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

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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 taxol, both P-gp substrates. Treatment of MRP1-overexpressing cells with tiopronin led to a significant reduction in MRP1 protein. Synthesis and screening of analogs 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 analogs.

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. 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 affects remission rates in patients suffering from leukemia, lymphoma, and a variety of solid tumors. ²

Supporting information. Supporting information is included as described in the text above, including assessment of **10** (Supporting Figures 1 and 2), calcein-AM P-gp efflux assay supporting the Rh123 data presented in Figure 3a, evidence for down-regulation of P-gp in long term culture with **1** (Supporting Figure S4), ethidium bromide loading control for Figure 5a (Supporting Figure S5), and structures of compounds described in Table 2 (Supporting Figure 6). This material is available free of charge via the Internet at http://pubs.acs.org.

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 non-resistant parental cells from which they were selected. This phenomenon, termed 'collateral sensitivity' (or MDR1-selectivity), has been demonstrated using compounds such as NSC73306 (11) and verapamil, which selectively kill P-gp-expressing cells and reduce P-gp expression at subtoxic concentrations. This strategy holds promise for overcoming or preventing clinical MDR. Collateral sensitivity is assessed most readily *in vitro* by determining the cytotoxicity (IC₅₀) of a compound against a parental line relative to its MDR subline.

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. 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 \longleftrightarrow 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) as a radioprotectant, 15 (vi) it protects against the toxicity of enediynes such as neocarzinostatin (NCS), 16 and (vii) it behaves as a neuroprotective agent against cerebral ischemia by scavenging the neurotoxic aldehyde 3-aminopropanal (currently in clinical trials). 17 , 18

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 analogs 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*-acetyl cysteine did not demonstrate significant collateral sensitivity. We also describe the synthesis of a number of 1 analogs 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 sub-lines 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 IC₅₀ for parental cells divided by its IC₅₀ for MDR cells. An RR value > 1 indicates that the compound kills MDR cells more effectively than parental cells - so-called collateral sensitivity. ⁴ The cytotoxicity of **1** against parental KB-3-1 cells is relatively low

 $(7.54\pm0.2~\text{mM})$, but it showed increasing cytotoxicity against the KB-8-5 ($5.68\pm0.48~\text{mM}$, 1.4-fold collaterally sensitive, hereafter termed 'selective'), KB-8-5-11 ($0.349\pm0.09~\text{mM}$, 13-fold selective) and KB-V1 cells ($0.147\pm0.02~\text{mM}$, 51-fold selective) (Table 1). Highly adriamycin-resistant KB-A1 cells expressing P-gp also showed sensitivity to 1 ($0.633\pm0.098~\text{mM}$, 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 \rightarrow 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 five days of incubation (Supporting 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 towards KB-V1 cells (Supporting 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).

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 towards 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) 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, taxol-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. 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 Figure S3). We also found that 1 did not stimulate or suppress the activity of P-gp using a P-gp ATPase assay (Promega, Wisconsin, USA), confirming 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 (IC₅₀ = 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 (IC₅₀ = 12.27 \pm 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 (IC₅₀ = 0.26 ± 0.04 mM) and plasmid control (IC₅₀ = 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]-(2dimethylcarbamoylethylsulfanyl)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) towards ABCG2-expressing H460 MX20 (IC $_{50}$ = 5.6 \pm 0.32 mM) cells compared with parental H460 cells (IC $_{50}$ = 5.34 \pm 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 *ABCB1* from 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 hours demonstrated that there was a marked reduction in the amount of *ABCB1* message within 48 hours, but no further reduction at 72 hours (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 hours 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 taxol respectively. Tiopronin-treated cells dosed with cisplatin (which is not a P-gp substrate) gave only a 1.5-fold sensitization compared with control non-treated 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 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 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 as well as disulfide compounds were tested against KB-3-1 and KB-V1 cells. The compounds stepronin, captopril, N-acetyl cysteine, pro-cysteine, 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 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 (IC₅₀ > 20 mM) against both KB-3-1 and KB-V1 cell lines despite only differing from 1 by the incorporation of a -CH₂-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-(2nitrobenzoic 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 analogs retaining the 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). Analogs 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 analogs 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 (Bu₃P) and methyl ester saponification with 2M sodium hydroxide (NaOH) of 2b-5b was carried out in a one-pot operation to afford the target analogs 2-5 in 42-85% yield. Tributylphosphine mediated disulfide reduction of 6b afforded 6, the methyl ester analog 1 in 85% yield.

