The Inhibitor Ko143 Is Not Specific for ABCG2

Lora D. Weidner, Sami S. Zoghbi, Shuiyu Lu, Suneet Shukla, Suresh V. Ambudkar, Victor W. Pike, Jan Mulder, Michael M. Gottesman, Robert B. Innis, and Matthew D. Hall

Molecular Imaging Branch, National Institute of Mental Health, Bethesda, Maryland (L.D.W., S.S.Z., S.L., V.W.P., R.B.I.); Karolinska Institutet, Department of Neuroscience, Stockholm, Sweden (L.D.W., J.M.); and Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland (S.S., S.V.A., M.M.G., M.D.H.)

Received April 24, 2015; accepted June 26, 2015

ABSTRACT

Imaging ATP-binding cassette (ABC) transporter activity in vivo with positron emission tomography requires both a substrate and a transporter inhibitor. However, for ABCG2, there is no inhibitor proven to be specific to that transporter alone at the blood-brain barrier. 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], a nontoxic analog of fungal toxin fumitremorgin C, is a potent inhibitor of ABCG2, although its specificity in mouse and human systems is unclear. This study examined the selectivity of Ko143 using human

embryonic kidney cell lines transfected with ABCG2, ABCB1, or ABCC1 in several in vitro assays. The stability of Ko143 in rat plasma was measured using high performance liquid chromatography. Our results show that, in addition to being a potent inhibitor of ABCG2, at higher concentrations ($\geq 1 \mu\text{M}$) Ko143 also has an effect on the transport activity of both ABCB1 and ABCC1. Furthermore, Ko143 was found to be unstable in rat plasma. These findings indicate that Ko143 lacks specificity for ABCG2 and this should be taken into consideration when using Ko143 for both in vitro and in vivo experiments.

Introduction

The treatment of brain disorders is often plagued with obstacles when it comes to maintaining optimal drug efficacy, in part due to the body's resistance to the respective treatments. Although several hypotheses exist regarding the mechanisms that underlie drug resistance, one of the most prominent involves the overexpression of ATP-binding cassette (ABC) transporters at the blood-brain barrier (BBB) (Loscher and Potschka, 2005a; Kwan et al., 2011). These membrane-bound ATP-driven efflux pumps serve to protect tissues by actively effluxing xenobiotics from cells into the extracellular space (Gottesman et al., 2002). For this reason, drug brain penetrance of transporter substrates is limited, and it is thought that increased transporter expression may exacerbate the situation (Gottesman et al., 2002; Loscher and Potschka, 2005b).

P-glycoprotein (ABCB1) was the first mammalian ABC transporter discovered and is also the most extensively studied. Increased ABCB1 expression has been observed in brain tissue from patients with epilepsy, brain tumors, and HIV, providing further evidence for its role in drug resistance (Tishler et al.,

1995; Langford et al., 2004). However, in the case of drug-resistant epilepsy (DRE), for example, whether ABC transporters are truly overexpressed remains uncertain partly due to a lack of appropriate control tissue samples. Excluding postmortem tissue, brain tissue samples from individuals whose condition is managed successfully by antiepileptic drugs are not available to determine whether observed increases in ABCB1 are unique only to those suffering from drug resistance (Loscher and Potschka, 2005a; Liu et al., 2012a).

Performing positron emission tomography (PET) scans in humans using a radiolabeled substrate and a nonradio-labeled selective inhibitor is a noninvasive alternative to measure ABC transporter activity (Kannan et al., 2009). ABCB1 function can be assessed with radioligands such as [^{11}C]N-desmethyl-loperamide or [^{11}C]verapamil, both of which are avid substrates (Kannan et al., 2010; Romermann et al., 2013). [^{11}C]N-Desmethyl-loperamide uptake into the brain before and after inhibition of ABCB1 with tariquidar can be quantified, giving insight into the activity of ABCB1 in healthy individuals in vivo (Kreisl et al., 2010). Recently, Feldmann et al. (2013) reported decreased uptake of [^{11}C]verapamil in the temporal lobe of individuals with DRE compared with medication-responsive epileptics and healthy controls. This was the first in vivo evidence of ABCB1 overexpression in patients with DRE.

This work was supported in part by the Intramural Research Program of the National Institutes of Health [National Institute of Mental Health and National Cancer Institute].

dx.doi.org/10.1124/jpet.115.225482.

ABBREVIATIONS: ABC, ATP-binding cassette; BBB, blood-brain barrier; CAM, calcein-AM; DCPQ, (2*R*)-anti-5-[3-[4-(10, 11-dichloromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy]quinoline trihydrochloride; DRE, drug-resistant epilepsy; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; FTC, fumitremorgin C; HEK, human embryonic kidney; HPLC, high performance liquid chromatography; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Ko143, [(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; MeCN, acetonitrile; MK571, 3-[[[3-[(1*E*)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid; MTX, mitoxantrone; P-18, purpurin-18; PET, positron emission tomography; PPA, pheophorbide a; rh123, rhodamine-123; TFA, trifluoroacetic acid.

Breast cancer resistance protein (ABCG2) is another ABC transporter widely coexpressed with ABCB1 and may be equally important in drug biodistribution (Sisodiya et al., 2006; Ito et al., 2011; Kannan et al., 2013). Unlike ABCB1, no specific substrate exists for ABCG2 that has been radiolabeled and administered in vivo. The [2-¹¹C-carbonyl]dantrolene, a substrate of ABCG2, was synthesized by Takada et al. (2010), but has not yet been tested in vivo. Therefore, the discovery of an ABCG2 substrate amenable to radiolabeling remains an active area of research (Wanek et al., 2013). Furthermore, to implement a PET imaging agent for ABCG2 function, a specific (nonradioactive) inhibitor of ABCG2 is required. The compound Ko143 [(3*S*,6*S*,12*aS*)-1,2,3,4,6,7,12,12*a*-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxypyrazino[1',2':1,6]pyrido[3,4-*b*]indole-3-propanoic acid 1,1-dimethylethyl ester] is reported to be a potent ABCG2 inhibitor and may be a candidate for application in PET imaging studies along with a suitable radiotracer. Derived from fumitremorgin C (FTC) as a nontoxic analog, Ko143 (Fig. 1A) has been used over the past decade to examine the interaction between ABCG2 and pharmaceutical drugs in vitro and in vivo (Allen et al., 2002). For example, Ko143 was found to elevate mouse brain levels of the ABCG2 substrate D-luciferin in a bioluminescence-based imaging strategy (Bakhsheshian et al., 2013b). Given the frequent use of Ko143, characterization of Ko143 in terms of specificity in both human and mouse cell lines is of particular significance given the known species differences of ABC transporters.

Therefore, the aim of this study was to establish the efficacy and specificity of the inhibitor Ko143 concerning three ABC transporters: ABCG2, ABCB1, and multidrug resistance-associated protein 1 (ABCC1). We determined the selectivity using several cell-based in vitro assays with multiple cell lines expressing either human or mouse ABCG2 and ABCB1 or human ABCC1. The hydrolysis of Ko143 by plasma esterases was examined, and the transporter inhibitory activity of Ko143 acid (Fig. 1B), the hydrolytic metabolite of Ko143, was assessed.

