Tariquidar Is an Inhibitor and Not a Substrate of Human and Mouse P-glycoprotein

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ABSTRACT

Since its development, tariquidar (TQR; XR9576; N-[2-[[4-[2-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]carbamoyl]-4,5-dimethoxyphenyl]quinoline-3-carboxamide) has been widely regarded as one of the more potent inhibitors of P-glycoprotein (P-gp), an efflux transporter of the ATP-binding cassette (ABC) transporter family. A third-generation inhibitor, TQR exhibits high affinity for P-gp, although it is also a substrate of another ABC transporter, breast cancer resistance protein (BCRP). Recently, several studies have questioned the mechanism by which TQR interfaces with P-gp, suggesting that TQR is a substrate for P-gp instead of a noncompetitive inhibitor. We investigated TQR and its interaction with human and mouse P-gp to determine if TQR is a

substrate of P-gp in vitro. To address these questions, we used multiple in vitro transporter assays, including cytotoxicity, flow cytometry, accumulation, ATPase, and transwell assays. A newly generated BCRP cell line was used as a positive control that demonstrates TQR-mediated transport. Based on our results, we conclude that TQR is a potent inhibitor of both human and mouse P-gp and shows no signs of being a substrate at the concentrations tested. These in vitro data further support our position that the in vivo uptake of [¹¹C]TQR into the brain can be explained by its high-affinity binding to P-gp and by it being a substrate of BCRP, followed by amplification of the brain signal by ionic trapping in acidic lysosomes.

Introduction

The ATP-binding cassette (ABC) transporters have a profound impact on therapeutic efficacy. These transmembrane transporters use ATP to pump small molecules out of cells, irrespective of the concentration gradient (Gottesman et al., 2002). As a result, expression of family members such as P-glycoprotein (P-gp; *ABCB1*) and breast cancer resistance protein (BCRP; *ABCG2*) at sites such as the blood-brain barrier or in multidrug-resistant (MDR) tumors can reduce drug accumulation (Gottesman et al., 2002; Loscher and Potschka, 2005). A number of small-molecule inhibitors of P-gp were developed with the

intention of reversing the efflux of chemotherapeutics from MDR cancer cells. Early (first-generation) inhibitors were existing pharmaceuticals already known to inhibit P-gp, such as verapamil and cyclosporin A (CsA). Second-generation inhibitors were simple derivatives of first-generation inhibitors lacking their primary pharmacologic activity. Subsequent medicinal chemistry efforts led to new chemical compounds known as third-generation, high-affinity (nanomolar IC₅₀) P-gp inhibitors. In clinical trials, however, inhibitors did not improve patient outcome, although there were some notable exceptions (Tamaki et al., 2011; Shaffer et al., 2012). More recently, P-gp inhibitors have been used in humans as part of positron emission tomography (PET) imaging in combination with radiolabeled substrates of P-gp to measure the function of the transporter at the blood-brain barrier (Kannan et al., 2009; Mairinger et al., 2011).

One such inhibitor used in cancer clinical trials and PET imaging protocols is tariquidar (TQR; XR9576; N-[2-[[4-[2-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]carbamoyl]-4,5-dimethoxyphenyl]quinoline-3-carboxamide) (Fox and Bates, 2007). Early work demonstrated its high inhibitory activity (K_D = 5.1 nM) (Martin et al., 1999). TQR was thought to specifically inhibit P-gp among ABC transporters, although later work revealed that it is also a substrate of

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ABBREVIATIONS: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; BIBW22 BS, 4-(N-(2-hydroxy-2-methylpropyl)ethanolamino)-2,7-bis(cis-2,6-dimethylmorpholino)-6-phenylpteridine; CsA, cyclosporin A; DCPQ, (2R)-anti-5-f3-[4-(10,11-dichloromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxyquinoline trihydrochloride; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester hydrate; MDR, multidrug resistant; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX, mitoxantrone; PBST, phosphate-buffered saline Tween 20; PET, positron emission tomography; P-gp, p-glycoprotein; Pp-18, purpurin-18; PSC833, 6-[(2S,4R,6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A; Rh123, rhodamine 123; TQR, tariquidar; XR9576, N-[2-[[4-[2-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]carbamoyl]-4,5-dimethoxyphenyl]quinoline-3-carboxamide.

TABLE 1
Effect of TQR on the cytotoxicity of paclitaxel in human and mouse P-gp cell lines

Cell Line	Cytotoxic Drug	Cytotoxicity Value (IC ₅₀)									
		Drug Alone		Drug + 1 nM TQR		Drug + 10 nM TQR		Drug + 100 nM TQR		Drug + 1 μ M TQR	
		IC ₅₀	RR	IC ₅₀	RR	IC ₅₀	RR	IC ₅₀	RR	IC ₅₀	RR
		<i>nM</i>		<i>nM</i>		<i>nM</i>		<i>nM</i>		<i>nM</i>	
KB-8-5-11	Paclitaxel	206 $\pm$ 51	52	228 $\pm$ 77	57	1 $\pm$ 0	0.25 ^a	1.5 $\pm$ 0.1	0.38 ^a	1 $\pm$ 0	0.25 ^a
KB-3-1	Paclitaxel	4 $\pm$ 0									
C3M	Paclitaxel	8169 $\pm$ 844	190	7960 $\pm$ 2903	185	8368 $\pm$ 2056	195	375 $\pm$ 263	8.8 ^b	2.2 $\pm$ 0.1	0.05 ^c
3T3	Paclitaxel	43 $\pm$ 2									

RR, resistance ratio (the quotient of the IC₅₀ value of the resistant cell line to that of the parental line).

