In Vitro Characterization of Axitinib Interactions with Human Efflux and Hepatic Uptake Transporters: Implications for Disposition and Drug Interactions.

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**Abbreviations:** A, apical; B, basolateral; BCRP, Breast Cancer Resistance Protein; CNS, Central Nervous System; CsA, Cyclosporine A; CYP, Cytochrome P450; DDI, Drug-Drug Interaction; FACS, Fluorescence Activated Cell Sorting; LC-MS/MS, Liquid Chromatography-tandem Mass Spectrometry; MDCK, Madin-Darby Canine Kidney cells-Type 2; MDCK-LE, MDCK-Low Efflux cells; MDR1, Multidrug Resistance Gene 1; OATP, Organic Anion Transporting Polypeptide; OAT, organic anion transporters; OCT, organic cation transporters; and OCTN, carnitine/organic cation transporter; P-gp, P-glycoprotein (or MDR1); TEER, Transepithelial Electrical Resistance; Papp, apparent permeability; TKI, Tyrosine Kinase Inhibitor; VEGF, Vascular Endothelial Growth Factor; VEGFR, Vascular Endothelial Growth Factor Receptor; WBA, whole-body autoradiography.
Abstract

Axitinib is an inhibitor of tyrosine kinase vascular endothelin growth factor receptors 1-3. ABC and SLC transport properties of axitinib were determined in selected cellular systems. Axitinib exhibited high passive permeability in all cell lines evaluated (Papp $\geq 6 \times 10^{-6}$ cm/sec). Active efflux was observed in Caco-2 cells and further evaluation in MDR1- or BCRP-transfected MDCK cells indicated that axitinib is at most only a weak substrate for P-glycoprotein (P-gp) but not breast cancer resistance protein (BCRP). Axitinib showed incomplete inhibition of P-gp-mediated transport of digoxin in Caco-2 cells and BCRP transport of topotecan in BCRP-transfected MDCK cells with IC$_{50}$ values of 3 $\mu$M and 4.4 $\mu$M, respectively. Axitinib (10 mg) did not pose a risk for systemic drug interactions with P-gp or BCRP per regulatory guidance. A potential risk for drug interactions through inhibition of P-gp and BCRP in the gastrointestinal tract was identified since an axitinib dose of 10 mg divided by 250 mL was greater than 10-fold the IC$_{50}$ for each transporter. However, a GastroPlus™ simulation that considered the low solubility of axitinib resulted in lower intestinal concentrations and suggested a low potential for gastrointestinal interactions with P-gp and BCRP substrates. OATP1B1 and OATP1B3 transfected HEK293 cells transported axitinib to a minor extent but uptake into suspended hepatocytes was not inhibited by rifamycin SV suggesting that high passive permeability predominates. Mouse whole-body autoradiography revealed that $[^{14}\text{C}]$axitinib-equivalents showed rapid absorption and distribution to all tissues except brain. This suggested efflux transport of axitinib may occur at the mouse blood brain barrier.
Introduction

Axitinib (AG-013736), N-methyl-2-[[3-[(1E)-2-(pyridin-2-yl)ethenyl]-1H-indazol-6-yl]sulfanyl]benzamide (Figure 1), is an orally dosed inhibitor of vascular endothelial growth factor receptors (VEGFR)-1, 2 and 3 (Hu-Lowe et al., 2008). The VEGFR regulates angiogenesis, which is the formation of new blood vessels from the existing vasculature. New blood vessels are necessary for rapid tumor growth (Folkman et al., 1971). Most solid tumors in breast, lung, renal, colorectal and liver tissues express high levels of VEGFR compared to normal tissues (Hanahan and Folkman, 1996). The mechanism of action for VEGFR inhibitors, such as axitinib, is to reduce vascularization of solid tumors, and consequently, minimize tumor growth. A phase I clinical trial investigated the tolerability, pharmacokinetics and clinical activity of axitinib (Rugo et al., 2005). The dose limiting toxicities were elevated blood pressure (an extension of pharmacology), hemoptysis, and stomatitis. Pharmacokinetics were studied over an oral dose range from 2 – 20 mg twice daily and T\textsubscript{max} and half-life ranged from 1.7-6.0 hr and 1.7-4.8 hr, respectively. Axitinib produced confirmed partial responses in three patients and a dose of 5 mg twice daily was selected for phase II studies. In subsequent clinical trials, axitinib was active in cancers of the kidney (Rixe et al., 2007) and thyroid (Cohen et al., 2008). A multicenter international phase III clinical trial confirmed that axitinib was well tolerated with a similar adverse event profile as sorafenib, demonstrating a significant increase in progression free survival in advanced renal cell carcinoma (RCC) patients compared to sorafenib (Rini et al., 2011). In early 2012, axitinib received approval for treatment of advanced RCC in patients who failed one prior systemic therapy by the United States Food and Drug Administration.