All analogs 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). Analogs **2-5** demonstrated collateral sensitivity towards P-gp-and MRP1-expressing cells (Table 3). While all analogs were active (RR > 1), there was a large variation in both the IC $_{50}$ values as well as the fold-selectivity between different drugresistant cell line pairs. Ser-tiopronin (**4**) was the most selective analog 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 $\bf 1$ (RR = 0.95) or its analogs $\bf 2$, $\bf 3$ and $\bf 5$ (RR = 1.2, 1.6, 1.56 respectively) (Table 3). Methylating the tiopronin carboxyl group to produce the non-acidic ester analog $\bf 6$ resulted in a significantly more cytotoxic compound (IC₅₀ = 0.51 mM against KB-3-1 versus 7.54 mM for $\bf 1$) perhaps due to greater cell entry of the neutral species. Selectivity was reduced against both KB-V1 and VP-16 cells compared with $\bf 1$, 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, taxol, colcemid and taxol 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** analogs (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 with a methyl group (**9**), or even insertion of a -CH₂-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 Figure S2) to yield the disulfide **10** led to a marked reduction in selectivity towards KB-V1 cells. **1** is known to be cell permeable, ^{25,26} and intracellular activity (at mM concentrations) has been reported for a number of modalities. ^{16,17,27}

In contrast, structural variation of $\bf 1$ by substitution of the glycine portion of the molecule (Figure 1) for other amino acids retained selectivity, and in the case of $\bf 4$ (replacement of -H with -CH₂OH) selectivity increased (RR = 74.7) for KB-V1 cells. Interestingly, conversion of the carboxyl group in $\bf 1$ to a methyl ester ($\bf 6$) 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 analogs with increased potency, replacing glycine in $\bf 1$ 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 $\bf 1$ or its analogs ($\bf 2$ - $\bf 6$), which may indicate that cells resistant to mitoxantrone are not sensitized to $\bf 1$.

The HeLa MDR Tet-off, 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 taxol (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 or metal poisoning. While the absolute cytotoxicity of 1 (high μ M 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 treating 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*-acetyl cysteine (NAC), buthionine sulfoximine (BSO), cysteine-glycine dipepetide, cystamine, cystine, DTT, N-ethyl maleimide, procysteine, glutathione, glycine and thiolactic acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA); *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, UK); and (isobutyrylamino)acetic acid from Oakwood Products (West Columbia, South Carolina, USA).

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 pre-coated aluminum-backed silica gel plates with fluorescent indicator, TLC visualization was achieved using 254 nm 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 multi-wavelength detectors using a Zorbax Stable Bond C-18 column ($4.6 \times$ 50 mm, 3.5 µm) with a flow rate of 0.5 mL/min or 1.0 mL/min. Solvent A was 0.05% trifluoroacetic acid (TFA) in water (H₂O), solvent B was 0.05% TFA in acetonitrile (ACN), and a linear gradient of 5% B to 95% B over 10 minutes 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 \geq 95% by the

