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The "Specific" P-Glycoprotein Inhibitor Tariquidar Is Also a Substrate and an Inhibitor for Breast Cancer Resistance Protein (BCRP/ABCG2)

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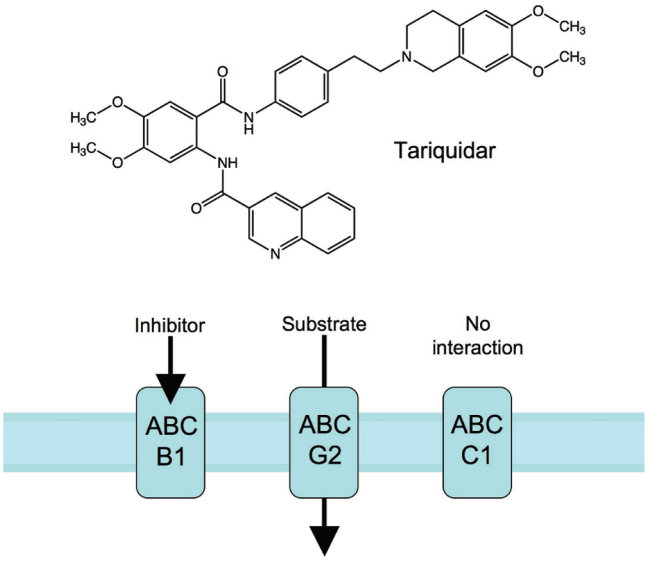
Abstract



Tariquidar was developed as a specific inhibitor of the efflux transporter ABCB1. Recent positron emission tomographic brain imaging studies using [^{11}C]-tariquidar to measure ABCB1 (P-gp, P-glycoprotein) density in mice indicate that the inhibitor may not be as specific as previously thought. We examined its selectivity as an inhibitor and a substrate for the human transporters P-gp, breast cancer resistance protein (BCRP, ABCG2), and multidrug resistance protein 1 (MRP1, ABCC1). Our results show that at low concentrations, tariquidar acts selectively as an inhibitor of P-gp and also as a substrate of BCRP. At much higher concentrations (≥100 nM), tariquidar acts as an inhibitor of both P-gp and BCRP. Thus, the *in vivo* specificity of tariquidar depends on concentration and the relative density and capacity of P-gp vs BCRP.

Keywords: Positron emission tomography, drug transporters, P-glycoprotein, breast cancer resistance protein, blood−brain barrier, transport inhibitors, tariquidar

Efflux transporters belonging to the ATP-binding cassette (ABC) family, which are highly expressed at the blood−brain barrier (BBB), prevent the entry of drugs and other substrates into the brain (*1*, *2*). Of these transporters, three of the most common ones at the blood−brain barrier are P-glycoprotein (P-gp, encoded by the gene *ABCB1*), multidrug resistance protein 1 (MRP1, encoded by the gene *ABCC1*), and breast cancer resistance protein (BCRP, encoded by the gene *ABCG2*). Changes in P-gp function and expression are hypothesized to play a role in neurological disorders, mediating drug-resistant epilepsy, drug effectiveness against HIV infection of the brain, and Alzheimer disease (*3*−*5*).

P-gp substrates radiolabeled for use in positron emission tomography (PET) can be used to measure the *in vivo* function of P-gp (*6*). For example, the substrate [^{11}C]*N*-desmethyl-loperamide (dLop) has very limited entry into the human brain but has high uptake in the brain after P-gp inhibition (*7*). However, substrate radiotracers cannot be used directly to measure the expression or density of P-gp at the blood−brain barrier, because they do not bind to the transporter in a classical receptor−ligand fashion (*6*). Instead, inhibitors known to bind to P-gp with high affinity have been radiolabeled and evaluated for their ability to measure P-gp density in the brain (*8*, *9*). Tariquidar (XR9576, *N*-[2-[[4-[2-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl)ethyl]phenyl]carbamoyl]-4,5-dimethoxyphenyl]quinoline-3-carboxamide, Figure 1) has been evaluated in animals because of its high binding affinity (K_D = 5 nM) to P-gp (*10*). However, the results from imaging studies are not straightforward. When [^{11}C]-tariquidar was injected at tracer doses into wild-type mice, the amount of radioactivity detected in the brain was negligible (*8*, *9*). The radiotracer also did not enter the brains of *abcb1a*/*b* knockout or *abcg2* knockout mice, but only entered the brains of triple knockout