^a*P* < 0.01 (α < 0.05, from initial IC₅₀ value of resistant cell line) by Student's two-tailed *t* test.

^b*P* < 0.001 (α < 0.05, from initial IC₅₀ value of resistant cell line) by Student's two-tailed *t* test.

^c*P* < 0.0001 (α < 0.05, from initial IC₅₀ value of resistant cell line) by Student's two-tailed *t* test.

BCRP (Kannan et al., 2011). During preclinical development, mechanistic characterization of TQR in Chinese hamster ovary cells expressing hamster P-gp indicated that TQR inhibited the transporter by blocking ATPase activity, and that TQR was not a substrate for P-gp (Martin et al., 1999). That report established the prevailing view on the mechanism of action of TQR, and subsequent work confirmed that [³H]TQR was not effluxed as a substrate from human P-gp-expressing cells (Kannan et al., 2011).

Several recent studies have contradicted earlier data and questioned the interaction of TQR with P-gp. First, Loo and Clarke (2014) reported that TQR stimulated the ATPase activity of a "Cys-less" mutant form of human P-gp. Second, two simultaneously published papers reported that, in PET studies with [¹¹C]TQR, only mice genetically lacking both BCRP and P-gp exhibited brain penetration of [¹¹C]TQR as compared with mice lacking either BCRP or P-gp (Bauer et al., 2010; Kawamura et al., 2010). Third, Bankstahl et al. (2013) reported that, in a transwell apparatus, cells expressing mouse or human P-gp accumulated more [³H]TQR in the presence of the P-gp inhibitor PSC833 (6-[(2*S*,4*R*,6*E*)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-*L*-valine-cyclosporin A). These results are more consistent with TQR behaving like a substrate of P-gp.

Given that earlier studies could not detect that TQR was a substrate of P-gp, we hypothesized that species differences may account for the divergent observations that exist in the literature. To this end, we assessed TQR's interaction with human and mouse P-gp utilizing techniques regularly used for assessing transporter interactions, with BCRP serving as a positive control. This included radiation and flow cytometry accumulation assays, ATPase assays in crude membranes, and cytotoxicity assays measuring the ability of TQR to inhibit P-gp. Finally, we generated an LLC-PK1 cell line expressing BCRP and assessed the transwell transport of TQR by polarized cells expressing P-gp or BCRP. Confirming the relationship between TQR and P-gp is vital not only for studies utilizing TQR as an inhibitor to study P-gp function, but also for PET imaging studies using [¹¹C]TQR to measure P-gp density in vivo.

Materials and Methods

Chemicals. [³H]TQR (70 Ci/mmol) was synthesized and purchased from American Radiolabeled Chemicals (St. Louis, MO). TQR was purchased from MedKoo Biosciences (Chapel Hill, NC). (2*R*)-anti-5-*f*3-[4-(10,11-dichloromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxyquinoline trihydrochloride (DCPQ) was provided by Dr. Victor W. Pike (National Institutes of Mental Health, Bethesda, MD). Purpurin-18 (Pp-18) was obtained from Frontier Scientific (Logan, UT). ((3*S*,6*S*,12*aS*)-1,2,3,4,6,7,12,12*a*-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,6]pyrido[3,4-*b*]indole-3-propanoic acid 1,1-dimethylethyl ester hydrate) was purchased from Tocris Bioscience (Minneapolis, MN). Zeocin and geneticin (G418) were purchased from Invitrogen

(Carlsbad, CA). Flavopiridol was obtained from the National Cancer Institute In Vitro Anticancer Drug Discovery Screen (Bethesda, MD). The anti-ABCG2 5D3-PE monoclonal antibody was purchased from eBioscience (San Diego, CA), whereas the anti-ABCG2 BXP21 and anti-ABCG2 BXP53 monoclonal antibodies were provided by Dr. Suresh Ambudkar (National Cancer Institute, Bethesda, MD). The anti-mouse IgG-2a-horseradish peroxidase monoclonal antibody was purchased from Cell Signaling Technologies (Danvers, MA). The anti-Na⁺/K⁺ ATPase monoclonal antibody rhodamine 123 (Rh123), CsA, mitoxantrone (MTX), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Cell Lines. The parental (control) and resistant (ABC transporter-expressing) cell lines used in this study were as follows (drug selection shown in parentheses): the human adenocarcinoma cell line KB-3-1 and its P-gp-expressing subline KB-8-5-11 (250 nM colchicine) (Shen et al., 1986), 3T3 and its mouse P-gp-expressing subline 3T3 C3M (1 μ g/ml colchicine) (Hall et al., 2011), and the human breast cancer cell line MCF-7 and its BCRP-expressing subline MCF-7 FLV10000 (10 μ M flavopiridol) (Robey et al., 2001). Cells were grown at 37°C in 5% CO₂ and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, and antibiotic.