analytical HPLC trace displaying only a single peak during analysis. Microwave $(\mu\lambda)$ irradiation was carried out in a CEM Discover Synthesizer with 150 watts max power. 1H and ^{13}C NMR spectra were recorded on a Varian spectrometer operating at 400 MHz and 100 MHz respectively. Chemical shifts are reported relative to either chloroform (δ 7.26), dichloromethane (δ 5.32) or deuterium oxide (δ 4.79) for 1H 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 (H₂O) (12 mL). The resulting reaction mixture was heated to 100 °C under microwave ($\mu\lambda$) irradiation for 30 minutes, after which TLC [1:1 hexane (Hex): ethyl acetate (EtOAc)] showed completion. The reaction was quenched by addition of a saturated aqueous solution of sodium thiosulfate (Na₂S₂O₃) and the mixture extracted with EtOAc twice. The combined organic layers were washed twice with Na₂S₂O₃ and once with brine, dried over magnesium sulfate (MgSO₄) and concentrated. The crude mixture was purified by re-crystallization from toluene, affording **2** (1.61 g, 70% yield) as white crystals. ¹H NMR (400 MHz, CDCl₃): δ 9.66 (bs, 2H), 3.57 (m, 2H), 1.50 (d, J = 7.2 Hz, 6H). ¹³C NMR (100 MHz, CDCl₃): 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 eq.), the amino acid methyl ester hydrochloride 2a-6a (2.05 eq.) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) (2.05 eq.) in EtOAc was cooled to 0 °C. To this cooled mixture was added slowly a solution of *N*-methylmorpholine (NMM) (5 eq.) in EtOAc. The resulting reaction mixture was allowed to stir at 0 °C for 5 minutes and at room temperature for 1-2 hours while being monitored by TLC.

Workup A—Reaction mixture was diluted EtOAc and H_2O , the phases separated and the organic layer washed twice with 1M HCl, once with brine, dried over MgSO₄ 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 rinsed with EtOAc. The filtrate was concentrated and the residue purified by flash column chromatography to afford the bisamide disulfide compound.

Bisamide disulfide 2b

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 hours. Workup A, flash column chromatography: silica gel, 75% EtOAc in hexanes to afford **2b** (714 mg, 95% yield) as a syrup. $^1\mathrm{H}$ NMR (400 MHz, CDCl₃): 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). $^{13}\mathrm{C}$ NMR (100 MHz, CDCl₃): 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

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 hours. Workup A, flash column chromatography:

silica gel, 40% EtOAc in hexanes to afford **3b** (803 mg, 94% yield) as a syrup. 1 H NMR (400 MHz, CDCl₃): 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, CDCl₃): 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(x2), 17.9(x2), 17.8, 17.2. MS m/z = 437.2 [M + H]⁺.

Bisamide disulfide 4b

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 hour. Workup B, flash column chromatography: silica gel, EtOAc to afford **4b** (501 mg, 73% yield) as a waxy solid. $^1\mathrm{H}$ NMR (400 MHz, CDCl₃): 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). $^{13}\mathrm{C}$ NMR (100 MHz, CDCl₃): δ 173.4, 172.3, 171.8, 170.9(x2), 63.3, 62.5, 62.4, 56.1, 55.1(x2), 52.8, 17.4, 16.7, 16.4. MS m/z=437.3 [M + Na]⁺.

Bisamide disulfide 5b

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 hour. Workup A, flash column chromatography: silica gel, 35% EtOAc in hexanes to afford **5b** (945 mg, 94% yield) as a syrup. $^1\mathrm{H}$ NMR (400 MHz, CDCl₃): 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). $^{13}\mathrm{C}$ NMR (100 MHz, CDCl₃): δ 171.9, 171.6, 171.4, 129.3, 129.2, 128.5(x2), 127.1, 53.6, 53.4, 52.4, 50.1, 49.4, 48.8, 47.7, 37.9, 37.7(x2), 37.6, 17.3, 16.7(x2). MS m/z=533.1 [M + H] $^+$.