Materials and Methods

Chemicals. [³H]Ko143 was synthesized by Moravsek Biochemicals (Brea, CA). The final product had a specific activity of 5.0 Ci/mmol and a concentration of 1.0 mCi/ml, whereas high performance liquid

chromatography (HPLC) indicated a radiochemical purity of 95.4%. Ko143 (purity >99%) was purchased from Tocris Bioscience (Minneapolis, MN), and pheophorbide a (PPA) and purpurin-18 (P-18) were obtained from Frontier Scientific (Logan, UT). Flavopiridol was obtained from the National Cancer Institute In Vitro Anticancer Drug Discovery Screen (Bethesda, MD). DCPQ [(2*R*)-anti-5-[3-[4-(10,11-dichloromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy]quinoline trihydrochloride] was provided by Dr. Victor W. Pike, National Institute of Mental Health (Bethesda, MD). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell Lines. Cultured cell lines were used to investigate the interaction between ABC transporters and Ko143. These included human embryonic kidney (HEK)-293 cells transfected with human ABCG2 (HEK G2), ABCB1 (HEK B1), ABCC1 (HEK C1), and plasmid control (HEK PC), which were provided by S. Bates (National Cancer Institute, Bethesda, MD) (Müller et al., 2002; Robey et al., 2003, 2011). HEK PC cells served as the parental line. Additional cell lines used to examine ABCG2 were the human breast cancer cell line MCF-7 and its ABCG2-expressing variant MCF-7/FLV10000, as well as the mouse embryonic fibroblast cell line MEF 3.8 and its Abcg2-expressing variant MEF M32 (mouse G2). The mouse embryonic fibroblast cell line 3T3 and its Abcb1a-expressing variant C3M (mouse B1) were used along with the HEK cell line to study ABCB1. A mouse Abcc1 cell line was not included due to a lack of a stably transfected cell line. HEK cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 2 mg/ml G418 (a gentamicin-related selecting agent) to ensure stable transporter expression. Occasionally, 4 μ M etoposide was added to HEK C1 cells to enforce ABCC1 expression. The remaining cell lines were grown in Dulbecco's modified Eagle's medium with the following supplementation for the resistant lines: 10 μ g/ml flavopiridol for MCF7/FLV10000, 32 nM mitoxantrone (MTX) for MEF M32, and 1 μ g/ml colchicine for C3M. Both culture mediums were supplemented with 10% fetal bovine serum (FBS), 5 mM glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin (Lee et al., 1998). All cell lines were grown at 37°C in 5% CO₂.

Animals. Blood from one Sprague-Dawley rat (530 g) was drawn via cardiac puncture and subsequently used for HPLC experiments. Animal experiments were performed in accordance with the *Guide for Care and Use of Laboratory Animals* (NIH, 2001) and were approved by the National Institute of Mental Health Animal Care and Use Committee.

Cytotoxicity Assay. To determine whether Ko143 interacts with ABCB1 and ABCC1, cytotoxicity assays were performed to measure the extent to which Ko143 inhibited efflux of transporter-specific cytotoxic substrates. Cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). The protocol was followed as described by Brimacombe et al. (2009), except with the following modifications. Cells were seeded at a density of 4000 cells/well in 100 μ l media. Serial dilutions of cytotoxic drugs were made in either EMEM or Dulbecco's modified Eagle's medium, and an additional 100 μ l drug-containing media was added to each well. The following transporter-substrate cytotoxic drugs were used: MTX for ABCG2, paclitaxel for ABCB1, and doxorubicin for ABCC1. The outcome measure was IC₅₀, which indicates the concentration of cytotoxic drug required to decrease cell viability by 50% compared with untreated control cells (Brimacombe et al., 2009). Resistance ratio was then calculated by dividing the mean IC₅₀ from three separate observations of the transporter-expressing cell line by that of the parental cell line.

Inhibition of Transporter Function. The efflux of transporter-specific fluorescent substrates was measured by flow cytometry as an additional method of determining the interaction between Ko143 and the ABC transporters. Cells expressing human or mouse ABCB1 and human ABCC1 were suspended in EMEM containing 10% FBS at a density of 2.0×10^5 cells/ml. The cells were then washed with Iscove's modified Dulbecco's medium containing 5% FBS and centrifuged to form a pellet. The cells were resuspended in media

Fig. 1. Structure of Ko143 (A) and Ko143 acid (B).

containing either the fluorescent ABCB1 substrate rhodamine-123 (rh123; 1.3 μM) or the fluorescent ABCC1 substrate calcein-AM (CAM; 0.25 μM) under the following conditions: no drug (untreated), inhibitor-treated (positive control), and Ko143-treated. Cyclosporin A (5 μM) and MK571 (3-[3-[(1*E*)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid; 50 μM) acted as the positive control inhibitors for ABCB1 and ABCC1, respectively. The cells were incubated for 45 minutes in the dark at 37°C, after which they were washed and resuspended in 300 μl 0.1% bovine serum albumin in phosphate-buffered saline (pH 7.4). The samples were then kept on ice until analysis (within 1 hour), which was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The geometric mean of fluorescence intensity was recorded for a total of 10,000 cells per sample in the FL-2 (rh123) or the FL-1 (CAM) channels. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

The flow cytometry experiments using ABCG2-expressing cells were performed as described above, except with the following modifications. The fluorescent substrates used were MTX, PPA, Hoechst 33342 (Hoechst), JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), and P-18 at a concentration of 5 μM . FTC (5 μM) acted as the positive control inhibitor. Cells under each condition were incubated for 30 minutes in the dark at 37°C. Data were recorded using a LSR II flow cytometer (BD Biosciences) using an excitation emission wavelength of 488/575 for JC-1. The excitation emission wavelengths for the remaining fluorescent substrates are outlined in Bakhsheshian et al. (2013a).

ATPase Assays. To determine how Ko143 interacts with ABCG2 and ABCB1, ATPase assays were performed using crude membranes isolated from Hi-five insect cells expressing ABCB1 or ABCG2. The protocol described in Kannan et al. (2011) was followed. Briefly, the membrane vesicles in ATPase assay buffer [50 mM 2-(*N*-morpholino) ethanesulfonic acid-Tris buffer (pH 6.8), 50 mM KCl, 5 mM sodium azide, 1 mM EGTA, 1 mM ouabain, 10 mM MgCl_2 , and 2 mM dithiothreitol] were incubated in varying concentrations of Ko143 with or without beryllium fluoride (0.2 mM beryllium sulfate plus 2.5 mM NaF). The ATP hydrolysis was measured by estimating the release of inorganic phosphate after incubation with 5 mM ATP, as described previously (Shukla et al., 2006).