All LLC PK1 porcine kidney cell lines were grown in Medium-199 (Gibco, Grand Island, NY) supplemented with 3% fetal bovine serum, glutamine, antibiotic, 500 μ g/ml G418 (for LLC-vector, LLC-EQ, and LLC-MDR1-WT cells, hereafter referred to as LLC-MDR1), or 500 μ g/ml zeocin (for LLC-BCRP-vector and LLC-BCRP cell lines). For all cell cultures, medium was removed and cells were grown in the same medium in the absence of drug selection 5–30 days before assays.

Generation of LLC-BCRP Cell Line. LLC-PK1 cells expressing human ABCG2 were generated by transient DNA transfection of LLC-PK1 cells (Fung et al., 2014a) with plasmids containing human ABCG2 cDNA (SAIC, Frederick, MD) and vector alone using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. After transfection, stable cells were isolated by colony cloning. At least 30 individual clones were isolated and were constantly selected by zeocin (500 μ g/ml) for 2 weeks for further analysis.

Cytotoxicity Assay. Cytotoxicity assays were performed to determine the ability of TQR to reverse the resistance of human and mouse P-gp to the cytotoxic substrate paclitaxel. The same assay was used to verify the presence of functional BCRP in the LLC-BCRP cells. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Cells were seeded at a density of 4000 cells per well in 100 μ l of medium. Serial dilutions of either paclitaxel or MTX (for LLC-BCRP cells) were made in Dulbecco's modified Eagle's medium, and an additional 100 μ l of medium containing inhibitor was added to each well (TQR for P-gp-expressing cells and Ko143 for LLC-BCRP cells). The outcome measure was half-maximal inhibitory concentration (IC₅₀), which indicates the concentration of cytotoxic drug required to decrease cell viability by 50% compared with untreated control cells (Brimacombe et al., 2009). The resistance ratio was then calculated from three separate experiments by dividing the mean IC₅₀ of the resistant cell line by that of the parental cell line's IC₅₀.

Flow Cytometry. Flow cytometry was used to measure the activity of P-gp in the presence of TQR, as well as the fluorescence of TQR itself, and confirm cell

Fig. 1. The effect of TQR on the accumulation of Rh123 (1.3 μ M) in cells expressing human (A) and mouse P-gp (B), and in human parental cells (C). Additional concentrations of TQR were tested in cells expressing human P-gp (A inset). Bars represent mean fluorescence from three experiments $\pm$ S.D. For each experiment, accumulation was defined as the mean peak fluorescence intensity in parental and P-gp-expressing cells without the addition of an inhibitor (white bars), as well as transporter-expressing cells with varying concentrations of TQR (shaded bars), with data normalized to accumulation in parental cells. DCPQ (5 μ M, white striped bars) was used as the positive control inhibitor.

surface expression of BCRP on the newly generated LLC-BCRP cell line. The experiments were conducted as previously described (Weidner et al., 2015) with the following modifications. The efflux of the fluorescent P-gp substrate Rh123 was measured to determine the extent to which TQR increased cellular accumulation. Cells expressing human (KB-3-1/KB-8-5-11) or mouse (3T3/C3M) P-gp were

incubated in medium containing Rh123 (1.3 μ M) under the following conditions: no drug (untreated), inhibitor-treated (positive control), and TQR-treated. DCPQ (5 μ M) acted as the positive control inhibitor. Measurements were conducted using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The geometric mean of fluorescence intensity was recorded for a total of 10,000 events per sample in the FL-2 channel.

For experiments measuring the fluorescence of TQR, cells were seeded at a density of 5×10^5 cells per sample and incubated in the dark at 37°C for 30 minutes with 2 μ M Rh123, 15 μ M Pp-18, or 1 μ M TQR, with or without the P-gp inhibitor CsA (1 μ M) or the BCRP inhibitor Ko143 (5 μ M). To measure the geometric mean of fluorescence of TQR, KB-3-1 cells were incubated in TQR concentrations from 0 to 50 μ M. Cells were then washed and incubated in substrate-free medium with the same inhibitor conditions at 37°C for 30 minutes. Cellular accumulation of fluorescent substrates was measured with an LSR II flow cytometer (BD Biosciences). Cells were gated for forward versus side scatter, and the geometric mean of fluorescence intensity of the substrate was measured for 20,000 events using excitation/emission wavelengths: TQR (355/530), Pp-18 (633/620), and Rh123 (488/530).

Cell surface expression of BCRP was measured by incubating trypsinized cells with anti-ABC2 5D3-PE monoclonal antibody (1 μ g per 200,000 cells) at 37°C for 45 minutes. In addition, to confirm the expression of BCRP in the LLC-BCRP cells, cells were suspended in medium with phosphoribide a (2.5 μ M) in the presence or absence of Ko143 (10 μ M) at 37°C for 45 minutes. The remainder of both protocols was followed as described earlier. All fluorescence-activated cellular sorting data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Confocal Microscopy. KB-3-1 cells were plated at a density of 10,000 cells/well in chambered coverslips and allowed to proliferate for 48 hours. Prior to imaging, the medium was aspirated and replaced with Iscove's modified Dulbecco's medium containing 20 nM LysoTracker Red DND-99 (LysoTracker Red; ThermoFisher, Grand Island, NY), 20 μ M TQR, or a combination of the two in which the cells were incubated for 30 minutes. Cells were then washed and resuspended in phosphate-buffered saline. Confocal images were acquired using a Zeiss LSM780 inverted microscope (Carl Zeiss, Oberkochen, Germany) with a C-Apochromat 40 $\times$ objective lens. For TQR and LysoTracker Red, excitation wavelengths were 355 (with a UV laser) and 561 nm, and fluorescence emission spectral detector windows were set at 420–550 and 585–690 nm, respectively. Images were acquired with an optical slice thickness of 1.0 and 0.10 μ m X-Y pixel size.