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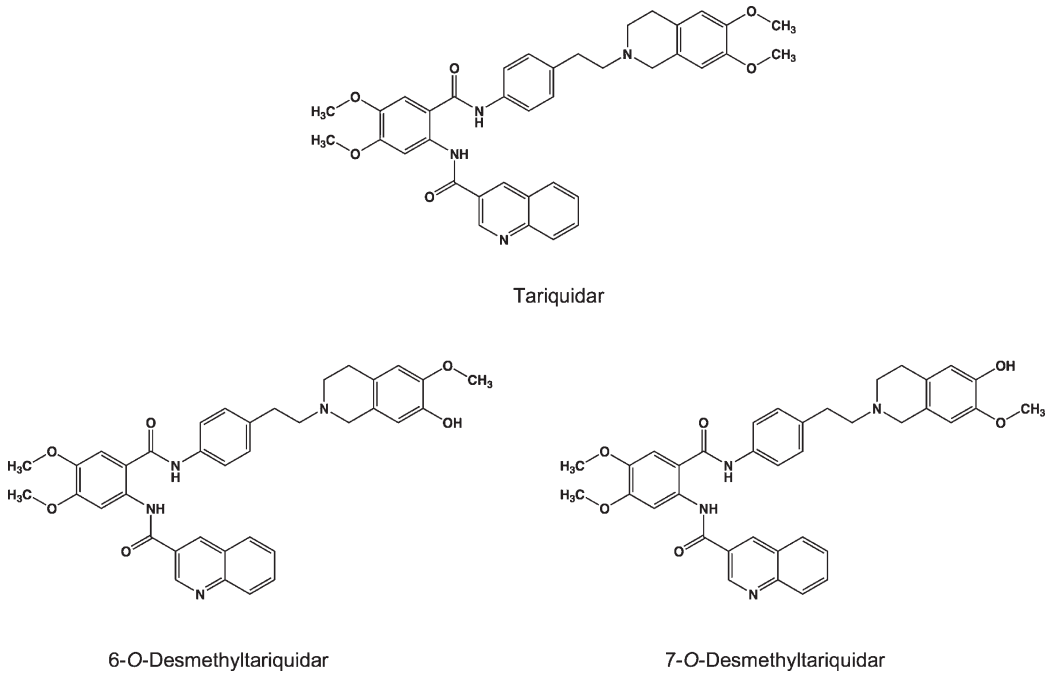

Figure 1. Structures of tariquidar (XR9576, *N*-[2-[[4-[2-(6,7-dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl)ethyl]phenyl]carbamoyl]-4,5-dimethoxyphenyl]quinoline-3-carboxamide), and 6- and 7-*O*-desmethyltariquidar.

abcb1a/b/abcg2 mice (*8*, *9*). Tariquidar has been reported to inhibit the function of BCRP at micromolar concentrations (*11*, *12*), but the imaging studies indicate that tariquidar may interact with BCRP at much lower concentrations, suggesting to us that it may be a substrate of BCRP.

Therefore, we sought to determine the selectivity of tariquidar at low concentrations both as an inhibitor and as a substrate for P-gp, MRP1, and BCRP. To avoid confounding results from species differences, we assessed selectivity using three pairs of human cell lines that overexpress each transporter. One advantage of this approach is that many engineered transporter-expressing cell lines available are developed from polarized cell lines with a high background of endogenous transporter expression, potentially interfering with interpretation of transport specificity. While a number of MRPs are believed to be expressed at the BBB (*2*), we examined MRP1-expressing cells as an exemplar given that MRPs often transport glutathione-conjugated or anionic compounds and show significant commonality in their substrates (*13*).