Uptake of [^3H]Ko143 in Cells. The specificity of Ko143 for ABCG2 was also measured using radioactivity uptake assays. Cells were seeded at a density of 2.5×10^5 cells/ml media per well in a 24-well plate. A time-course assay was performed to determine the time at which a stable level of [^3H]Ko143 was reached, as follows: media containing [^3H]Ko143 was added to each well and subsequently aspirated at specific time points between 0 and 45 minutes. Three wells received nonradioactive media to account for background signal, whereas another three were reserved for cell counts to standardize

accumulation. After media was removed, cells were washed in phosphate-buffered solution (pH 7.4), and 100 μl 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 fmol/ 10^6 cells.

Subsequent accumulation assays were performed over 30 minutes. Media containing [^3H]Ko143 was added to parent and transporter-expressing cells with and without the addition of a transporter-specific inhibitor. The cells were incubated at 37°C for 30 minutes before the radioactive media was aspirated, and the remainder of the protocol was followed, as previously described. The following inhibitors were used: Ko143 (1 μM for ABCG2), FTC (5 μM for ABCG2), DCPQ (5 μM for ABCB1), and MK571 (50 μM for ABCB1).

Stability of Ko143 in Rat Plasma. Reversed-phase chromatography was used to determine the stability of Ko143 over time in rat plasma. A mobile phase composition of 80% MeOH:20% H_2O :0.1% Et_3N was found to be optimal to give an elution time (t_R = 9.47 minutes) sufficiently long for Ko143 to be resolved from any polar metabolites (t_R = 4.38 minutes). Whole blood was collected with a heparin-coated syringe by means of a cardiac puncture. The blood was then centrifuged at 1800g for 5 minutes, and the resulting plasma supernatant was collected into separate tubes. Plasma was immediately distributed into tubes for the following conditions: plasma plus 1 mM Ko143, plasma plus 50 μl saturated NaF solution (an esterase inhibitor) and 1 mM Ko143, and DMSO plus 1 mM Ko143 (negative control). Ko143 was added to the appropriate tubes at time 0, and samples were taken at 5, 10, 15, 30, and 60 minutes at room temperature. The addition of 1 mM Ko143 to the plasma resulted in a saturated concentration in which the samples were spun down, and the clear supernatant was removed and subsequently used for experimentation. We elected to start with an excessive concentration of Ko143 to ensure detectability of metabolites as well as the parent. For each time point, samples were immediately transferred to tubes containing acetonitrile (MeCN) to stop the reaction and deproteinize the samples. The samples were subsequently centrifuged for 1 minute at 10,000g. A total of 100 μl clear supernatant was injected onto a X-terra Prep C_{18} column (7.8 $\times$ 300 mm; Waters, Milford, MA) and eluted at 4 ml/min. Absorbance was determined at 228 nm wavelength.

Synthesis of Ko143 Acid. Hydrolysis of *t*-butyl ester in trifluoroacetic acid (TFA) (Turkman et al., 2011) was adapted for the preparation of Ko143 acid. Ko143 (7.93 mg) and TFA (300 μl) were added to a Pyrex glass tube fitted with rubber septa and N_2 gas balloon and heated in an oil bath at 50°C for 15 minutes. TFA from the resulting brown solution was removed by a stream of N_2 gas. The residue was dissolved in MeCN (400 μl), filtered through a small plug of silica gel to obtain a light yellow solution. Evaporation of MeCN under a stream of N_2 gas gave a crude product (7.94 mg) as a light

TABLE 1
Effect of Ko143 on the cytotoxicity of transporter-specific substrates in human cell lines

Cell Line	Cytotoxic Drug	Cytotoxicity Value (IC_{50})											
		Drug Alone		Drug + 1 nM Ko143		Drug + 10 nM Ko143		Drug + 100 nM Ko143		Drug + 1 μM Ko143		Drug + 5 μM Ko143	
		IC_{50}	RR	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>		<i>nM</i>	
HEK ABCG2	MTX	138 $\pm$ 20	28	98 $\pm$ 8.3	20 ^a	54 $\pm$ 1	11 ^b	1 $\pm$ 0.3	0.2 ^c	2 $\pm$ 0	0.4 ^c	1.4 $\pm$ 0.3	0.3 ^c
HEK PC	MTX	5 $\pm$ 0											
HEK ABCB1	Paclitaxel	1243 $\pm$ 40	414	—		—		1205 $\pm$ 43	402	393 $\pm$ 37	131 ^d	67 $\pm$ 13	22 ^d
HEK PC	Paclitaxel	3 $\pm$ 1											
HEK ABCC1	Doxo	2481 $\pm$ 75	92	—		—		3136 $\pm$ 226	116 ^b	652 $\pm$ 292	24 ^c	222 $\pm$ 27	8 ^d
HEK PC	Doxo	27 $\pm$ 5											

Doxo, doxorubicin; RR, resistance ratio (the quotient of the IC_{50} value of the resistant cell line to that of the parental line).

^a P < 0.05 (α < 0.05, from initial IC_{50} value of resistant cell line) by Student's two-tailed *t* test.

^b P < 0.01 (α < 0.05, from initial IC_{50} value of resistant cell line) by Student's two-tailed *t* test.

^c P < 0.001 (α < 0.05, from initial IC_{50} value of resistant cell line) by Student's two-tailed *t* test.

^d P < 0.0001 (α < 0.05, from initial IC_{50} value of resistant cell line) by Student's two-tailed *t* test.

TABLE 2

Effect of Ko143 on the cytotoxicity of transporter-specific substrates in mouse cell lines

Cell Line	Cytotoxic Drug	Cytotoxicity Value (IC ₅₀)									
		Drug Alone		Drug + 1 nM Ko143		Drug + 10 nM Ko143		Drug + 100 nM Ko143		Drug + 1 μ M Ko143	
		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>	
Mouse ABCG2	MTX	0.23 $\pm$ 0.02	46	0.15 $\pm$ 0.01	30 ^a	0.09 $\pm$ 0.02	18 ^b	0.001 $\pm$ 0	0.2 ^c	0.003 $\pm$ 0	0.6 ^c
Parent	MTX	0.005 $\pm$ 0									
Mouse ABCB1	Paclitaxel	19 $\pm$ 1	826	19 $\pm$ 1	826	19 $\pm$ 1	826	19 $\pm$ 1	826	16 $\pm$ 0.3	696 ^a
Parent	Paclitaxel	0.023 $\pm$ 0									

RR, resistance ratio (the quotient of the IC₅₀ value of the resistant cell line to that of the parental line).^a $P < 0.01$ ($\alpha < 0.05$, from initial IC₅₀ value of resistant cell line) by Student's two-tailed t test.^b $P < 0.001$ ($\alpha < 0.05$, from initial IC₅₀ value of resistant cell line) by Student's two-tailed t test.^c $P < 0.0001$ ($\alpha < 0.05$, from initial IC₅₀ value of resistant cell line) by Student's two-tailed t test.

yellow solid. The crude product (5.0 mg) was dissolved in MeCN (2 ml) and divided into four portions. Each portion of the solution (0.5 ml) was premixed with 0.5% aqueous triethylamine solution (pH = 9, 0.5 ml), and then injected on a semipreparative column (Luna, C18, 10 μ m, 250 $\times$ 10 mm), eluted at 6 ml/min with a mixture of 0.1% *aq.* TFA (A) and MeCN (B), with eluent monitored for absorbance at 298 nm. The initial composition of B was at 35% for 1 minute and increased to 80% after 10 minutes. HPLC fractions at 6.5–7.5 minutes were collected and combined. After removal of solvent, the residue was dissolved in dichloromethane (400 μ l) and transferred to a small glass vial (4 ml). Dichloromethane was removed under a stream of N₂ gas, then under high vacuum to afford Ko143 acid (1.44 mg, >98% pure). Liquid chromatography–mass spectrometry (m/z 414.2 for M+H⁺) and analytical HPLC (t_R = 5.3 minutes) analyses confirmed the identity of the acid product.