Uptake of [3 H]Tariquidar in Cells. Radioactivity assays with [3 H]TQR were used to determine the interaction of TQR with human and mouse P-gp. Cells were seeded at a density of 2.5×10^5 cells/ml of medium per well in a 24-well plate. Medium containing [3 H]TQR (3 nM) was added to parent and P-gp-expressing cells with and without the addition of 1 μ M cold TQR. Six wells received nonradioactive medium to account for background signal, whereas another six were reserved for cell counts to standardize accumulation. Cells were incubated in radioactive medium for 30 minutes at 37°C. Medium was then removed, cells were washed in phosphate-buffered solution (pH 7.4), and 100 μ l of trypsin was added to each well for 90 minutes. Radioactivity was then measured using a liquid scintillation counter. After correction for cell counts, radioactivity was expressed as percent accumulation compared with untreated control cells.

ATPase Assays. To determine whether TQR affects human and mouse P-gp differently, ATPase assays were performed using crude membrane extracts from High-five insect cells expressing either human or mouse P-gp. Due to the fact that wild-type mouse mdr1a cDNA is toxic in the bacterial cells needed for the cloning process, an M107L point mutation was introduced, which reduced bacterial toxicity but retained functionality (Pluchino et al., 2015). The protocol described in Kannan et al. (2011) was followed. In brief, the membrane vesicles in ATPase assay buffer [50 mM MES-Tris buffer (pH 6.8), 50 mM KCl, 5 mM sodium azide, 1 mM EGTA, 1 mM ouabain, 10 mM MgCl₂, and 2 mM dithiothreitol] were incubated in varying concentrations of TQR with or without 0.3 mM sodium orthovanadate. ATP hydrolysis was measured by estimating the release of inorganic phosphate after incubation with 5 mM ATP, as described previously (Shukla et al., 2006).

Protein Extraction and Immunoblot Analysis. Total protein extraction from cell culture and protein concentration estimation methods was reported previously (Fung et al., 2014b). For SDS-PAGE, protein samples were loaded onto a 3–8% Tris-acetate gel (Invitrogen). Separated proteins were transferred to a nitrocellulose membrane by iBlot (Invitrogen) following the manufacturer's instructions. For Western blotting, the membrane was first blocked in phosphate-

Fig. 2. Accumulation of [^3H]TQR in cells expressing human and mouse P-gp. Accumulation in P-gp-expressing cells (black bars) is compared with the parental cell line (white bars) before and after the addition of cold TQR (1 μM). This is shown in cells expressing both human P-gp (KB-8-5-11) and mouse P-gp (C3M). Bars represent the average of three observations $\pm$ S.D. * $P < 0.05$; *** $P < 0.001$ ($\alpha < 0.05$, from baseline accumulation in resistant cell line) by one-way analysis of variance. ns, not significant.

buffered saline Tween 20 (PBST) with 20% milk for 30 minutes, then incubated overnight at 4°C with anti-ABCG2 BXP21 (1:2000) and anti-ABCG2 BXP53 (1:2000) monoclonal antibodies diluted in PBST plus 5% milk. The membrane was then washed with medium plus PBST and incubated with mouse anti-IgG-2a-horseradish peroxidase antibody (1:20,000) for 1 hour. The ABCG2 proteins were visualized by enhanced chemiluminescence (GE, Fairfield, CT) + reagent. To measure relative loading between samples, the membrane was reprobed with monoclonal Na/K ATPase antibody (1:5000).

Trans epithelial Drug Transport Assay. Cells were initially grown at a density of 2×10^6 cells/24-mm well on 0.4- μm pore size transwell polycarbonate filters (Corning Inc., Corning, NY). Cells were cultured for 7 days with medium changed once every 2 days. To confirm the quality of cell monolayers, transepithelial electrical resistance was measured with an epithelial volt ohmmeter (World Precision Instruments, Sarasota, FL). Cell monolayers with transepithelial electrical resistance values lower than 200 Ω were not used for the assay. Two hours before the transport experiment, fresh medium was added to the wells (2 ml in the basolateral and apical sides). Transport across the cell monolayer was conducted by adding [^3H]TQR (6.25 nM) to either the apical or basolateral side of the monolayer. For the P-gp inhibition assay, P-gp inhibitor DCPQ (1 μM) was added to the apical and basolateral chambers for 20 minutes before adding [^3H]TQR. Samples (50 μl) were taken from the opposite side of the cell monolayer at 0, 15, 90, 135, and 335 minutes. Radioactivity was then measured using a liquid scintillation counter. The efflux ratio ($B \rightarrow A/A \rightarrow B$), in folds, was calculated by dividing the transepithelial drug efflux rate of ($B \rightarrow A$) by ($A \rightarrow B$) to evaluate drug transporter-mediated directional efflux.

Statistical Analysis. Data are expressed as the mean $\pm$ S.D. from three observations for fluorescence accumulation assays, cytotoxicity assays, and radiation accumulation assays, and from two observations for transwell assays. After the data were tested for homogeneity of variance, statistical significance was evaluated by Student's *t* test (unpaired, two-tailed, $\alpha = 0.05$) and by a two-way analysis of variance followed by the Bonferroni post-*t* test ($\alpha = 0.05$).