The inhibition activity of tariquidar for each transporter was indicated by its ability to inhibit efflux of a fluorescent substrate in the presence of increasing concentrations of tariquidar. We found that at concentrations ≥100 nM, tariquidar inhibited both P-gp and BCRP but did not inhibit MRP1. Accumulation of the fluorescent substrate calcein-AM in *ABCB1*-expressing cells treated with 100 nM and 1 μM tariquidar increased 14-fold ($P < 0.001$) and 19-fold ($P < 0.001$), respectively (Figure 2A). Most P-gp is inhibited at 100 nM, consistent with results from Callaghan and co-workers showing the same effect on paclitaxel accumulation in P-gp-expressing cells (*10*). At the same concentrations, tariquidar also increased the accumulation of the fluorescent substrate mitoxantrone in *ABCG2*-expressing cells by 4-fold ($P < 0.001$) and 8-fold ($P < 0.001$), respectively (Figure 2B). In this system, these data indicate that tariquidar inhibits both transporters with similar potency because at 100 nM, it restored accumulation to 56% of control for P-gp and 84% of control for BCRP. Extrapolated to an *in vivo* situation, the potency of tariquidar as an inhibitor of P-gp and BCRP may vary according to expression levels. Tariquidar did not increase accumulation of substrate in *ABCC1*-expressing cells (Figure 2C). As positive controls, inhibition of each transporter was demonstrated with a known inhibitor (Figure 2). Accumulation of fluorescent substrates in the parental lines of each pair did not change in the absence or presence of the inhibitors (data not shown).

To confirm this effect, we also assessed tariquidar's selectivity as an inhibitor by measuring its effect on the resistance of cells to cytotoxic substrates of each transporter (Table 1). At 100 nM, tariquidar decreased the resistance of *ABCB1*-expressing cells to doxorubicin by 30-fold ($P < 0.001$) and of *ABCG2*-expressing cells to mitoxantrone by 2-fold ($P < 0.05$). The same concentration of tariquidar did not change the resistance of

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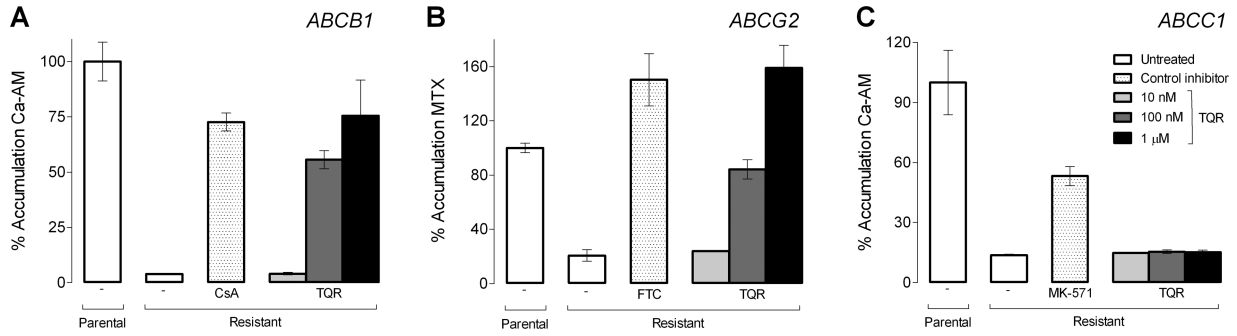

Figure 2. The ability of tariquidar to inhibit the function of three ABC transporters (P-gp, BCRP, and MRP1) as measured by the uptake of the fluorescent substrates calcein-AM and mitoxantrone in three pairs of human cell lines. At ≥100 nM, tariquidar inhibits both (A) P-gp and (B) BCRP function, but not (C) MRP1 function. Mean fluorescence intensity ± SD from three observations, reported as accumulation of fluorescent substrate, is normalized to accumulation in untreated parental cells. Abbreviations: calcein-AM (Ca-AM), mitoxantrone (MTX), fumitremorgin C (FTC, BCRP inhibitor; 5 μM), cyclosporin A (CsA, P-gp inhibitor; 10 μM), tariquidar (TQR), MK-571 (MRP1 inhibitor).