Bisamide disulfide 6b

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 hours. Workup B, flash column chromatography: silica gel, 75% EtOAc in hexanes to afford **6b** (385 mg, 77% yield) as a syrup. ¹H NMR (400 MHz, CDCl₃): 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). ¹³C NMR (100 MHz, CDCl₃): δ 172.4, 172.2, 171.0(x2), 52.5, 49.6, 47.8, 41.4, 17.2, 16.7. MS m/z = 353.1 [M + H]⁺.

Synthesis of analogs 2-5

A solution of the racemic bisamide disulfide compound **2b-5b** (1 eq.) in 20% H_2O in tetrahydrofuran (THF) (v:v) was thoroughly degassed by bubbling argon through the solution for 5-10 minutes. Tributylphosphine (Bu₃P) (3.5 eq.) was added slowly and the resulting reaction mixture allowed to stir at room temperature for 5 minutes and monitored by TLC. The reaction mixture was diluted with ethanol (EtOH), 2M sodium hydroxide (NaOH) added, and stirring continued for 1 hour.

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 discarded. The aqueous layer was acidified with 1M HCl and extracted twice with EtOAc, the combined organic layers were dried over $MgSO_4$ and concentrated to afford the 1 analog.

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 discarded. The aqueous layer was acidified with 1M HCl and concentrated under reduced pressure. The solid residue was extracted with EtOH and the insoluble salts filtered, the filtrate was concentrated to afford the 1 analog.

Ala-tiopronin (2)

Bisamide disulfide **2b** (700 mg, 1.84 mmol), 20% H₂O in THF (v:v, 10 mL), Bu₃P (1.30 g, 6.44 mmol). TLC (1:1 Hex:EtOAc) 5 minutes. EtOH (4 mL), 2M NaOH (5 mL), 1 hour. Workup A to afford Ala-Tiopronin analog **2** (271 mg, 42% yield) as a fluffy solid. ¹H NMR (400 MHz, D₂O): δ 4.35 (q, J = 7.2 Hz, 1H), 3.67-3.58 (m, 1H), 1.49-1.42 (m, 6H). ¹³C NMR (100 MHz, D₂O): δ 176.4(x2), 48.8, 36.2, 20.4, 15.9. MS m/z = 178.1 [M + H]⁺.

Val-tiopronin (3)

Bisamide disulfide **3b** (800 mg, 1.83 mmol), 20% $\rm H_2O$ in THF (v:v, 10 mL), $\rm Bu_3P$ (1.30 g, 6.44 mmol). TLC (1:1 Hex:EtOAc) 5 minutes. EtOH (5 mL), 2M NaOH (5 mL), 1 hour. Workup A to afford Val-Tiopronin analog **3** (577 mg, 77% yield) as a white solid. $^1\rm H$ NMR (400 MHz, CD₂Cl₂): 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). $^{13}\rm C$ NMR (100 MHz, CD₂Cl₂): 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)

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 minutes. EtOH (3 mL), 2M NaOH (3 mL), 1 hour. Workup B to afford Ser-Tiopronin analog **4** (305 mg, 65% yield) as a white solid. ¹H 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). ¹³C 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)

Bisamide disulfide **5b** (460 mg, 0.863 mmol), 20% H₂O in THF (v:v, 6 mL), Bu₃P (611 mg, 3.02 mmol). TLC (1:1 Hex:EtOAc) 10 minutes. EtOH (2 mL), 2M NaOH (2 mL), 1 hour. Workup A to afford Phe-Tiopronin analog **5** (371 mg, 85% yield) as a thick syrup. ¹H NMR (400 MHz, CDCl₃): 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). ¹³C NMR (100 MHz, CDCl₃): δ 174.6, 173.3, 135.5, 129.4, 128.6, 127.3(x2), 53.3, 37.9, 37.2, 21.8(x2). MS m/z = 254.1 [M + H]⁺.