Results

Tariquidar as an Inhibitor of P-gp. We first examined whether TQR was equally effective as an inhibitor of mouse and human P-gp. Using MTT cytotoxicity assays, we determined the effect of increasing TQR concentrations on cells expressing human (KB-8-5-11) and mouse P-gp (C3M) by measuring the sensitization of these cell lines to the P-gp-specific cytotoxic substrate paclitaxel. The IC_{50} of paclitaxel significantly decreased in the presence of 10 nM ($P < 0.01$), 100 nM ($P < 0.001$), and 1 μM ($P < 0.001$) TQR in cells expressing human P-gp compared with cells treated with paclitaxel alone (Table 1). In cells expressing mouse P-gp, the IC_{50} decreased after 100 nM and 1 μM

TQR (both $P < 0.001$) (Table 1). The disparity in response can be attributed to the inherent differences between human and mouse P-gp, as well as the basal P-gp expression in the mouse parental 3T3 cells. Treatment with 1 nM TQR had no effect on cellular sensitivity to paclitaxel. We also determined the inherent cytotoxicity of TQR and found the IC_{50} value to be $\approx 50 \mu\text{M}$ in human and mouse cells irrespective of P-gp expression (data not shown).

The ability of TQR to inhibit P-gp was also measured via accumulation of the fluorescent P-gp substrate Rh123 using flow cytometry. Whereas the coincubation of 10 nM TQR had no effect on accumulation of Rh123, 100 nM restored accumulation of Rh123 in cells expressing human P-gp to that of the parent cells ($P < 0.0001$; Fig. 1A). Concentrations of TQR from 10–100 nM were then examined, and it was found that 40 nM significantly increased cellular accumulation of Rh123 in these cells as compared with untreated cells ($P < 0.05$; Fig. 1A inset), and an IC_{50} of 74 nM was calculated. A similar pattern of accumulation was seen in cells expressing mouse P-gp, with 1 μM TQR resulting in maximal uptake of Rh123 ($P < 0.001$; Fig. 1B). A decrease in accumulation of Rh123 in human KB-8-5-11 cells was seen at higher concentrations (1 and 10 μM TQR; Fig. 1A), and the effect was also observed in the parental KB-3-1 cells (Fig. 1C). Given that Rh123 is a mitochondrial dye, it is possible that TQR accumulates in the mitochondria at higher concentrations, depolarizing and displacing Rh123. We previously reported a similar effect for TQR in lysosomes (Kannan et al., 2011).

Interactions of TQR with P-gp. Having established that TQR inhibits mouse and human P-gp, we next examined the interaction of P-gp with TQR to determine whether we could observe any substrate-like interaction between TQR and P-gp. In a previous report, we found that KB-8-5-11 cells expressing human P-gp bound more [^3H]TQR (3 nM) than the parental cell line (KB-3-1), consistent with specific binding to P-gp (Kannan et al., 2011). Here, we confirmed this observation and assessed the cellular accumulation of [^3H]TQR in mouse cells (Fig. 2). In cells expressing human P-gp, baseline binding was 7-fold higher ($717 \pm 71 \text{ fmol}/10^6 \text{ cells}$) than in parental cells ($100 \pm 8 \text{ fmol}/10^6 \text{ cells}$; $P < 0.001$). It has been suggested that addition of P-gp inhibitor in this experiment would reveal that TQR is in fact a substrate of P-gp (Bankstahl et al., 2013). Coincubation of 1 μM “cold” TQR displaced binding of [^3H]TQR from KB-8-5-11 cells ($365 \pm 15 \text{ fmol}/10^6 \text{ cells}$), but increased it in KB-3-1 parental cells ($340 \pm 302 \text{ fmol}/10^6 \text{ cells}$) to equivalent levels. The same pattern of binding was observed in C3M cells expressing mouse P-gp, with a 2-fold higher binding ($228 \pm 44 \text{ fmol}/10^6 \text{ cells}$) than parental cells ($100 \pm 14 \text{ fmol}/10^6 \text{ cells}$; $P < 0.001$), which was reversed with addition of 1 μM cold TQR (resistant cells: $163 \pm 8 \text{ fmol}/10^6 \text{ cells}$; control cells: $188 \pm 12 \text{ fmol}/10^6 \text{ cells}$).

Fig. 3. ATPase activity of human (closed squares) and mouse P-gp (open circles) in the presence of increasing TQR concentrations. Data represent fold stimulation, and each point is the mean from three separate experiments $\pm$ S.D.

Fig. 4. The inherent fluorescent characteristics of TQR. (A) Accumulation of TQR in lysosomes in KB-3-1 cells as imaged with confocal microscopy. Scale bar indicates 20 μm . Flow cytometry experiments showing that the geometric mean of fluorescence increases with increasing concentrations of TQR (B), and showing the accumulation of TQR in cells expressing human P-gp and human BCRP before (white bars) and after the addition of an inhibitor (black bars) (C). Rh123 (2 μM) was used as the specific fluorescent substrate in the P-gp-expressing cells, whereas Pp-18 was used as the specific fluorescent substrate in BCRP-expressing cells. The inhibitors used were CsA (1 μM) and Ko143 (5 μM) for P-gp and BCRP, respectively. Data were normalized to accumulation in cells plus inhibitor from three experiments $\pm$ S.D. *** P < 0.001; **** P < 0.0001 (α < 0.05, from baseline accumulation in resistant cell line) by one-way analysis of variance.