Table 1. Effect of Tariquidar on the Cytotoxicity of Drugs Effluxed by Three ABC Transporters, P-gp, BCRP, and MRP1

		cytotoxicity value			
		drug alone		drug +100 nM tariquidar	
cell line	cytotoxic drug	IC_{50}[a] (nM)	RR[b]	IC_{50}[a] (nM)	RR[b]
B1 resistant (P-gp)	doxorubicin	4498 ± 398	121[c]	132 ± 11	4[c]
B1 parental	doxorubicin	37 ± 6		27 ± 8	0.7
G2 resistant (BCRP)	mitoxantrone	1058 ± 135	24[c]	549 ± 37	13[d]
G2 parental	mitoxantrone	43 ± 5		25 ± 5	0.6
C1 resistant (MRP1)	doxorubicin	704 ± 290	2	667 ± 39	2
C1 parental	doxorubicin	337 ± 23		373 ± 81	1

[a] Mean ± SD from three independent experiments. [b] RR = resistance ratio, which is the quotient of the IC_{50} value of each cell line to that of the parental line not treated with tariquidar. [c] $P < 0.001$ by one-way analysis of variance followed by Bonferroni post *t*-test ($\alpha = 0.05$). [d] $P < 0.05$ by one-way analysis of variance followed by Bonferroni post *t*-test ($\alpha = 0.05$).

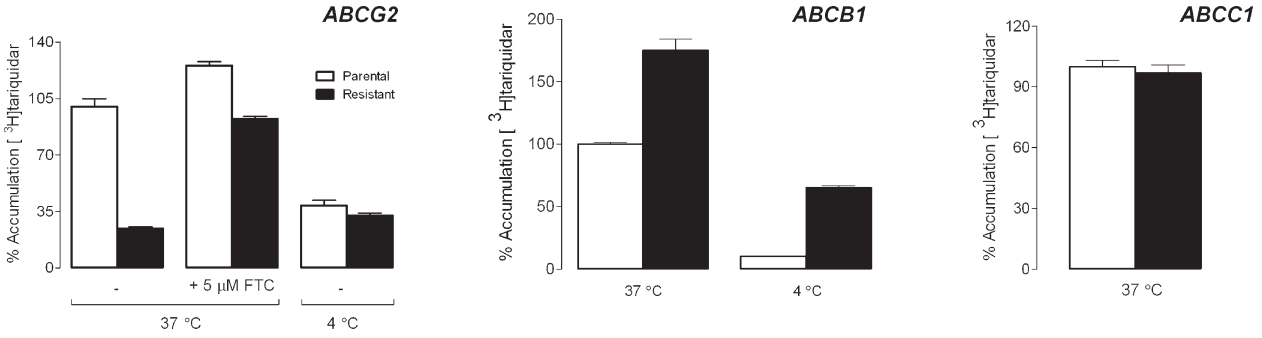

Figure 3. Substrate activity of tariquidar for three ABC transporters as measured by the accumulation of [^{3}H]tariquidar in human cells consisting of parental (white bar) and resistant ABC transporter-expressing (black bar) lines. (A) Tariquidar is a substrate for BCRP and (B) binds to P-gp, but (C) has no activity at MRP1. The accumulation of [^{3}H]tariquidar in cells was assayed 240 min after the addition of [^{3}H]tariquidar (1 nM). Bars represent mean ± SD of six observations.

ABCC1-expressing cells to its substrate doxorubicin ($P = 0.84$). Tariquidar alone did not induce cytotoxicity at concentrations < 20 μM (data not shown).

The selectivity of tariquidar as a substrate for each transporter was assessed by measuring the cellular accumulation of [^{3}H]tariquidar. We found that tariquidar is a substrate for human BCRP, but not for P-gp or MRP1. Parental cells accumulated 4-fold more [^{3}H]tariquidar than *ABCG2*-expressing cells (Figure 3, $P < 0.001$). Treatment with the BCRP inhibitor fumitremorgin C (5 μM) increased accumulation of [^{3}H]-tariquidar 4-fold (Figure 3, $P < 0.001$). Accumulation also increased slightly in parental cells, because H460 cells endogenously express a baseline level of

