7*, 659–673.

(25) Till, U.; Bergmann, I.; Breddin, K.; Heptinstall, S.; Losche, W.; Mazurov, A.; Pescarmona, G. Sulfhydryl/Disulfide-Status of Blood Platelets: A Target for Pharmacological Intervention? In *Prostaglandins in Clinical Research: Cardiovascular System*; Schror, K., Sinzinger, H., Eds.; Alan R. Liss, Inc.: New York, NY, 1989; pp 341−345.

(26) Toshioka, N.; Mita, I.; Chiba, T. Absorption, Distribution, Metabolism and Excretion of 35S-Thiola in Rats. In *Proceedings of International Symposium on Thiola*; Santen Pharmaceutical Co., Ltd.: Osaka, Japan, 1970; pp 1−8.

(27) Schunck, C.; Miura, K. F.; Obe, G. Thiopronin reduces the frequencies of neocarzinostatin-induced chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary cells. *Mutat. Res.* **1998**, *412*, 207–212.

(28) Huff, L. M.; Lee, J. S.; Robey, R. W.; Fojo, T. Characterization of gene rearrangements leading to activation of MDR-1. *J. Biol. Chem.* **2006**, *281*, 36501–36509.

(29) Brimacombe, K. R.; Hall, M. D.; Auld, D. S.; Inglese, J.; Austin, C. P.; Gottesman, M. M.; Fung, K. L. A dual-fluorescence high-throughput cell line system for probing multidrug resistance. *Assay Drug Dev. Technol.* **2009**, *7*, 233–249.

(30) Akiyama, S.; Fojo, A.; Hanover, J. A.; Pastan, I.; Gottesman, M. M. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somat. Cell Mol. Genet.* **1985**, *11*, 117–126.

(31) Tachiwada, T.; Chen, Z. S.; Che, X. F.; Matsumoto, M.; Haraguchi, M.; Gotanda, T.; Sumizawa, T.; Furukawa, T.; Nishiyama, K.; Seki, N.; Yamamoto, M.; Nakagawa, M.; Akiyama, S. Isolation and characterization of arsenite-resistant human epidermoid carcinoma KB cells. *Oncol. Rep.* **2007**, *18*, 721–727.

(32) Shen, D. W.; Akiyama, S.; Schoenlein, P.; Pastan, I.; Gottesman, M. M. Characterisation of high-level cisplatin-resistant cell lines established from a human hepatoma cell line and human KB adenocarcinoma cells: cross-resistance and protein changes. *Br. J. Cancer* **1995**, *71*, 676–683.

(33) Callaghan, R.; van Gorkom, L. C.; Epand, R. M. A comparison of membrane properties and composition between cell lines selected and transfected for multi-drug resistance. *Br. J. Cancer* **1992**, *66*, 781–786.

(34) Cabral, F.; Abraham, I.; Gottesman, M. M. Isolation of a Taxol-resistant Chinese hamster ovary cell mutant that has an alteration in alpha-tubulin. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4388–4391.

(35) Cabral, F.; Sobel, M. E.; Gottesman, M. M. CHO mutants resistant to colchicine, colcemid or griseofulvin have an altered beta-tubulin. *Cell* **1980**, *20*, 29–36.

(36) Cardarelli, C. O.; Aksentijevich, I.; Pastan, I.; Gottesman, M. M. Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. *Cancer Res.* **1995**, *55*, 1086–1091.

(37) Schneider, E.; Horton, J. K.; Yang, C. H.; Nakagawa, M.; Cowan, K. H. Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance. *Cancer Res.* **1994**, *54*, 152–158.

(38) Henrich, C. J.; Robey, R. W.; Bokesch, H. R.; Bates, S. E.; Shukla, S.; Ambudkar, S. V.; Dean, M.; McMahon, J. B. New inhibitors of ABCG2 identified by high-throughput screening. *Mol. Cancer Ther.* **2007**, *6*, 3271–3278.