Statistical Analysis. Data are expressed as mean $\pm$ S.D. from three observations for the inhibition, cytotoxicity, ATPase, and radioactivity accumulation assays, whereas the data for HPLC were drawn from one observation. Statistical significance was evaluated for the cytotoxicity assays via Student's t test (unpaired, two-tailed, $\alpha = 0.05$) and for inhibition and radioactivity accumulation assays by a one-way analysis of variance, followed by the Bonferroni post- t test ($\alpha = 0.05$).

Results

HEK-293 cells transfected with human ABCG2, ABCB1, ABCC1, or plasmid control were used to examine the specificity of Ko143. This ensured stable expression of individual ABC transporters (Müller et al., 2002; Robey et al.,

2003, 2011), and, because each cell line was transfected with only one transporter, concern over low levels of other transporters interfering with the results was minimal. To confirm functional transporter expression, we examined the resistance of each cell line to a transporter-specific cytotoxic substrate (Table 1). Resistance was indicated by a higher IC₅₀ value for each cytotoxic drug. Compared with HEK PC, HEK G2 was 28 times more resistant to MTX, HEK B1 was 414 times more resistant to paclitaxel, and HEK C1 was 140 times more resistant to doxorubicin. In murine cell lines compared with their respective parental cells, mouse G2 cells were 46 times more resistant to MTX and mouse B1 cells were 826 times more resistant to paclitaxel (Table 2). Using a cytotoxicity assay, we determined the toxicity of Ko143 against the HEK cells to ensure that our results were not associated with increased cell death due to Ko143. Concentrations higher than 5 μ M were found to be toxic to all HEK cell lines after incubation for 72 hours (data not shown).

At Higher Concentrations, Ko143 Is Not Specific for ABCG2. We examined the inhibitory effect of Ko143 by measuring the sensitization of each cell line to a transporter-specific cytotoxic substrate. As expected, concentrations as low as 10 nM Ko143 significantly decreased (2.5-fold) the IC₅₀ of MTX for HEK G2 cells ($P < 0.01$; Table 1) and mouse G2 cells ($P < 0.001$; Table 2) compared with untreated cells. HEK B1 and HEK C1 cells were sensitized by 1 μ M Ko143 to paclitaxel ($P < 0.0001$) and doxorubicin ($P < 0.001$), respectively, whereas 5 μ M Ko143

Fig. 2. The effect of Ko143 on the accumulation of a fluorescent substrate of ABCG2, ABCB1, or ABCC1. Fluorescent substrates used were MTX (5 μ M) for ABCG2, rh123 (1.3 μ M) for ABCB1, and CAM (0.25 μ M) for ABCC1. Bars represent mean fluorescence from three experiments $\pm$ S.D. For each experiment, accumulation was defined as mean peak fluorescence intensity in parental and transporter-expressing cells without the addition of an inhibitor (white bars), as well as transporter-expressing cells with varying concentrations of Ko143 (gray bars), and normalized against accumulation in parental cells. Positive control inhibitors (striped bars) used were FTC (5 μ M for ABCG2), cyclosporin A (CSA; 5 μ M for ABCB1), and MK571 (50 μ M for ABCC1). *** $P < 0.001$, **** $P < 0.0001$ ($\alpha < 0.05$, from baseline accumulation in resistant cell line) by one-way analysis of variance.

Fig. 3. Accumulation of the fluorescent substrate rh123 (1.3 μ M) in cells expressing human ABCB1 (A) and mouse B1 (B). For each experiment, accumulation was defined as mean peak fluorescence intensity in parental and transporter-expressing cells without the addition of an inhibitor (white bars), as well as transporter-expressing cells with varying concentrations of Ko143 (shaded bars), and with the addition of the positive control inhibitor cyclosporin A (CSA; striped bars; 5 μ M). Data normalized to accumulation in parental cells 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.

sensitized both cell lines 18-fold (P < 0.0001; Table 1). Mouse B1 cells were also sensitized to paclitaxel by 1 μ M Ko143 compared with untreated resistant cells (P < 0.01; Table 2).

Flow cytometry was used to examine efflux of fluorescent substrates of each transporter. Higher concentrations of Ko143 could be used for these assays because of the short incubation time (≤ 45 minutes). All concentrations of Ko143 tested increased the accumulation (due to inhibition of efflux) of MTX in HEK G2 cells (P < 0.0001) at least 3.5-fold compared with baseline accumulation in these cells (Fig. 2). Accumulation of the ABCB1 substrate rh123 increased significantly in HEK B1 cells after administration of 20 μ M (P < 0.001), 50 μ M (P < 0.0001), and 100 μ M (P < 0.0001) Ko143, compared with untreated HEK B1 cells (Fig. 2). No effect was observed at lower concentrations (data not shown). In mouse B1 cells, we observed a greater increase of rh123 accumulation with 20 (2-fold increase, P < 0.0001) and 50 (3-fold increase, P < 0.0001) μ M Ko143 (Fig. 3). Ko143 also inhibited ABCC1 in HEK C1 cells as indicated by a 4-fold and 7-fold increase in accumulation of CAM after 10 and 20 μ M (P < 0.0001) were administered, respectively (Fig. 2). Positive control inhibitors were used as indicators of maximal ABC transporter inhibition. Compared with untreated cells, 5 μ M FTC resulted in a 3-fold higher accumulation of MTX in both HEK cells and mouse G2 cells, 5 μ M cyclosporin A resulted in an 11-fold higher accumulation of rh123 in HEK cells and a 4-fold higher accumulation in mouse B1 cells, and 50 μ M MK571 resulted in a 7-fold higher accumulation of CAM in HEK cells (Fig. 2).

Ko143 Inhibits Both Human and Mouse ABCG2. To identify possible differences between species, we measured

the effect of Ko143 on the accumulation of five different fluorescent substrates (5 μ M) in HEK G2 and mouse G2 cells. Of the five tested substrates, significant differences in Ko143 inhibitory IC_{50} were observed between human and mouse cell lines for MTX, Hoechst, and P-18; no significant differences were found for PPA or JC-1 (Fig. 4; Table 3).