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BCRP (*14*). To confirm that tariquidar is a substrate of BCRP, we also measured accumulation of [^{3}H]tariquidar in a pair of human embryonic kidney cell lines transfected with an empty vector plasmid (HEK 293, parental) and with *ABCG2* (HEK 293 ABCG2, resistant). The parental line accumulated 4-fold more [^{3}H]tariquidar than the resistant line ($P < 0.001$; data not shown); when the resistant line was treated with 5 μM fumitremorgin C, accumulation increased 2-fold ($P < 0.001$; data not shown). Because we have found that tariquidar is trapped in the intracellular lysosomes of cells (unpublished results) (*15*), we also measured the accumulation of [^{3}H]tariquidar at 4 °C, a temperature at which energy-dependent processes, such as acidification of lysosomes, are abrogated (*16*, *17*). As expected, the accumulation of [^{3}H]tariquidar decreased 3-fold in parental cells (Figure 3, $P < 0.001$), almost reaching parity with the accumulation in *ABCG2*-expressing cells.

We confirmed that tariquidar is not a substrate for P-gp (*10*). *ABCB1*-expressing cells "accumulated" almost 2-fold more [^{3}H]tariquidar than parental cells (Figure 3, $P < 0.001$), and we hypothesized that this increased "accumulation" was due to binding of [^{3}H]-tariquidar to P-gp. Given that cellular accumulation of tariquidar in cells is dependent on lysosomal trapping (mentioned above), we tested the hypothesis that binding to P-gp was occurring by measuring accumulation at 4 °C to eliminate the lysosomal trapping component of cellular accumulation. Under these conditions, [^{3}H]tariquidar accumulation decreased in both cell lines at 4 °C (Figure 3). However, the absolute amount of tariquidar measured in *ABCB1*-expressing cells due to binding (compared with accumulation in parental cells) was equivalent at both temperatures (1094 ± 81 fmol/10^6 cells at 37 °C; 1100 ± 136 fmol/10^6 cells at 4 °C). Assuming 1:1 binding of tariquidar to P-gp, 1100 fmol/10^6 cells equates to ~660 000 molecules of P-gp per KB-8-5-11 cell, which is consistent with published observations (*18*). Finally, tariquidar was not found to be a substrate of MRP1, because accumulation between parental and resistant cells was not different (Figure 3C, $P = 0.16$).

The interaction of tariquidar as a substrate for the BCRP transporter was further explored by measuring the ATPase activity of BCRP in crude membranes in the presence of tariquidar. Tariquidar stimulated ATPase activity to 2.5-fold the basal activity, and the concentration required for 50% stimulation of ATP hydrolysis was 138.4 ± 21.4 nM (Figure 4). This is consistent with tariquidar being a substrate of BCRP, with an affinity similar to other reported avid substrates of BCRP (*19*). This is in contrast to P-gp, where tariquidar has been shown by Callaghan and co-workers to potently inhibit

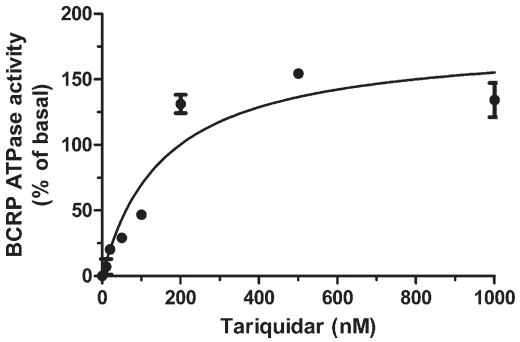

Figure 4. BCRP ATPase activity measured in the presence of tariquidar. Tariquidar stimulates ATPase activity to 2.5-fold the basal activity, demonstrating a direct substrate interaction with BCRP (concentration required for 50% stimulation = 138.4 ± 21.4 nM). The basal activity was subtracted to calculate percent stimulation in the presence of indicated concentrations of tariquidar. The average from three experiments is shown here, and the error bars represent SE.

ATPase activity (IC_{50} = 5.1 nM) (*10*). The affinity for BCRP also corresponds well with its activity as a competitive inhibitor of BCRP (Figure 2B), where cells treated with 100 nM tariquidar were seen to inhibit BCRP efflux activity to about 50% of maximal inhibition.