In the presence of increasing TQR concentrations, the ATPase activity of P-gp decreased below the basal rate for both human and mouse P-gp (Fig. 3). One micromolar TQR elicited a 50% decrease in ATP hydrolysis. This observation is consistent with that previously reported for TQR with membranes derived from cells expressing high levels of hamster P-gp (Martin et al., 1999).

Given the inherent fluorescence of TQR, we were able to examine its uptake directly using confocal microscopy (excitation = 420 nm, emission = 550 nm). In KB-3-1 cells, TQR fluorescence is observed as punctate staining (Fig. 4A, green, top left). We have previously reported that TQR is a weak base that competes with other weak bases for lysosomal trapping, and the fluorescent lysosome stain Lysotracker Red also demonstrates punctate staining (Fig. 4A, red, top right) that colocalizes with TQR (Fig. 4A, merge, bottom left). P-gp-expressing cells demonstrate similar accumulation of TQR with no qualitative difference in accumulation observable (not shown). To identify a concentration of TQR appropriate for assessing cellular accumulation

by flow cytometry, we assessed increasing concentrations of TQR in KB-3-1 cells, and found that the geometric mean of fluorescence was positively correlated to concentration (Fig. 4B) and detectable at 20 μM . We then compared the uptake of TQR in cells expressing either human P-gp or BCRP before and after transporter inhibition as well as the uptake of positive control fluorescent substrates of P-gp and BCRP (Fig. 4C). In KB-8-5-11 cells, there was no difference in TQR fluorescence before and after inhibition with CsA, whereas there was a marked difference in the uptake of the P-gp substrate Rh123 under the same conditions (P < 0.001). In cells expressing human BCRP (MCF-7 FLV10000), the accumulation of TQR increased after incubation with Ko143 (P < 0.001), consistent with the characterization of TQR as a BCRP substrate. A strong effect was observed for the positive control BCRP substrate Pp-18 under the same conditions (P < 0.0001).

Transwell Transport of [^3H]TQR. We used transwell assays to determine whether [^3H]TQR is transported by human P-gp and BCRP. We used LLC-PK1 cells transfected with human P-gp (LLC-MDR1), as well as

Fig. 5. The LLC-BCRP transfected cell line expresses functional human BCRP. (A) Western blot confirms expression of BCRP in the LLC-BCRP cells (lane 3) as compared with the vector alone (lane 2) and a positive control BCRP-expressing cell line (lane 1). (B) Cell surface expression measured by 5D3-PE shows higher expression of BCRP in the LLC-BCRP cells (white) compared with the vector cells (black). (C) Accumulation of pheophorbide in LLC-BCRP cells before (white) and after administration of 10 μ M Ko143 (black). The shift of the black histogram to the right indicates an increase in intracellular accumulation of pheophorbide due to the inhibition of BCRP. (D) Cytotoxicity curves show a resistance of the LLC-BCRP cell line to mitoxantrone (open squares) as compared with LLC-vector cells (open circles), which can be reversed with administration of 10 μ M Ko143 (closed circles).

a functional mutant P-gp-expressing cell line (LLC-EQ) and an empty vector-transfected control cell line (LLC-vector) that we have previously reported (Fung et al., 2014a,b). To assess and compare transport of [3 H] TQR by BCRP, we generated an LLC-PK1 cell line transfected with human BCRP (termed LLC-BCRP). To confirm the expression of BCRP in the newly generated LLC-BCRP line, a Western blot was performed using two monoclonal anti-BCRP antibodies. We found expression of BCRP in the LLC-BCRP cell line, but not in LLC-vector cells [cell lysate from a BCRP-expressing cell line was used as a positive control in lane 1 (Fig. 5A)]. Cell surface immunolabeling with a 5D3-PE anti-BCRP antibody was used to assess the cell surface expression of BCRP, which showed increased expression in the LLC-BCRP cells compared with the LLC-vector cells (Fig. 5B). To determine functionality, flow cytometry experiments with the fluorescent BCRP substrate pheophorbide were conducted, which showed that BCRP-mediated efflux was reversed in the presence of Ko143 (Fig. 5C). MTT cytotoxicity assays mirrored these results; the LLC-BCRP cells were resistant to MTX ($IC_{50} = 9.8 \mu$ M) compared with LLC-vector ($IC_{50} = 2.2 \mu$ M). Resistance in the LLC-BCRP line was reversed in the presence of Ko143 ($IC_{50} = 1.0 \mu$ M; Fig. 5D).

The susceptibility of compounds to be transported by P-gp or BCRP can be assessed using transporter-expressing polarized LLC-PK1 cells

grown in permeable transwell filters (Taub et al., 2005). Consistent with the fact that TQR is a substrate of BCRP (Kannan et al., 2011), the basolateral to apical transport of [3 H]TQR was significantly higher than in the apical to basolateral direction with LLC-BCRP cells (Fig. 6; Table 2). The addition of inhibitors Ko143 (1 μ M) or elacridar (10 μ M) resulted in equal concentrations of [3 H]TQR on either side (Fig. 6; Table 2). However, in LLC-MDR1 cells, no appreciable transport was measured in either direction (Fig. 6; Table 2), and the addition of inhibitor DCPQ (1 μ M; Fig. 6; Table 2) or TQR (5 μ M; Table 2) had no effect. Similarly, no transport of [3 H]TQR was detected with LLC-vector cells (Table 2) or with LLC-EQ cells (Fig. 6; Table 2). The LLC-MDR1 cells transported the positive control substrate [3 H]paclitaxel, resulting in a 5.3-fold basolateral-to-apical/apical-to-basolateral transport (not shown).