Axitinib is a member of the tyrosine kinase inhibitors (TKIs) which, as a class, have similarities in absorption, metabolism and transport characteristics. Many (but not all) have
good to complete absorption with fraction absorbed values greater than 0.3 with some in the 0.6 to 1.0 range (Hartmann et al., 2009). Most are substrates for cytochrome P450s and the efflux transporters, P-gp and BCRP. Axitinib is metabolized primarily by cytochrome P450 (CYP3A) with minor contributions from CYP1A2, CYP2C19, and UGT1A1 (Zientek, 2010; Pithavala et al., 2012). Metabolism and efflux may contribute to the variable absorption, distribution and elimination of these medicines. Focusing on the transporter aspect, tyrosine kinase inhibitors (such as imatinib, erlotinib, sunitinib and lapatinib) are inhibitors of efflux transporters (P-gp and BCRP) and, to varying degrees, interact with organic anion-transporting polypeptides (OATP), organic anion transporters (OAT), organic cation transporters (OCT), and carnitine/organic cation transporter (OCTN) (van Erp et al., 2009; Dohse et al., 2010). OATP transporters can be rate limiting in the clearance of drugs (Shimizu et al., 2005) and may contribute to patient variability especially if OATP1B1 is involved due to polymorphisms associated with this hepatic uptake transporter (Nishizato et al., 2003). The aim of this work was to investigate the role of efflux and uptake transporters in the disposition and drug interaction potential of axitinib. Poller et al. (2011) previously demonstrated that axitinib is a substrate for P-gp and BCRP in rodent knockout and cell-based in vitro models. Early studies conducted in our lab suggested differing results for these efflux transporters and the work herein attempts to understand this discrepancy as well as to examine other transporter-related characteristics. Also, with ongoing interest in DDI potentials relating to P-gp efflux and the significance of these effects, proposals utilizing modeling and simulation are beginning to be included in regulatory filings for new drugs. We examined GastroPlus™, a drug absorption and physiologic-based pharmacokinetic modeling and simulation software program in this work to better understand the pH-dependent solubility of axitinib and how this characteristic may relate to the DDI potential.
Material and Methods

Chemicals. Chemicals and compounds used in these studies were purchased from Sigma-Aldrich (St. Louis, MO) with the exceptions being topotecan (Toronto Research Chemicals Inc.(North York, ON, Canada) and Ko143 (Tocris Bioscience (Ellisville, MO)), respectively) and axitinib and talinolol which were synthesized by Pfizer, Inc. Axitinib has exhibited instability to light when in solution so to minimize degradation during studies solutions were protected from light. Caco-2 and MDCK cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MDR1-transfected MDCK cells were obtained externally (Schinkel et al., 1995; Evers et al., 2000), BCRP-transfected MDCK cell stocks and low efflux MDCK cells selected for low expression of canine Mdr1 (MDCK-LE) were prepared internally at Pfizer, Inc. (Xiao et al., 2006; Di et al., 2011). Organic anion transporting polypeptide (OATP)-transfected HEK293 cells (and non-transfected wild-type HEK293 cells) were obtained from Prof. Dietrich Keppler (Konig et al., 2000a; Konig et al., 2000b). Cell culture components and buffers including Dulbecco’s Modified Eagle Medium (DMEM), penicillin/streptomycin (10,000/10,000), Minimum Essential Medium with Non Essential Amino Acids (MEM-NEAA), Minimum Essential Medium-α (MEM-α), Modified Eagle Medium with Earle Salts (MEM with Earle Salts, cat.# 11090-081), Glutamax-I, Hanks’ Balanced Salt Solution (HBSS), Williams’ Medium E (WEM), Trypsin-EDTA, and materials for Western blot work were purchased from Life Technologies (Carlsbad, CA) and Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA) for antibodies (Mdr(G-1) and Mdr (C-19)). Fetal bovine serum (FBS) and heat inactivated FBS were obtained from Sigma-Aldrich (St. Louis, MO).