Our results show that at low concentrations, tariquidar is an inhibitor of P-gp and a substrate of BCRP. At high concentrations (≥100 nM), it is an inhibitor of both P-gp and BCRP. Tariquidar has been reported to inhibit BCRP at high concentrations (~1 μM) (*10*−*12*) but not at the concentrations that inhibit P-gp. This inhibition of BCRP is most likely competitive, derived from tariquidar's activity as a substrate, reported here for the first time. This finding has implications for the clinical use of tariquidar in cancer chemotherapy (for which tariquidar was developed and has been evaluated in clinical trials). The use of tariquidar as an adjuvant to chemotherapeutics in an attempt to reverse drug resistance in tumors yielded negative results in numerous clinical trials (*6*, *20*, *21*). In clinical trials, the inhibitor was used at a dose of 2 mg/kg i.v., resulting in a plasma C_{max} of 2.3 μM (*22*), a concentration at which tariquidar would be able to inhibit both P-gp and BCRP (in contrast, PET radiotracer concentrations are generally in the picomolar range). We speculate that the modified pharmacokinetics and resulting side effects reported in some trials may have been exacerbated in part because tariquidar was inhibiting the physiological functions of BCRP in addition to P-gp. For example, the decrease in maximum tolerated dose of chemotherapeutics used in combination with tariquidar was due to enhanced bone marrow toxicity, a tissue in which BCRP plays a key role in protecting hematopoetic stem cells (*20*).

An interesting observation from our results is that binding of tariquidar at low concentrations to P-gp did not inhibit transporter function. Tariquidar at 1 nM in

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radioaccumulation experiments bound to human P-gp (Figure 3), but partial inhibition of function required concentrations >10 nM (Figure 2). A likely explanation is the presence of "spare receptor" function in the cells, as has been demonstrated for P-gp *in vitro* and *in vivo* (*23*). In brief, P-gp works so rapidly and with such high capacity that even when a sizable percentage of transporters are blocked (e.g., 60−80%), the remaining functional transporter (20−40%) can still effectively preclude entry of substrate. Therefore, the net effect of inhibitors on the function of transporters, similar to that of enzymes and receptors, depends on the relative concentrations of the inhibitor and its target protein.

However, if our results indicate that tariquidar binds to P-gp at low concentrations (1 nM), why did positron emission tomographic (PET) imaging studies using [^{11}C]tariquidar at similar concentrations observe no binding to mouse P-gp in the mouse brain *in vivo* (*8*, *9*)? One explanation is that P-gp and tariquidar have too low a "binding capacity" to be visualized using PET. The specific *in vivo* binding signal ("binding capacity") is the product of receptor density and affinity of the radioligand (*24*, *25*). In this case, the density of P-gp in mouse brain or the affinity of [^{11}C]tariquidar may be too low to visualize P-gp. Although low binding capacity is the simplest explanation for low *in vivo* brain uptake of [^{11}C]tariquidar, its interaction as a substrate for BCRP might create transiently low concentrations of the radioligand in the immediate environment of P-gp, depending on the distance between these two transporters in the lipid membranes of the blood−brain barrier. In any case, the low *in vivo* binding signal reported in mouse brain (*8*, *9*) and the cross reactivity to human BCRP demonstrated in this paper suggest that [^{11}C]tariquidar will not be useful to measure the density of P-gp in human subjects using PET.

In conclusion, tariquidar is not a specific inhibitor of P-gp in human cells. Instead, it interacts with P-gp as a noncompetitive inhibitor and with BCRP as a substrate. That is, at low concentrations relative to BCRP, tariquidar acts merely as a substrate, but at higher concentrations relative to BCRP, it also acts as a competitive inhibitor. Thus, the net *in vivo* effects of tariquidar depend not only on its concentration but also on the concentrations of P-gp and BCRP.

Methods

Chemicals

[*O*-methyl-^{3}H]Tariquidar (American Radiolabeled Chemicals, Inc., St. Louis, MO) was synthesized by [$^{3}H_3$]methylation of *O*-desmethyl-tariquidar. [*O*-methyl-^{3}H]Tariquidar had a radiochemical purity of 99.6% by high-performance liquid chromatography analysis, a specific activity of 3.0 GBq/μmol, and a concentration of 37 MBq/mL. All other chemicals were purchased from Sigma Aldrich (St. Louis, MO), unless specified otherwise.