Tiopronin-Me Ester (6)

A solution of bisamide disulfide **6b** (380 mg, 1.08 mmol) in 20% H₂O in THF (v:v, 10 mL), was thoroughly degassed by bubbling argon through the solution for 10 minutes. Bu₃P (763 mg, 3.77 mmol) was added slowly and the resulting reaction mixture stirred for 10 minutes under argon. TLC (EtOAc) showed complete reduction. Reaction mixture was concentrated under reduced pressure and the residue was subjected to flash column chromatography: silica gel, 1:1 Hex:EtOAc to afford the Tiopronin-Me Ester analog **6** (332 mg, 85% yield) as a thick syrup. ¹H NMR (400 MHz, D₂O): 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). ¹³C NMR (100 MHz, D₂O): 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 human cervical epithelial adenocarcinoma cell line KB-3-1 (a HeLa derivative) and its P-gp-expressing MDR sub-lines 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 sub-line MCF-7/VP16; the human lung carcinoma cell line H460 and its ABCG2-expressing MDR sub-line 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% CO₂ 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, California, USA). The H460 lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium from Life Technologies (Carlsbad, California, USA) 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: taxol (50 ng/mL), MCF-7/VP16: etoposide (4 µM), and H460/MX20: mitoxantrone (20 nM). 19, 28 The parental Clontech Hela "Tet-off" and the HeLa "MDR Tet-off" (American Type Culture Collection, Manassas, Virginia, USA) derived cell lines were grown in high glucose (DMEM) media 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, California, USA). Media 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 hours. Stock solutions of compounds (3 M) were prepared in $\rm H_2O$ and then two-fold serially diluted in media to give a range of final tissue culture concentrations of 20 mM to 78 μ M. After 72 h, cell viability was examined. Cytotoxicity (IC₅₀) 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 IC₅₀ of the parental cell line by that of the transporter-expressing cell line. An RR value >1 indicates that the MDR cell population is collaterally sensitive to the tested drug, while an RR <1 indicates the MDR cells are resistant to the drug relative to the parental cell line.

ABCB1 and ABCC1 mRNA Analysis

 1×10^6 MDR Tet-off cells were seeded for 24 hours before treating (in the absence of doxycycline), either with or without 1 (1 mM) for 8, 24 or 48 hours. Cells were then trypsinized, rinsed with PBS three times and the total RNA purified using an RNeasy mini kit according to the manufacturer's instructions (Qiagen, Germantown, Maryland, USA). First strand cDNA was prepared from 1 μg total RNA using the High Capacity cDNA reverse transcription kit (Applied Biosciences, Foster City, California, USA) 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, California, USA). 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, Maryland, USA). The cDNA was biotinylated using the Brightstar Psoralen-Biotin Kit (Ambion, Austin, Texas, USA). 2 µg of total RNA from MDR Tet-off cells was resolved on a non-denaturing 1% TAE agarose gel. Equal loading of RNA in the gel was confirmed by ethidium bromide staining showing 18S and 28S rRNA bands (Supporting Figure S5). Northern blots were performed using NorthernMax membranes and detection with BrightStar BioDetect according to the manufacturers instructions (Ambion, Austin, Texas, USA).

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 (5x) buffer, loaded onto a 3-8% NuPAGE® Novex ® Tris-Acetate gel (Invitrogen Corp., Carlsbad, CA, USA), 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 either P-gp, MRP1 or 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, 1:10,000, Pierce Biotechnology, Rockford, Illinois, USA) for 60 min at 21 °C, and washed again. Each blot was immunoprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Ambion, Austin, Texas, USA) as a loading control.