Discussion

Despite the minimal literature on the interaction of TQR with P-gp, there exists confusion in the field as to whether TQR is a nontransported inhibitor or a substrate of P-gp. Using multiple in vitro assays and a range of concentrations, our results clearly show that TQR is an effective inhibitor of both human and mouse P-gp in vitro, consistent

Fig. 6. Directional transport of $[^3\text{H}]\text{TQR}$ is not P-gp-dependent. Basolateral-to-apical (solid line) and apical-to-basolateral (dashed line) transport of $[^3\text{H}]\text{TQR}$ across LLC-BCRP, LLC-MDR1, and LLC-EQ monolayers. Effluxed $[^3\text{H}]\text{TQR}$ concentrations are plotted as a function of time. $[^3\text{H}]\text{TQR}$ was added to either the apical or basolateral side of the monolayer. An aliquot (50 μL) of sample was taken at indicated time points and processed as in the *Materials and Methods* section. AB, apical-to-basolateral; BA, basolateral-to-apical; DMSO, dimethylsulfoxide.

with a similar characterization using hamster P-gp reported by Martin et al. (1999). None of the methods used provided any indication that TQR is a substrate of P-gp, whereas we have provided additional evidence that TQR is a substrate of BCRP.

The interaction of TQR with P-gp has been previously examined in several ways. There are a great deal of *in vivo* and *in vitro* data concerning the phenotypic alterations related to the functional inhibition of P-gp by TQR—for example, by imaging radiolabeled substrates, accumulation of fluorescent substrates in MDR cell lines or circulating lymphocytes, or sensitization of MDR xenografts or MDR cell lines to substrate chemotherapeutics (Fox and Bates, 2007).

The primary study on the mechanism of TQR is that by Martin et al. (1999), who examined the interaction of TQR with hamster P-gp in P-CH³B30 cells, a P-gp-expressing subline of the AuxB1 Chinese hamster ovary cell line. TQR was shown to inhibit P-gp-mediated cellular efflux of $[^3\text{H}]\text{vinblastine}$ ($\text{EC}_{50} = 487 \text{ nM}$) and $[^3\text{H}]\text{paclitaxel}$ ($\text{EC}_{50} = 25 \text{ nM}$). Critically, the authors tested whether TQR was a substrate of P-gp. Steady-state accumulation of $[^3\text{H}]\text{TQR}$ was equivalent in parent and P-gp-expressing cells over the range 2–150 nM (and was not affected by the P-gp inhibitor elacridar). At lower doses (10, 5 nM), the CH³B30 cells bound more $[^3\text{H}]\text{TQR}$ than parent cells, suggesting specific binding. This specific binding was shown in membrane preparations

from CH³B30 cells (compared with AuxB1 cells), with a calculated K_D of 5.1 nM ($n = 7$), a rapid association rate (10 times faster than vinblastine), and a slow dissociation rate. It is noteworthy that the experimental replicates were numerous—TQR is highly lipophilic with a clog P of 6.1 (Egger et al., 2007), and this causes high binding to plastic and the need for a large number of replicates (Callaghan, 2013).

The data presented here are consistent with those of Martin et al. (1999) for hamster P-gp; in cells expressing mouse or human P-gp, we observed greater cell binding than in parental cells with 3 nM $[^3\text{H}]\text{TQR}$ (Fig. 2), with a relatively high background due to lysosomal trapping (Kannan et al., 2011). Displacement of $[^3\text{H}]\text{TQR}$ (3 nM) by a high concentration of cold TQR is not clean—displacement is observed in P-gp-expressing cells, but TQR is elevated in parental cells to parity with the P-gp-expressing cells (Fig. 2). We believe this is related to high levels of cold TQR that block binding sites on plastic and elevate the free (non-plastic-bound) fraction of $[^3\text{H}]\text{TQR}$, although other possibilities exist, such as competition for lysosomal degradation. Consistent with our findings, displaceable specific binding has been observed before with the BCRP inhibitor $[^3\text{H}]\text{Ko143}$ (Weidner et al., 2015), and with the P-gp inhibitor $[^3\text{H}]\text{BIBW22 BS}$ (4-(N-(2-hydroxy-2-methylpropyl)ethanolamino)-2,7-bis(cis-2,6-dimethylmorpholino)-6-phenylpteridine) (Liu et al., 1996).

ATPase assays showed that TQR suppressed basal ATPase activity in membranes expressing human or mouse P-gp, with an IC_{50} of approximately 100 nM (Fig. 3). Martin et al. (1999) reported the same effect with an IC_{50} value of $43 \pm 9 \text{ nM}$ against hamster P-gp. However, a recent study by Loo and Clarke (2014) reported that TQR stimulates the ATPase activity of “Cys-less” human P-gp. The authors suggested that TQR acts by trapping P-gp in a closed conformation, preventing substrate transport while stimulating ATP hydrolysis. This effect has not been observed in our work with wild-type human or M107L P-gp.