Ko143 Stimulates ABCB1 ATPase Activity. We used an ATPase assay to determine the ATPase activity of ABCG2 and ABCB1 in the presence of increasing Ko143 concentrations. At a concentration of 50 nM Ko143, we observed a 4-fold decrease in the ATPase activity of ABCG2, whereas 1 μ M Ko143 elicited a 6-fold decrease (IC_{50} = 9.7 nM; Fig. 5). This is consistent with the effect of high-affinity inhibitors on ABC transporters. In contrast, we observed a biphasic effect on ABCB1, with a stimulation of ATPase activity of ABCB1. For example, 10 μ M Ko143 resulted in peak orthophosphate levels 2-fold higher than basal, and at 100 μ M stimulation decreased from V_{max} (IC_{50} = 2.7 μ M; Fig. 5). The ATPase activity of ABCC1 was not measured as this transporter exhibits very low basal activity in insect cell membranes.

Accumulation of [3 H]Ko143 in ABCG2 Cells Is Displaced by ABCG2 Inhibitors. Radiolabeled Ko143 was used to determine whether Ko143 is transported by ABCG2, ABCB1, or ABCC1. We compared accumulation of [3 H]Ko143 in HEK G2, HEK B1, and HEK C1 cells to HEK PC cells. Cellular binding of [3 H]Ko143 in HEK G2 cells (838.7 ± 74.3 fmol/ 10^6 cells) was 2-fold higher than binding to HEK PC cells (359.5 ± 15.7 fmol/ 10^6 cells) and was displaced after incubation with 5 μ M FTC (333.3 ± 41 fmol/ 10^6 cells;

Fig. 4. Accumulation of five fluorescent substrates of ABCG2 measured in cells expressing human and mouse ABCG2 in the presence of Ko143. Dose-effect curves of fluorescence displayed as the mean fluorescence intensity across three observations $\pm$ S.D., normalized to the maximal fluorescence (10 μ M Ko143). Each fluorescent compound was tested at 5 μ M: MTX, PPA, Hoechst 33342 (Hoechst), JC-1, and P-18.

TABLE 3
Effect of Ko143 on human- and mouse-mediated ABCG2 efflux

Cell Line	Ko143 IC ₅₀				
	MTX	PPA	Hoechst	JC-1	P-18
Human ABCG2	13 ^a	74	100 ^a	112	516 ^b
Mouse ABCG2	484	126	684	29	754

Hoechst, Hoechst 33342.

^a $P < 0.0001$ ($\alpha < 0.05$, between human and mouse lines) by Student's two-tailed t test.

^b $P < 0.001$ ($\alpha < 0.05$, between human and mouse lines) by Student's two-tailed t test.

$P < 0.0001$), whereas HEK PC binding remained unaffected (349.5 ± 14.6 fmol/ 10^6 cells) (Fig. 6A). In a separate experiment, 1 μ M Ko143 also effectively decreased [3 H]Ko143 binding 2-fold ($P < 0.01$) in HEK G2 cells (Fig. 6B). We repeated the assay with additional pairs of cells expressing human ABCG2 (MCF7/FLV10000; Fig. 6C) and mouse G2 (Fig. 6D) with their respective parent cell lines and found similar 2-fold decreases in [3 H]Ko143 binding after incubation with 5 μ M FTC or 1 μ M Ko143 (all $P < 0.0001$). We found no difference in binding of [3 H]Ko143 to HEK B1 cells (Fig. 6E), HEK C1 cells (Fig. 6F), or mouse B1 cells (data not shown) when compared with parental cells or after incubation with 5 μ M DCPQ (HEK B1 and mouse B1), or 50 μ M MK571 (HEK C1).

Ko143 Metabolite Does Not Inhibit the Function of ABC Transporters. Because Ko143 is a *tert*-butyl ester that would be susceptible to enzyme-mediated hydrolysis in plasma, we examined the rate of hydrolysis of the parent compound in rat plasma using HPLC. Ko143 quickly metabolized in rat plasma, with 50% degradation at ~ 12 minutes and no remaining parent compound at 60 minutes (Fig. 7A). Addition of the esterase inhibitor NaF completely halted the hydrolysis of Ko143 (Fig. 7A). Based on this observation, we examined whether the hydrolytic product of Ko143 that would result from the loss of the *tert*-butyl ester group had any inhibitory effect on transporters. The carboxylic acid derivative was synthesized from Ko143 (Fig. 1B), and we measured the effect of transporter inhibition using flow cytometry in the same manner as for Ko143 (Fig. 7, B–D). In HEK G2 cells, only the highest concentration of the Ko143 acid tested (100 μ M)

significantly increased MTX accumulation ($P < 0.01$) compared with untreated cells (Fig. 7B). No differences in accumulation of rh123 or CAM were observed at concentrations of Ko143 acid ranging from 1 to 100 μ M in HEK B1 or HEK C1 cells, respectively (Fig. 7, C and D).

Discussion

Our results indicate that, although Ko143 effectively inhibits ABCG2 at nanomolar concentrations, it also has an effect on ABCB1 and ABCC1 at higher micromolar concentrations. Few studies have been conducted in which the IC₅₀ of Ko143 was determined for transporters other than ABCG2. Allen et al. (2002) reported that the EC₅₀ of Ko143 was 1.0 ± 0.3 μ M for ABCB1-mediated paclitaxel resistance and 2.0 ± 0.4 μ M for ABCC1-mediated etoposide resistance. A literature and transporter database search performed by Matsson et al. (2009) flagged Ko143 as an inhibitor of ABCB1. However, they and Kuhnle et al. (2009) were unable to measure the exact IC₅₀ of Ko143 on cells expressing human ABCB1 using CAM efflux assays. Data from the present study suggest that this was probably due to the fact that the highest concentration of Ko143 used in both studies was 10 μ M. Nevertheless, Szolomajer-Csikós et al. (2013) also used a CAM efflux assay and found the IC₅₀ of Ko143 to be 8.74 ± 0.29 μ M for ABCB1 and 9.13 ± 1.07 μ M for ABCC1. In addition, 800 nM Ko143 partially inhibited basolateral to apical transport of amprenavir in cells overexpressing ABCB1 (Liu et al., 2014).

Because the in vivo use of Ko143 has predominately been in mice and rats, we repeated the cytotoxicity and flow cytometry experiments in cells expressing mouse ABCB1. Although the cytotoxicity data were similar to that found for human ABCB1-expressing cells (Table 2), the uptake of rh123 into mouse B1 cells was much greater than observed in HEK cells expressing human ABCB1 (Fig. 3). Testing Ko143 in vivo was outside the scope of our own study, but Liu et al. (2012b) found that, although the brain concentration of Ko143 was below the lower limit of detection in wild-type mice following a 3 mg/kg Ko143 dose, it was approximately three times higher in *Abcb1a/b*-knockout and *Abcb1a/b/Abcg2*-knockout mice than in *Abcg2*-knockout mice. Based on this, the authors hypothesized that Ko143 was a dual substrate of ABCG2 and ABCB1 (Liu et al., 2012b).