Tariquidar

Tariquidar was prepared according to a known procedure starting from commercially available 4,5-dimethoxy-2-nitrobenzoic acid, 4-nitrophenethyl bromide, and 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (*26*, *27*).

6- and 7-*O*-Desmethyltariquidar

A literature method for aryl methyl ether demethylation was used (*28*). Thus, a mixture of tariquidar (0.24 g, 0.37 mmol), dodecanethiol (0.23 g, 1.1 mmol), and NaOH (0.030 g, 0.76 mmol) in *N*-methylpyrrolidinone (NMP; 2 mL) was heated at 130 °C for 8 h. The mixture was then cooled to room temperature, diluted with water (15 mL), and acidified (pH ≈ 6) by addition of 1 N HCl. The yellow precipitate was collected by vacuum filtration. The aqueous phase was further extracted with dichloromethane (2 × 25 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to dryness. The residue and the previously collected precipitate were combined and purified by column chromatography on silica gel using 5% MeOH in CH_2Cl_2 as the solvent to give a yellow solid. ^{1}H NMR analysis showed that this yellow solid was a mixture of isomers of *O*-desmethyltariquidar (73 mg). The solid was further purified by HPLC on a reverse phase column (Luna, 10 μm, 10 mm × 250 mm; Phenomenex) eluted with a gradient of 0.25 mM aqueous ammonia (solvent A) and acetonitrile (solvent B) at 4.5 mL/min, with A starting at 80% decreasing to 50% over 15 min, and finally to 30% A over 35 min. The collected product fraction (t_R = 40.1 min; yield, 34 mg, 15%) was found by ^{1}H NMR analysis to be a 1:1 mixture of the 6-*O*-desmethyl and 7-*O*-desmethyl-tariquidar. Thus, at room temperature, the two isomers were distinguished by different chemical shifts for the singlets representing the two aryl protons in the tetrahydroisoquinolinyl ring. Four singlets of equal intensity were seen in the range δ 6.52−6.57, two for each isomer. Other resonances in the recorded ^{1}H NMR spectrum were not distinguishable for the two isomers. Spectra run at higher temperatures (50 and 75 °C) showed no coalescence of peaks and therefore no evidence of conformational exchange. Mp 184−188 °C. ^{1}H NMR (400 MHz, $CDCl_3$, RT): δ 12.48 (broad s, 2 H), 9.54 (d, J = 2.4 Hz, 2 H), 8.77 (d, J = 2.4 Hz, 2 H), 8.63 (s, 2 H), 8.17 (d, J = 8.4 Hz, 2 H), 8.00 (d, J = 7.6 Hz, 2 H), 7.89 (s, 2 H), 7.83 (dt, J_1 = 1.2 Hz, J_2 = 6.8 Hz, 2 H), 7.64 (dt, J_1 = 1.2 Hz, J_2 = 7.2 Hz, 2 H), 7.54 (dd, J_1 = 2.0 Hz, J_2 = 8.4 Hz, 4 H), 7.29 (d, J = 8.4 Hz, 4 H), 7.1 (s, 2 H), 6.67 (s, 1 H), 6.60 (s, 1 H), 6.58 (s, 1 H), 6.52 (s, 1 H), 4.01 (s, 6 H), 3.90 (s, 6 H), 3.85 (s, 3 H), 3.84 (s, 3 H), 3.65 (s, 2 H), 3.62 (s, 2 H), 2.75−2.94 (m, 16 H). ^{13}C NMR (DMSO-d_6, RT): δ 166.9, 162.9, 151.5, 148.6, 148.2, 146.0, 145.8, 144.7, 144.4, 144.3, 136.5, 136.2, 135.5, 133.8, 131.5, 129.3, 128.7, 127.6, 127.4, 126.8, 126.5, 126.1, 125.1, 124.3, 121.5, 115.0, 114.1, 113.1, 112.0, 111.9, 110.2, 105.2, 59.5, 56.0, 55.6, 55.1, 54.9, 50.6, 32.4, 28.2, 28.0. LC-MS [M + 1]$^{+}$: 633. HRMS (ESI, [M + 1]$^{+}$): calcd for $C_{37}H_{36}N_4O_6$, 633.2713; found, 633.2704.