Resensitization Assay

KB-V1 cells were grown in **1** at 0.1mM and then 1mM and finally 5 mM of **1** over six 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, taxol 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 pre-treated with a P-gp inhibitor (positive control, 200 nM tariquidar), 1 (20 mM) or media (negative control) for 10 min in a 37°C water bath. Cells were isolated by centrifugation, resuspended in 1 mL IMDM and incubated with rhodamine 123 (4 μ M). Cells were then incubated in the dark for 45 min in a 37 °C water bath, centrifuged, resuspended in 300 μ L of 0.1% bovine serum albumin in 1x 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, California, USA). FACS data were analyzed using FlowJo software (Tree Star, Inc., Ashland, Oregon, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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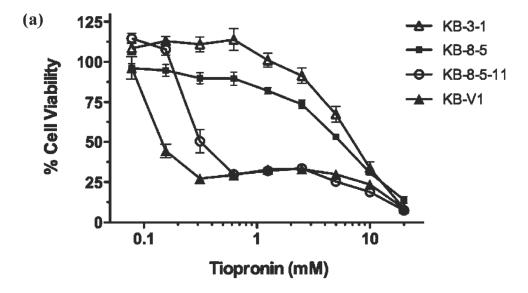
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Figure 1.

Chemical structures of tiopronin (1, [(2-mercaptopropanoyl)amino]acetic acid) and analogs. 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.



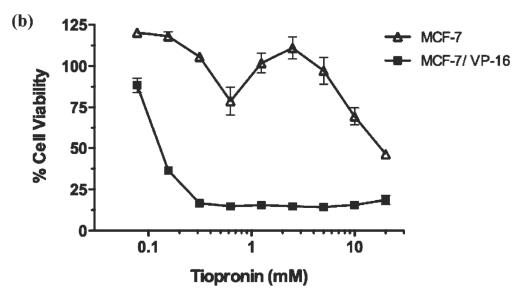
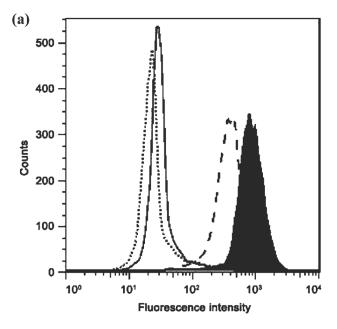


Figure 2. Collateral sensitivity of MDR cell lines to 1. (a) Dose-response curves of 1 against the P-gp-expressing sub-lines KB-V1, KB-8-5-11 and KB-8-5 and their parental KB-3-1 human adenocarcinoma cell line treated with 1 for 72 h. P-gp expression is KB-V1 > KB-8-5-11 > KB-8-5, and P-gp expression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



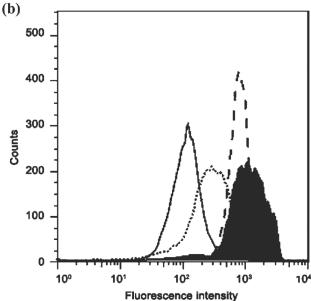


Figure 3. The effect of 1 on the transporter function of P-gp and MRP1. (a) 1 does not interfere with P-gp function. Cell lines were incubated with the P-gp substrate rhodamine 123 (4 μ M) alone (solid line) or in the presence of 1 (20 mM, dotted line) or with the positive control P-gp inhibitor tariquidar (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 μ M) alone (solid line) or in the presence of tiopronin (20 mM, dotted line) or with the positive control MRP1 inhibitor MK-571 (50 μ M, dashed line) and compared with the fluorescence of the parental cell line MCF-7 (black filled histogram).

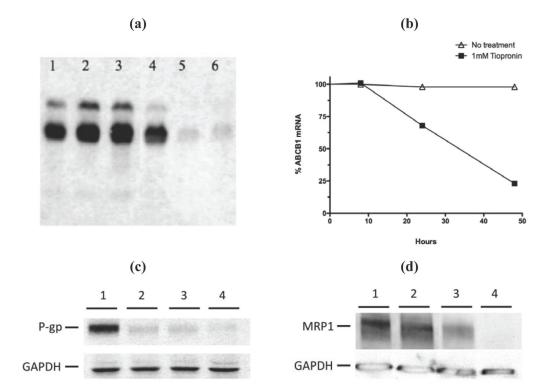


Figure 4.