Our ATPase data for Ko143 with ABCB1 demonstrate that this compound interacts with ABCB1, but do not directly confirm that Ko143 is a substrate of ABCB1. Increased ATPase activity could be the indirect outcome of a different interaction between Ko143 and ABCB1, for example, a possible allosteric interaction. If Ko143 were a substrate of ABCB1, decreased accumulation of [3 H]Ko143 would be observed in transporter-expressing cells versus parental control cells, and diminished accumulation should be restored with the addition of an ABCB1-specific inhibitor. For example, Kannan et al. (2011) showed this substrate-transporter interaction with [3 H]tariquidar and ABCG2 in which cellular accumulation of [3 H]tariquidar in ABCG2-expressing cells increased to that of the parent after the addition of 5 μ M FTC. However, in the present study, [3 H]Ko143 did not behave as a substrate of human or mouse ABCB1 or human ABCC1 (Fig. 6, E and F).

Despite the interactions between Ko143 and other ABC transporters, the extant literature shows that Ko143 is currently the ABCG2 inhibitor of choice. Our results regarding

Fig. 5. ATPase activity of ABCG2 and ABCB1 in the presence of Ko143. Data points represent the beryllium fluoride-sensitive basal activity normalized to basal activity (100%) to calculate percent increase or decrease of activity in the presence of indicated concentration of Ko143. Each point is the mean from three separate experiments $\pm$ S.D.

Fig. 6. Accumulation of [³H]Ko143 in parental cells and HEK G2 cells (A and B), MCF7/FLV10000 cells (C), mouse G2 cells (D), mouse B1 cells (E), and HEK C1 cells (F). Accumulation is represented in untreated cells (black bars) and cells after administration of a positive control inhibitor (striped bars): Ko143 (1 μM for ABCG2), FTC (5 μM for ABCG2), DCPQ (5 μM for ABCB1), and MK571 (50 μM for ABCC1). Bars represent the average of three observations ± S.D. ***P* < 0.01, *****P* < 0.0001 (*α* < 0.05, from baseline accumulation in resistant cell line) by one-way analysis of variance.

Ko143's interaction with ABCG2 are in close agreement with the original study by Allen et al. (2002), who reported reversal of ABCG2-mediated resistance of MTX and topotecan with 25 nM Ko143 in human and mouse cell lines. We also determined the IC₅₀ of Ko143 inhibition of ABCG2 in human and mouse cell lines using multiple known ABCG2 fluorescent substrates (Fig. 4; Table 3), confirming efficacy in both species (Polgar et al., 2008; Strouse et al., 2010; Bakhsheshian et al., 2013a).

Ko143 is an ester (Fig. 1A), and therefore susceptible to hydrolysis by plasma esterases, which results in an acid as the major metabolite (Fig. 1B). We wondered whether this metabolite had any effect on the activity of ABCG2, ABCB1, or ABCC1, or even whether this acid was solely responsible for the observed interactions. We observed a rapid loss of the Ko143 HPLC peak in rat plasma, which was undetectable at 60 minutes in vitro (Fig. 7A). The pan-esterase inhibitor NaF completely prevented metabolism and resulted in steady levels

of Ko143 over time (Fig. 7A), indicating that enzymatic hydrolysis was responsible for the degradation. There is conflicting literature regarding the stability of Ko143; Zander et al. (2013) found that Ko143 was unstable ex vivo in mouse plasma, but detectable in vivo for up to 120 minutes in mice administered with 10 mg/kg (i.p.) Ko143. This could be due to a reservoir effect of poorly soluble Ko143 in vivo. Conversely, as part of a cassette dosing study, Liu et al. (2014) could not detect Ko143 in plasma, brain, or cerebrospinal fluid at multiple time points (0.25, 1, and 3 hours) after 3 mg/kg dosing, and concluded that it was likely due to rapid clearance.

We then synthesized the acid metabolite of Ko143 (see *Materials and Methods*) to determine whether Ko143 acid alone affected the efflux of fluorescent substrates. Other than relatively weak inhibition of efflux in HEK G2 cells, Ko143 acid had no effect on increased cellular accumulation of transporter-specific substrates (Fig. 7, B–D). Thus, the observed effect of

Fig. 7. The effect of the hydrolysis product of Ko143 on the uptake of fluorescent substrates in cells expressing ABCG2 (B), ABCB1 (C), or ABCC1 (D). White bars represent cells not treated with an inhibitor, and shaded bars represent cellular accumulation of transporter-specific fluorescent substrates in the presence of increasing concentrations of Ko143. The striped bars indicate accumulation in transporter-expressing cells after administration of a positive control inhibitor: Ko143 (1 μ M for ABCG2), DCPQ (5 μ M for ABCB1), and MK571 (50 μ M for ABCC1). Bars represent mean fluorescence intensity from three observations normalized to accumulation in parental cells $\pm$ S.D. Stability of the parent compound in rat plasma over 60 minutes before (black circles) and after administration of NaF (open squares) as measured by HPLC (A). Each data set normalized to 5-minute time points. ** $P < 0.01$ ($\alpha < 0.05$, from baseline accumulation in resistant cell line) by one-way analysis of variance. AUC, area under the curve.

Ko143 with the transporters studied in this work was primarily due to the parent compound and not to the hydrolysis product. This reinforces the importance of the stability data, given that Ko143 is not present in rat plasma after 60 minutes and the acid has essentially no effect on efflux.

We found that cellular accumulation of [3 H]Ko143 was approximately 2-fold higher in cells expressing human (Fig. 6, A–C) and mouse (Fig. 6D) ABCG2 than in their respective parent cell lines. Coaddition of either 5 μ M FTC or 1 μ M (cold) Ko143 competitively displaced Ko143 ABCG2 binding, decreasing accumulation to that of the parent (Fig. 6, A–D). The same pattern of accumulation was observed by Kannan et al. (2011) with the high-affinity ABCB1 inhibitor [3 H]tariquidar in cells expressing ABCB1, leading the authors to conclude that the higher accumulation in resistant cells was due to reversible binding of the inhibitor to the transporter. Furthermore, Liu and colleagues demonstrated specific binding of the ABCB1 inhibitor [3 H]BIBW22 BS, showing higher binding to ABCB1-expressing CEM/VLB^{1.0} cells compared with the parent CEM cells (Liu et al., 1996). We conclude that this elevated accumulation is due to specific binding of [3 H]Ko143 to ABCG2.

Although performing in vivo experiments was outside the realm of this study, we can extrapolate our findings to predict the interactions of Ko143 in vivo. Ko143 was previously found to have a plasma protein binding of 92–95% (Zhang et al.,

2011), and, based on this, the theoretical unbound plasma concentration in a 20 g mouse after a 10 mg/kg dose is 10.6 μ M (assuming a 95% plasma protein binding). At this concentration, Ko143 would effectively inhibit ABCB1 and ABCC1 in addition to ABCG2 according to our in vitro results, but, given how quickly Ko143 is metabolized in plasma, it is safe to assume that after a relatively short time only ABCG2 would be affected.