Solubility Determinations. Several solubility studies were conducted to guide the conduct and interpretation of transport studies and modeling in GastroPlus™. The solubility of axitinib under
experimental conditions and at equilibrium were determined in HBSS transport buffer in the presence of 0.5 or 1% DMSO. Axitinib was added to HBSS transport buffer in DMSO at a concentration of 10 µM and incubated at 37°C for 3 hours for simulated use. Equilibrium solubility in HBSS buffer was determined by adding 1 mg axitinib to 2 mL HBSS containing either 0.5 or 1% DMSO and incubating at 37°C for 2-3 days. Following the incubation the samples were centrifuged to separate undissolved drug and the solution concentration of axitinib determined by HPLC/UV.

Equilibrium solubility determinations were assessed over a range of pH values to generate input values for GastroPlus™. The measurements were made in 20 mM sodium phosphate and 20 mM sodium acetate buffer for pH >2 and in dilute hydrochloric acid solution for pH<2 (0.06 M HCl and 0.1 M HCl for pH 1.7 and 1.1, respectively). Excess solid was equilibrated for 24 hours with stirring at 37°C. Samples were filtered through Millipore HV 0.45 µm filters and supernatant was collected and concentration quantitated by HPLC. A calibration curve was used over a range of 0.2 – 70 µg/mL.

Permeability and Transporter Evaluations

Caco-2 Cell Permeability. Caco-2 cells were cultivated under aseptic conditions at 37°C in an atmosphere of 90% relative humidity, 95% air and 5% CO2 in culture medium consisting of D-MEM, 10% FBS, 1% penicillin/streptomycin solution and 1% MEM-NEAA in 175 cm² culture flasks and passaged when cells reached 75-85% confluency with Trypsin-EDTA solution (0.25%). Caco-2 cells at passages 30 through 50 were seeded onto Corning Costar 24-well cell culture devices (Sigma-Aldrich, St. Louis, MO). The 24-well cultures were maintained with medium being replaced every three to four days and used in experiments on days 21-23 post-seeding. One day prior to the study, the base plate was exchanged for a fresh one and media was
changed. Cell monolayers were rinsed in HBSS, pH 7.4, and equilibrated in this buffer for 1 hour prior to experiments then the plates were placed on the deck of a Tecan Genesis robotic system (Tecan, Durham, North Carolina, USA). To begin the experiment, 300 µL of dosing solution was added to the apical (A) chamber for apical to basolateral (AB) or 750 µL to the basolateral (B) chamber for basolateral to apical (BA) transport studies with blank buffer added to the receiver chambers (800 µL or 300 µL for AB and BA studies, respectively). Samples were collected at five minutes and at two hours for donor and at one hour and two hours for receiver samples. The receiver chambers were replenished with fresh HBSS after the one hour sampling. Donor samples were diluted 10x with fresh HBSS following collection. The samples were analyzed by LC-MS/MS. Following the study, cell monolayers were incubated for one hour with buffer containing 0.1 mg/ml Lucifer yellow (dosed on the apical side, monitor for appearance on the basolateral side) to verify monolayer integrity was not compromised during the study using a criteria of <1% transported.