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 hours (lanes 1 and 4), 48 hours (lanes 2 and 5) or 72 hours (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 hours 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 hours 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 hours 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.

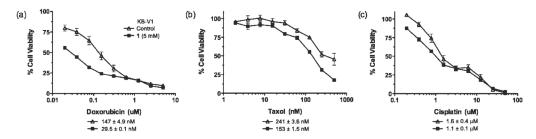


Figure 5. Long term culture of KB-V1 cells with 1 leads to the partial reversal of the MDR phenotype and consequent resensitization of the cells to chemotherapeutics. Dose response curves of KB-V1 cells that have previously been selected for growth in, and cultured either in the absence (line with triangles)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

Scheme 1.

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Determination of cytotoxicity (IC50, mM) of 1 and collateral sensitivity (RR) of parental and MDR sub-lines (indented)

Table 1

KB-3-1	mechanism	Selection	(IC ₅₀ , mM)	RR^a	Ref
			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	6.0	32
СНО			6.90 ± 1.30		33
C5	P-gp	colchicine-selected	8.27 ± 1.04	8.0	33
10001	ı		17.2 ± 0.33		34
10193	β-tubulin mutant	colcemid-selected	10.11 ± 1.63	1.7	35
10576	α-tubulin mutant	taxol-selected	$4.84 \pm 0.74 \ 9.20 \pm 2.12$	3.6	34
NIH 3T3					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	9.0	29
MCF-7			12.27 ± 2.34		37
VP-16	MRPI	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	9.0	21
H460			5.34 ± 0.24		38
TX50	P-gp	taxol-selected	0.94 ± 0.25	5.7	28

	mechanism	Selection	Cytotoxicity (IC50, mM)	RR ^a Ref	Ref
MX20	ABCG2	mitoxantrone-selected	5.60 ± 0.32	0.95 38	38

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 $\label{eq:Table 2} \textbf{Cytotoxicity of 1} \ \text{and analogs, and sulfur containing compounds against parenta KB-3-1 human} \ \text{adenocarcinoma cells and the P-gp-expressing sub-line KB-V1 (IC}_{50}, mM)}^a$

	Cytotox	icity (IC ₅₀ , mM)	
	IC ₅₀ KB 3-1	IC ₅₀ 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-acetyl cysteine	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
Disulphide compoun	ds		
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
Disulphide/Thiol rea	ctive		
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 IC50 against parental cells divided by its IC50 against resistant cells. IC50 values are mean \pm SD from three independent experiments. Structures of the thiol compounds in this table are shown in Supporting Information Figure S6.

Table 3

sub-line KB-V1; MCF-7 human breast cancer cells and the MRP1-expressing sub-line MCF-7/VP-16; and the human large cell lung cancer cell line H460 Cytotoxicity (IC₅₀ mM) of analogs 1-6 against parental and drug-resistant cell line pairs: KB-3-1 human adenocarcinoma cells and the P-gp-expressing and its ABCG2-expressing sub-line $\mathrm{H}460/\mathrm{MX}20^a$

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				Cytotoxi	Cytotoxicity (IC50, mM)	<u> </u>			
		P-gp			MRP1		W.	ABCG2	
	KB-3-1	KB-V1	RR	MCF-7	VP-16	RR	H460	MX20	RR
_	7.54±0.2	0.15 ± 0.02	51.0	51.0 12.27±2.34	0.29±0.17 42.5	42.5	5.34 ± 0.24	5.60 ± 0.32	0.95
7	18.25 ± 2.61	0.49 ± 0.02	37.1	20.2 ± 2.58	0.51 ± 0.05 39.5	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	1
ĸ	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 ± 0.13	1.56
9	0.51 ± 0.26	0.08 ± 0.02 6.2	6.2	0.22 ± 0.14	0.14 ± 0.01 1.6	1.6	0.23 ± 0.03	0.25 ± 0.03	0.90

^qIC₅₀ values are 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).

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