Currently, there is no reliable method of imaging ABCG2 activity in humans in vivo. In transgenic mice expressing the luciferase gene, activity was measured using the ABCG2 substrate D-luciferin before and after Ko143 administration (Bakhsheshian et al., 2013b). Because bioluminescence is not an option in the CNS of humans, a specific ABCG2 substrate that can be efficiently radiolabeled is needed instead. To this end, Wanek and colleagues developed a PET imaging paradigm to measure ABCG2 activity in vivo without the need for a specific ABCG2 radiolabeled substrate (Wanek et al., 2012). Mice with genetic or pharmacologic (achieved with 8 mg/kg tariquidar) ABCB1 knockout were imaged with the dual ABCB1/ABCG2 substrate [11 C]tariquidar before and after injection of 10 mg/kg Ko143. The increase in [11 C]tariquidar signal after administration of Ko143 was attributed to ABCG2 inhibition, resulting in an indirect measure of ABCG2 activity. Although this method can be easily executed in mice, it remains uncertain whether complete inhibition of ABCB1 is achievable in humans. The ideal

situation would be to have a substrate and inhibitor specific to the transporter of interest, but this has proved difficult given the overlapping substrate affinity of ABC transporters present at the BBB. As demonstrated in this study, Ko143 is not a specific inhibitor of ABCG2 at in vivo concentrations, which places greater importance on finding a specific ABCG2 substrate amenable to radiolabeling. If the substrate does not interact with any of the other transporters at the low concentrations required for PET imaging, then the promiscuity of Ko143 is not an issue as long as it effectively inhibits ABCG2 at the BBB.

It should be noted that the option remains to use radiolabeled Ko143, given that Ko143 is specific for ABCG2 at the low concentrations needed for PET imaging. By placing a carbon-11 in the methoxy group, [¹¹C]Ko143 might be used to measure ABCG2 density in humans at the BBB and blood-tumor barrier. However, as Kannan et al. (2013) have noted, a radiolabeled inhibitor would need an exceptionally high affinity (in the picomolar range) for the transporter to be able to measure local changes in transporter density, due to the high density of endothelial cells throughout the brain.

The results of this study demonstrate that the inhibitor Ko143 is not specific for ABCG2. At micromolar concentrations, it interacts with both human and mouse ABCB1 and human ABCC1. Although the acid metabolite of Ko143 has no appreciable effect on transporter-mediated efflux, the instability of Ko143 in rat and mouse plasma should be considered when utilizing this inhibitor in vivo. Further characterization is required in wild-type and ABCB1/ABCG2 knockout mice to better understand the specificity of Ko143 in vivo. In addition, cellular disposition of Ko143 may be affected by uptake transporters at the BBB and future studies will need to be conducted to determine the extent of this impact. Given these results, finding a specific substrate or modulator for use with PET imaging becomes even more important to image ABCG2 activity in humans.

Acknowledgments

This work was a collaboration among National Institute of Mental Health, National Cancer Institute, and Karolinska Institutet. The authors thank Ioline Henter and George Leiman for editorial assistance.

Authorship Contributions

Participated in research design: Weidner, Hall, Innis, Pike, Gottesman, Shukla, Ambudkar, Mulder.

Conducted experiments: Weidner, Hall, Zoghbi.

Contributed new reagents or analytic tools: Lu, Zoghbi.

Performed data analysis: Weidner, Hall, Shukla.

Wrote or contributed to the writing of the manuscript: Weidner, Hall, Innis, Pike, Gottesman, Shukla, Ambudkar, Mulder.