**MDR1-MDCK and BCRP-MDCK-LE Cell Permeability and Efflux Transport Determination.** The *in vitro* assessment of axitinib included the determination of efflux across monolayers of Madin Darby Canine Kidney (MDCK) cells transfected with human Multidrug Resistance 1 gene (MDR1 or P-gp) and Breast Cancer Resistance Protein (BCRP). BCRP was transfected into MDCK cells that were selected for low expression of canine P-gp and is referred to as BCRP-MDCK-LE. MDCK-LE cells are a clonal isolate of MDCK wild-type cells selected for low background efflux activity (Di et al., 2011). All cells were maintained similarly to Caco-2 cells described in the above section with the exceptions that these cells were grown on 96-well Millicell cell culture devices (Millipore, Billerica, MA) and maintained on media prepared with MEM-α supplemented with 1% MEM-NEAA, 1% Glutamax-I, 1% penicillin/streptomycin and
10% FBS. AB and BA flux were determined and the calculated BA/AB ratios of the transfected cells was used to assess the potential of axitinib to be a substrate for one or more of these efflux transporters. Experiments were conducted at 37°C, 95% air, 5% CO₂ with axitinib dosed either on the apical (for AB determinations) or the basolateral (for BA) compartment with donor samples collected at time zero and donor and receiver samples collected at 60 minutes incubation. Digoxin and topotecan were used as positive controls for MDR1 and BCRP transport, respectively at 2 µM. The marker compound atenolol was measured for monolayer integrity in each well with Papp <1.5 x 10⁻⁶ cm/sec as the criteria for data acceptance.

**OATP-transfected HEK293 Cell-Substrate Evaluation.** HEK293 cells and cells transfected by the individual OATP transporters, OATP1B1, OATP1B3 and OATP2B1 were maintained in growth medium containing MEM with Earle Salts supplemented with heat inactivated FBS (10%), penicillin/streptomycin (1%) and, for the transfected cell lines, G418 (geneticin) at 400 µg/ml. Cells were seeded onto 24-well BD Biocoat poly-D-lysine coated plates (BD Biosciences, Bedford, MA) at a cell density of 2.5 x 10⁵ cells/ml, 1 ml per well. Medium was replaced daily and on the second day post-seeding, the media was changed with medium containing MEM+10% FBS+Pen/Strep and 10 mM sodium butyrate. On day three, the cells were first washed (3 x 1 ml) with pre-warmed HBSS, pH 7.4, and uptake experiments were then conducted. HBSS spiked with 4 µM of test compound (250 µl) was added to rinsed plated cells and incubated for four minutes at 37°C followed by rapid aspiration of dosing solution and successive rinses (3X) with ice-cold buffer. Test compound was extracted from the cell layer with 0.75 mL 75/25 % (v,v) acetonitrile/H₂O including 0.01 µM buspirone as internal standard with a brief scraping of the cells to maximize recovery of the sample followed by transfer to a polypropylene deep 96-well plate and centrifugation for 10 minutes at ~2500 rpm. Supernatant
(240 μL) was transferred to a shallow 96-well plate (deep and shallow 96-well plates; Nalge Nunc International, Rochester, NY, USA) for bioanalytical evaluation by LC-MS/MS. To account for passive uptake and non-specific binding, the uptake study was performed in parallel using the non-transfected control cell line, HEK293.

**Hepatocyte Suspension Active Uptake Assay.** Cryopreserved human hepatocytes (Lot RCP, Celsis In vitro Technologies) were quickly thawed at 37°C and immediately suspended in warm Williams’ Medium E. Suspended hepatocytes were centrifuged at 50 x g for three minutes at room temperature. The cell pellet was resuspended in two milliliters (per vial) of Krebs-Henseleit Buffer (KH, Sigma) and diluted to a final density of 2 x 10⁶ viable cells/mL. Aliquots (200 μL) were transferred to round bottom tubes and placed on ice. Stock solutions of axitinib and rosuvastatin (both test compounds), and rifamycin SV (inhibitor) were prepared in DMSO. Hepatocytes were pre-warmed for three minutes in a 37°C water bath with gentle agitation, then 200 μL of pre-warmed dosing solution (KH buffer spiked with the appropriate stock solution of axitinib or rosuvastatin with and without rifamycin SV) was added to start the incubation. At time points 0.5, 1, and 1.5 minutes, 100 μL was taken from the incubation mixture and transferred to a centrifuge tube containing oil (mineral & silicone mix) with a bottom layer of 2 M ammonium acetate and immediately centrifuged for 10 seconds at 14,000 rpm. Hepatocytes are drawn through the liquid layers and pelleted to the bottom of the tube. After centrifugation, the tubes were placed on ice. Centrifuge tubes were cut just below the oil layer and the remaining ammonium acetate was removed by aspiration. Cell pellets were resuspended in 50μL Milli-Q® water and transferred to a 96 well plate followed by addition of methanol (100 μl) containing internal standard (buspirone 0.03 μM). Following vortexing and centrifugation at 2000 rpm for 10 minutes supernatant was transferred to a new 96 well plate for LC-MS/MS.
analysis. Standard curves for axitinib and rosuvastatin were prepared in matrix utilizing excess suspended hepatocytes in order to calculate concentration values for each sample.