Cell Lines

Four pairs of cell lines were cultured as previously described (*12*, *14*). These pairs were the human adenocarcinoma cell line KB-3-1 and its *ABCB1*-expressing variant KB-8-5-11;

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the human breast cancer cell line MCF-7 and its *ABCC1*-expressing variant MCF-7/VP16; the human adenocarcinoma cell line H460 and its *ABCG2*-expressing variant H460/MX20; and the human embryonic kidney cell line HEK293 and its *ABCG2*-transfected variant HEK293 G2. All culture media were supplemented as previously reported (*29*), and cell lines were grown at 37 °C in 5% CO_2.

Inhibition of Transporter Function

The selectivity of tariquidar as an inhibitor for each transporter was measured by the uptake of a fluorescent substrate in the presence of increasing concentrations of tariquidar. The assay was performed as previously described (*14*) but with the following modifications. Three conditions for each transporter were tested: untreated (negative control), known inhibitor-treated (positive control), and tariquidar-treated. Cells were pretreated for 10 min at 37 °C with either a known inhibitor or media containing 10 nM, 100 nM, or 1 μM tariquidar. Following pretreatment, cells were resuspended in media containing the same concentration of inhibitor or tariquidar used during pretreatment. In addition, cells were incubated with a fluorescent substrate specific to each transporter: 0.25 μM calcein-AM for P-gp and for MRP1 or 5 μM mitoxantrone for BCRP (*30*). The cellular accumulation of each fluorescent substrate was recorded as before (*14*).

Cytotoxicity Assay

Tariquidar's selectivity as an inhibitor of each ABC transporter was assessed by measuring its effect on the resistance of cells to cytotoxic substrates for each transporter. Cytotoxicity (IC_{50}) was measured and defined as previously described (*14*, *31*). Resistance ratios (RR) are also reported for each cell line, determined by dividing the IC_{50} of the resistant cell line by that of the parental cell line.

Cellular Accumulation of [^{3}H]Tariquidar

The substrate selectivity of tariquidar (at nanomolar concentrations) for the three ABC transporters was measured as the accumulation of [^{3}H]tariquidar in three pairs of cell lines (one pair for each transporter). The assay was carried out as previously described (*14*). Accumulation of [^{3}H]tariquidar (1 nM) in the cell lines was measured after 240 min to allow stable uptake of the compound. For the purposes of standardizing accumulation, the number of attached cells was counted in three wells per plate using a Cellometer automatic cell counter (Nexcelcom, Lawrence, MA). After correction for adsorption of [^{3}H]tariquidar to the wells and for cell count, radioactivity was standardized to that measured in control cells of each pair.

BCRP ATPase assay

The beryllium fluoride-sensitive ATPase activity of ABCG2 in membrane vesicles of High Five insect cells was measured as previously described (*32*). The membrane vesicles (10 μg of protein) were incubated with varying concentrations of tariquidar in the presence and absence of beryllium fluoride (BeFx; 0.2 mM beryllium sulfate and 2.5 mM NaF) in ATPase assay buffer (50 mM MES, pH 6.8, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, 2 mM dithiothreitol, 1 mM ouabain, and 10 mM $MgCl_2$) for 5 min. The reaction was started by the addition of 5 mM ATP and was stopped by the addition of 0.1 mL of 5% SDS solution after 20 min. The amount of inorganic phosphate released and the BeFx-sensitive ATPase activity of ABCG2 was determined as described previously (*32*, *33*).

Statistical Analysis

Data are expressed as mean ± SD from three observations for the fluorescent inhibition assay, from nine observations for the cytotoxicity assay, and from six observations for the radioaccumulation assay. For the BCRP ATPase assay, data are expressed as mean ± SE. Data were evaluated for statistical significance using the one and two way analysis of variance followed by the Bonferronni post *t*-test (unpaired, two-tailed, $\alpha = 0.05$).

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Author Contributions

All authors contributed toward design of the research, in interpretation of results, and in writing the paper. Synthesis and characterization was performed by S.T. All other experiments and data analysis were performed by P.K., S.S., and M.D.H.

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