References

- Allen JD, van Loeveijzen A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Koomen GJ, and Schinkel AH (2002) Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* 1:417–425.
- Bakhsheshian J, Hall MD, Robey RW, Herrmann MA, Chen JQ, Bates SE, and Gottesman MM (2013a) Overlapping substrate and inhibitor specificity of human and murine ABCG2. *Drug Metab Dispos* 41:1805–1812.
- Bakhsheshian J, Wei BR, Chang KE, Shukla S, Ambudkar SV, Simpson RM, Gottesman MM, and Hall MD (2013b) Bioluminescent imaging of drug efflux at the blood-brain barrier mediated by the transporter ABCG2. *Proc Natl Acad Sci USA* 110:20801–20806.
- Brimacombe KR, Hall MD, Auld DS, Ingles J, Austin CP, Gottesman MM, and Fung KL (2009) A dual-fluorescence high-throughput cell line system for probing multidrug resistance. *Assay Drug Dev Technol* 7:233–249.
- Feldmann M, Asselin M-C, Liu J, Wang S, McMahon A, Anton-Rodriguez J, Walker M, Symms M, Brown G, Hinz R, et al. (2013) P-glycoprotein expression and function in patients with temporal lobe epilepsy: a case-control study. *Lancet Neurol* 12:777–785.
- Gottesman MM, Fojo T, and Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2:48–58.
- Ito K, Uchida Y, Ohtsuki S, Aizawa S, Kawakami H, Katsukura Y, Kamiie J, and Terasaki T (2011) Quantitative membrane protein expression at the blood-brain barrier of adult and younger cynomolgus monkeys. *J Pharm Sci* 100:3939–3950.
- Kannan P, Brimacombe KR, Zoghbi SS, Liow JS, Morse C, Taku AK, Pike VW, Hallidin C, Innis RB, Gottesman MM, et al. (2010) N-desmethyl-loperamide is selective for P-glycoprotein among three ATP-binding cassette transporters at the blood-brain barrier. *Drug Metab Dispos* 38:917–922.
- Kannan P, John C, Zoghbi SS, Hallidin C, Gottesman MM, Innis RB, and Hall MD (2009) Imaging the function of P-glycoprotein with radiotracers: pharmacokinetics and in vivo applications. *Clin Pharmacol Ther* 86:368–377.
- Kannan P, Pike VW, Hallidin C, Langer O, Gottesman MM, Innis RB, and Hall MD (2013) Factors that limit positron emission tomography imaging of p-glycoprotein density at the blood-brain barrier. *Mol Pharm* 10:2222–2229.
- Kannan P, Telu S, Shukla S, Ambudkar SV, Pike VW, Hallidin C, Gottesman MM, Innis RB, and Hall MD (2011) The “specific” P-glycoprotein inhibitor Tariquidar is also a substrate and an inhibitor for breast cancer resistance protein (BCRP/ABCG2). *ACS Chem Neurosci* 2:82–89.
- Kreisl WC, Liow JS, Kimura N, Seneca N, Zoghbi SS, Morse CL, Herscovitch P, Pike VW, and Innis RB (2010) P-glycoprotein function at the blood-brain barrier in humans can be quantified with the substrate radiotracer 11C-N-desmethyl-loperamide. *J Nucl Med* 51:559–566.
- Kühnle M, Egger M, Müller C, Mahringer A, Bernhardt G, Fricker G, König B, and Buschauer A (2009) Potent and selective inhibitors of breast cancer resistance protein (ABCG2) derived from the p-glycoprotein (ABCB1) modulator tariquidar. *J Med Chem* 52:1190–1197.
- Kwan P, Schachter SC, and Brodie MJ (2011) Drug-resistant epilepsy. *N Engl J Med* 365:919–926.
- Langford D, Grigorian A, Hurford R, Adame A, Ellis RJ, Hansen L, and Masliah E (2004) Altered P-glycoprotein expression in AIDS patients with HIV encephalitis. *J Neuropathol Exp Neurol* 63:1038–1047.
- Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, Pastan I, and Dey S (1998) HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* 37:3594–3601.
- Liu JY, Thom M, Catarino CB, Martinian L, Figarella-Branger D, Bartolomei F, Koepp M, and Sisodiya SM (2012a) Neuropathology of the blood-brain barrier and pharmacoresistance in human epilepsy. *Brain* 135:3115–3133.
- Liu X, Cheong J, Ding X, and Deshmukh G (2014) Use of cassette dosing approach to examine the effects of P-glycoprotein on the brain and cerebrospinal fluid concentrations in wild-type and P-glycoprotein knockout rats. *Drug Metab Dispos* 42:482–491.
- Liu X, Ding X, Deshmukh G, Liederer BM, and Hop CE (2012b) Use of the cassette-dosing approach to assess brain penetration in drug discovery. *Drug Metab Dispos* 40:963–969.
- Liu Z, Lheureux F, Pouliot J-F, Heckel A, Bamberger U, and Georges E (1996) BIBW22 BS, potent multidrug resistance-reversing agent, binds directly to P-glycoprotein and accumulates in drug-resistant cells. *Mol Pharmacol* 50:482–492.
- Löscher W and Potschka H (2005a) Drug resistance in brain diseases and the role of drug efflux transporters. *Nat Rev Neurosci* 6:591–602.
- Löscher W and Potschka H (2005b) Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Prog Neurobiol* 76:22–76.
- Matsson P, Pedersen JM, Norinder U, Bergström CA, and Artursson P (2009) Identification of novel specific and general inhibitors of the three major human ATP-binding cassette transporters P-gp, BCRP and MRP2 among registered drugs. *Pharm Res* 26:1816–1831.
- Müller M, Yong M, Peng X-H, Petre B, Arora S, and Ambudkar SV (2002) Evidence for the role of glycosylation in accessibility of the extracellular domains of human MRP1 (ABCC1). *Biochemistry* 41:10123–10132.
- National Institutes of Health (2001) *Guide for the Care and Use of Laboratory Animals*, 8th ed, National Academies Press, Washington, DC.
- Polgar O, Robey RW, and Bates SE (2008) ABCG2: structure, function and role in drug response. *Expert Opin Drug Metab Toxicol* 4:1–15.
- Robey RW, Honjo Y, Morisaki K, Nadjem TA, Runge S, Risbood M, Poruchynsky MS, and Bates SE (2003) Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. *Br J Cancer* 89:1971–1978.
- Robey RW, Lin B, Qiu J, Chan LL, and Bates SE (2011) Rapid detection of ABC transporter interaction: potential utility in pharmacology. *J Pharmacol Toxicol Methods* 63:217–222.
- Römermann K, Wanek T, Bankstahl M, Bankstahl JP, Fedorowicz M, Müller M, Löscher W, Kuntner C, and Langer O (2013) (R)-[(11)C]verapamil is selectively transported by murine and human P-glycoprotein at the blood-brain barrier, and not by MRP1 and BCRP. *Nucl Med Biol* 40:873–878.
- Shukla S, Robey RW, Bates SE, and Ambudkar SV (2006) The calcium channel blockers, 1,4-dihydropyridines, are substrates of the multidrug resistance-linked ABC drug transporter, ABCG2. *Biochemistry* 45:8940–8951.
- Sisodiya SM, Martinian L, Scheffer GL, van der Valk P, Scheper RJ, Harding BN, and Thom M (2006) Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathol Appl Neurobiol* 32:51–63.
- Strouse JJ, Ivinskii-Steel I, Njus HM, Foutz TD, Yao T, Weiner WS, Schroeder CE, Simpson DS, Maki BE, Li K, et al. (2010) Selective efflux inhibition of ATP-binding cassette sub-family G member 2, in *Probe Reports from the NIH Molecular Libraries Program*. National Center for Biotechnology Information, Bethesda, MD.
- Szolomajer-Csikós O, Beéry E, Kósa L, Rajnai Z, Jani M, Hetényi A, Jakab KT, Krajcsi P, and Tóth GK (2013) Synthesis and ABCG2 inhibitory activity of novel fumitremorgin C analogs—specificity and structure activity correlations. *Med Chem* 9:494–509.
- Takada Y, Ogawa M, Suzuki H, and Fukumura T (2010) Radiosynthesis of [2-(11)C-carbonyl]dantrolene using [(11)C]phosgene for PET. *Appl Radiat Isot* 68:1715–1720.

- Tishler DM, Weinberg KI, Hinton DR, Barbaro N, Annett GM, and Raffel C (1995) MDR1 gene expression in brain of patients with medically intractable epilepsy. *Epilepsia* **36**:1–6.
- Turkman N, Gelovani JG, and Alauddin MM (2011) Radiosynthesis of N5-[18F]fluoroacetylornithine (N5-[18F]FAO) for PET imaging of ornithine decarboxylase (ODC) in malignant tumors. *J Labelled Comp Radiopharm* **54**:33–37.
- Wanek T, Kuntner C, Bankstahl JP, Mairinger S, Bankstahl M, Stanek J, Sauberer M, Filip T, Erker T, Müller M, et al. (2012) A novel PET protocol for visualization of breast cancer resistance protein function at the blood-brain barrier. *J Cereb Blood Flow Metab* **32**:2002–2011.
- Wanek T, Mairinger S, and Langer O (2013) Radioligands targeting P-glycoprotein and other drug efflux proteins at the blood-brain barrier. *J Labelled Comp Radiopharm* **56**:68–77.
- Zander SA, Beijnen JH, and van Tellingen O (2013) Sensitive method for plasma and tumor Ko143 quantification using reversed-phase high-performance liquid chromatography and fluorescence detection. *J Chromatogr B Analyt Technol Biomed Life Sci* **913–914**:129–136.
- Zhang J, Chen Y, Fu T, Yang Y, Li Y, Liu H, Cheng Z (2011) Use or misuse of Ko143 for Bcrp chemical knockout mice. *Chemical and Pharmaceutical Structure Analysis: 2nd Symposium*; 2011 April 13–16; Shanghai, China.

Address correspondence to: Lora D. Weidner, Molecular Imaging Branch, National Institute of Mental Health, National Institutes of Health, 10 Center Drive, Room B1D43, Bethesda, MD 20892. E-mail: lora.deutch@nih.gov