**Caco-2 Digoxin IC\textsubscript{50} Determination.** The inhibition of digoxin efflux across Caco-2 cell monolayers by axitinib was assessed using methods as described previously (Cook et al., 2010). Briefly, Caco-2 cells (passage 26) were plated in 24-well transwell plates containing 1 \( \mu \)m pore size inserts (Becton Dickinson, Cowley, U.K.), at a seeding density of \( 1.6 \times 10^5 \) cells/mL and seeding volume of 250 \( \mu \)L. The assay was carried out on day 22 post seeding, with media replaced every other day. Trans-epithelial electrical resistance (TEER) levels were measured prior to the experiment to ensure monolayer integrity. The efflux of \([^3 \text{H}]-\text{digoxin (5 } \mu \text{M})\) was determined after a 2 hour incubation in the presence of increasing concentrations of axitinib. The same experiment was repeated using ketoconazole as a positive control inhibitor. IC\textsubscript{50} values were fitted as described in the Data Analysis section.

**BCRP-MDCK-LE Topotecan IC\textsubscript{50} Determination.** The receiver concentrations of the AB and BA transport were measured to determine the net secretory flux of topotecan (known BCRP substrate) by itself and in the presence of Ko143 (known BCRP inhibitor) or axitinib to determine inhibition of BCRP efflux. Permeability of atenolol was determined, which served as the monolayer integrity marker. The donor compartment consisted of topotecan at a single concentration alone of 2 \( \mu \)M and in the presence of increasing concentrations of Ko143 or axitinib. The receiver compartments consisted of blank HBSS and the matching donor concentrations of Ko143 or axitinib. Atenolol was added to each well at 10 \( \mu \)M on the donor compartment. The study was conducted at 37\(^\circ\)C and 5\% CO\textsubscript{2} using a Beckman Biomek FX automated robotic system functioning to load the donor and receiver solutions to the Millicell 96-well transport plates (Millipore) to start the incubation and to collect donor samples at the time...
zero and donor and receiver samples following 60 minute (substrate assays) or 120 minute (inhibitor assay) incubations. Samples were assayed by LC-MS/MS.

**LC-MS/MS Methods.** The compounds of interest were introduced onto a Synergi Polar-RP (2 X 30 mm, 4 µm; Phenomenex, Torrance, CA) HPLC column with a CTC PAL autosampler (Leap Technologies, Carrboro, NC) and an integrated HPLC pumping system (Shimadzu Scientific Instruments, Columbia, MD). These compounds were then eluted and detected by an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) fitted with a TurboIon Spray interface. Mobile phase A was 0.1% aqueous formic acid, mobile phase B was 0.1% formic acid in acetonitrile and the flow rate was 0.8 ml/min. The initial condition for the HPLC gradient was 99:1 (% v/v, A:B). This was held for 0.75 min. From 0.75 to 2.5 min, the mobile phase composition changed linearly to 5:95 (% v/v, A:B). This condition was held until 2.6 min. The gradient was returned in a linear fashion to 99:1 (% v/v, A:B) from 2.6 to 2.8 min and re-equilibrated until 3.5 min. The injection volume was 10 µl. Multiple reaction monitoring was used to detect the compounds using the following transitions (267→90, atenolol; 388→356, axitinib; 1203→86, cyclosporin A; 782→652, digoxin; 470→415, Ko143; 482→258, rosvastatin; 364→309, talinolol; 422→378, topotecan). The peak area ratio of the analyte to the internal standard was determined for each injection and used to determine the biological response under consideration for the particular assay.