We examined TQR using other transporter assays in an attempt to tease out possible TQR–P-gp substrate interactions. By utilizing TQR’s inherent fluorescence, we were able to directly measure its uptake into cells using flow cytometry (Fig. 4B). No change in the efflux of TQR was observed with inhibitor, although in cells expressing BCRP, TQR behaved as a weak substrate (Fig. 4C). We also conducted transwell assays using

TABLE 2
Ratios of BA/AB transport of $[^3\text{H}]\text{TQR}$ by LLC cells

Cells	Condition	BA/AB (Fold)
LLC-vector	DMSO	1.1 $\pm$ 0.1
LLC-vector	DCPQ (1 μM)	1.0 $\pm$ 0.2
LLC-vector	TQR (5 μM)	0.9 $\pm$ 0.1
LLC-BCRP	DMSO	2.1 $\pm$ 0.1
LLC-BCRP	Ko143 (1 μM)	1.1 $\pm$ 0.1
LLC-BCRP	Elacridar (10 μM)	1.3 $\pm$ 0.1
LLC-MDR1	DMSO	1.2 $\pm$ 0.2
LLC-MDR1	DCPQ (1 μM)	1.2 $\pm$ 0.1
LLC-MDR1	TQR (5 μM)	1.1 $\pm$ 0.1
LLC-EQ	DMSO	1.0 $\pm$ 0.1

BA/AB, basolateral-to-apical/apical-to-basolateral; DMSO, dimethylsulfoxide.

[³H]TQR in LLC-PK1 cells transfected with human P-gp or BCRP. The LLC-BCRP cells were generated in this study (Fig. 5), whereas the LLC-MDR1, LLC-vector, and LLC-EQ cells have previously been characterized (Fung et al., 2014a,b). Although BCRP-transfected cells transported [³H]TQR, P-gp and vector-transfected cells did not (Fig. 6; Table 2).

Two PET studies using [¹¹C]TQR have shown an increase in radioactivity in the brains of Mdr1a/b^(-/-)Bcrp1^(-/-) mice compared with wild-type (WT), Mdr1a/b^(-/-), and Bcrp1^(-/-) mice (Bauer et al., 2010; Kawamura et al., 2010). Although these results were counterintuitive at the time, the authors concluded that [¹¹C]TQR was a nontransported inhibitor of P-gp because of existing in vitro data and data from another study, which showed increased [¹¹C]TQR signal in a rat model of P-gp overexpression compared with naïve rats (Kuntner et al., 2009). The authors followed up with a study evaluating the interaction of TQR and elacridar in vivo and in vitro (Bankstahl et al., 2013). Because the in vivo results echoed those of the previous study, it was concluded that TQR must be a substrate of P-gp. In vitro experiments assessing cellular accumulation of [³H]TQR in a transwell apparatus were reported (but the actual transwell data from the experiment were not disclosed), showing increased accumulation with addition of a P-gp inhibitor. However, this phenomenon, observed by the authors, can be easily explained by nonlinear kinetics, which we address later. We have found the opposite effect for TQR accumulation in P-gp-expressing cells (this study) using several different cell-based assays.

We have previously described the possible behavior of a radiolabeled P-gp inhibitor in vivo (Kannan et al., 2013), and the data outlined in this study further support our position. P-gp and BCRP have distinct mechanisms to block brain uptake of TQR: P-gp binds TQR, whereas BCRP transports it. Both of these mechanisms (binding and transport) have some spare capacity relative to the low concentrations of [¹¹C]TQR in typical PET experiments. That is, removing only P-gp or BCRP has little effect, as the spare capacity of the remaining transporter is able to compensate. However, in the presence of higher concentrations of nonradioactive TQR, the spare capacity is exhausted. For P-gp, higher concentrations of TQR saturate the binding sites and competitively displace [¹¹C]TQR; for BCRP, higher concentrations lead to substrate inhibition, allowing [¹¹C]TQR to enter the brain. The spare capacity of both P-gp and BCRP derives in part from their very high local concentrations in the capillary, which is estimated to be 40 nM for P-gp within the vascular volume of the capillary (Kannan et al., 2013). When the spare capacity of both transporters is exhausted (e.g., by high concentrations of nonradioactive tariquidar) or when both transporters are removed (e.g., genetic knockout), uptake of [¹¹C]TQR can be easily measured, because the radioactive signal is amplified by ionic trapping of [¹¹C]TQR in lysosomes.

The data outlined here present a comprehensive evaluation of the interaction of TQR with P-gp. Using cytotoxicity, flow cytometry, ATPase, radioactive accumulation, and transwell assays, we have systematically shown that TQR is a potent inhibitor of human and mouse P-gp. More importantly, we have shown that, at the concentrations detectable by the aforementioned assays, TQR does not behave as a substrate of P-gp. This is of particular importance given the use of [¹¹C]TQR in PET studies to measure the density of P-gp in vivo.

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Authorship Contributions

Participated in research design: Weidner, Fung, Mulder, Innis, Gottesman, Hall.

Conducted experiments: Weidner, Fung, Kannan, Moen, Kumar, Hall.

Performed data analysis: Weidner, Fung, Kannan, Moen, Kumar, Hall.

Wrote or contributed to the writing of the manuscript: Weidner, Fung, Kannan, Moen, Kumar, Mulder, Innis, Gottesman, Hall.

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