**GastroPlus™ Simulations.** GastroPlus™ version 6.0 (Simulations Plus, Lancaster, CA, USA) was used for the estimation of the mass of drug in solution in various regions of the GI tract using the following input parameters; human P_{eff} 2.4 x 10^{-4} cm/sec, MW 386.47, Log D pH 6.5 4.15, simulated intestinal fluid solubility 9.6 x 10^{-4} mg/mL, Particle size 4µm (Particle size standard deviation of 3 was applied), precipitation time 900 sec, Diff. Coeff. 0.75 cm^2/sec x 10^5.
Particle radius 2 µm, and Drug particle density 1.2 g/mL. GastroPlus™ simulation outputs estimated the dissolved axitinib mass (mg) at 0.08, 0.16, 0.24, 0.88, 1.04, 2, 4, 6, 8, 10, and 12 hours post dose in the stomach, duodenum, jejunum 1 (upper), and jejunum 2 (lower) for each dosing condition. To provide a conservative estimation of intestinal concentration, the sum of dissolved compound in stomach contents, duodenum, jejunum 1 (upper), and jejunum 2 (lower) across a 12-hour time window were used to determine the amount of drug in solution and determine a maximum concentration in micromolar. The dissolved mass across the various GI regions was then summed to estimate the total dissolved mass at each time point and converted to concentration (µM). This was accomplished using the following equation:

\[
Total\ Concentration\ (\mu M) = \frac{(\text{Sum}_\text{mgs} / 250\ mL) \times 1000\ mL/L \times 1000\ \mu M/mM)}{(\text{Molecular\ Weight})}
\]

This provided the most conservative estimate of dissolved drug.

**Whole-Body Autoradiography in Mice.** The tissue distribution of $^{14}$C-axitinib was determined in male and female pigmented mice (strain B6C3F1/Crl BR, Charles River (UK) Ltd, Margate, Kent) by whole-body autoradiography (WBA) at 1, 4, 8, 24 and 48 hours after oral administration. All procedures performed on these animals were in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee or through an ethical review process. $^{14}$C-Axitinib was administered orally by gavage at a nominal dose level of 50 mg/kg (105 $\mu$Ci/kg). At the times listed above, animals were sacrificed by cold-shock (following deep anesthesia under halothane, each animal was placed in a restraining device and rapidly frozen by total immersion in a hexane-solid carbon dioxide mixture (approximately -80°C) for 30 min, then transferred to storage at -20°C). For all animals, the legs, tail and whiskers were trimmed off and each frozen carcass was set in a block of frozen aqueous 2% (w/v) carboxymethylcellulose. The block was mounted onto the stage of a Leica CM3600 NT cryomicrotome maintained at about -20°C and sagittal sections (nominal...
thickness 30 µm) were obtained (Milnes Packaging Group, Brighouse, West Yorks, UK). Imaging was conducted using FUJI imaging plates (type BAS-MS, Raytek Scientific Ltd., Sheffield, England).

**Data Analysis.** Apparent permeability values (Papp), flux, net secretory flux, and IC₅₀ values were calculated as previously reported (Fenner et al., 2009). The drug interaction risk assessment was conducted according to the USFDA and EMA guidance documents (Anonomous, 2012c; Anonomous, 2012b). The nomenclature proposed by Zhang et al. (2008) was used to refer to the concentration of inhibitor in systemic circulation [I₁] and gastrointestinal tract [I₂]. When concentrations derived from GastroPlus™ simulation were used in the assessment, we referred to this as [Iₑₓ].
Results

Solubility Determination

The equilibrium solubility characteristics of axitinib as a function of pH are shown in Figure 2. Axitinib is highly soluble at low pH but solubility declines rapidly as pH increases above 2.0. At the range near neutral pH, the solubility of axitinib was < 1 µM. A series of solubility studies were conducted in order to ensure transport study data were reported using concentration ranges where axitinib was in solution. Additional solubility studies were performed to simulate experimental conditions during transport studies. When 10 µM axitinib was prepared in 0.5 or 1% DMSO in HBSS buffer (pH 7.4), and incubated at 37°C for 3 hours, the resulting solution concentration was 4.2 ± 2.2 µM and 2.4 ± 0.5 µM (mean ±SD), respectively. When the equilibrium solubility of axitinib was determined in 0.5 or 1% DMSO in HBSS buffer (pH 7.4), the resulting solution concentration was 0.31 ± 0.02 µM and 0.38 ± 0.06 µM (mean ±SD), respectively. This suggested that under the conditions used for transport studies, axitinib forms a supersaturated solution when solubilized using DMSO solvent even though the equilibrium solubility was considerably lower. Considering these results, data were reported for transport studies approximately up to 5 µM even though the experiments may have included higher concentrations.

Permeability and Efflux

The in vitro cellular permeability and efflux of axitinib was initially determined using Caco-2 cell monolayers. The Papp (AB), Papp (BA) and efflux ratio of axitinib (1 µM) in Caco-2 cells was 17x10⁻⁶ cm/s, 160x10⁻⁶ cm/s and 9.7, respectively. This suggested that axitinib has high passive permeability with some efflux activity.

P-gp and BCRP Substrate Evaluation
Evaluation of axitinib as a substrate for P-gp and BCRP was conducted in bidirectional transport studies in MDR1-transfected MDCK and BCRP-transfected MDCK-LE cell monolayers (Tables 1 and 2). MDR1-MDCK cell transport studies using axitinib showed weak efflux over a concentration range from 1-5 µM with no concentration dependence. The weak efflux observed with axitinib in MDR1-MDCK cells was inhibited by CsA. The MDR1 cell system was verified using the positive control digoxin to demonstrate MDR1 efflux and inhibition by CsA. BCRP-MDCK-LE cells transport studies using axitinib showed no efflux activity over a concentration range from 1-5 µM. The BCRP cell system was verified using the positive control topotecan to demonstrate BCRP efflux and inhibition by Ko143. In our laboratory, transfection of wild type MDCK cells with BCRP resulted in an increased expression of native canine Mdr1 in comparison to the non-transfected MDCK cells. Whereas, BCRP-MDCK-LE cells, no canine Mdr1 signal was observed as confirmed by western blot analysis (Figure 3). This enabled BCRP substrate activity to be evaluated without being confounded by canine Mdr1 expression. Taken together results suggested that axitinib is a weak substrate of P-gp and is not a substrate of BCRP.

**P-gp and BCRP Inhibition Evaluation**

P-gp inhibition was evaluated in the Caco-2 monolayer cell line with digoxin as the probe substrate. This is an accepted model to evaluate P-gp inhibition according to regulatory guidance (Anonomous, 2012b). The concentration range of axitinib in these studies ranged from 0.1 to 75 µM but evaluation of inhibition above 7 µM was limited, presumably due to poor solubility in pH 7.4 HBSS buffer. This resulted in inhibition curves not achieving maximal inhibition (Figure 4A). To better fit the inhibition curve, positive control inhibitor data for ketoconazole was utilized to define the maximum inhibition of digoxin flux (Figure 4B).
DMD #51193

Determination of an IC$_{50}$ value for axitinib as described by Fenner and coworkers was then calculated from the resulting data (Fenner et al., 2009). Axitinib decreased P-gp mediated digoxin transport by 67% at 7 µM, resulting in an IC$_{50}$ value of 3.0 µM by utilizing ketoconazole to define maximum inhibition. BCRP inhibition was evaluated in BCRP-MDCK-LE cells with topotecan as the probe substrate at a concentration range from 0.032 to 20 µM. Above 20 µM axitinib compromised monolayer integrity was observed as evidenced by elevated Lucifer yellow permeability post-experiment (data not shown). Axitinib decreased BCRP mediated efflux of topotecan to a maximum of 49% of nominal efflux at 5.6 µM which did not decrease further at 10 or 20 µM presumably due to poor solubility (Figure 5A). Ko143, the positive control inhibitor of BCRP, completely inhibited topotecan efflux with an IC$_{50}$ of 0.08 µM (Figure 5B). An IC$_{50}$ value of 4.4 µM for axitinib in BCRP mediated topotecan transport was estimated utilizing Ko143 to define maximum inhibition.

**OATP1B1, OATP1B3 and OATP2B1 Evaluation**

To determine whether axitinib is a substrate for human hepatic uptake transporters, OATP1B1, OATP1B3 and OATP2B1 in vitro uptake studies were performed. Axitinib (concentration range from 0.5 to 4 µM) was not a substrate of OATP2B1 at any concentration evaluated. Axitinib, was a substrate for both OATP1B1 and OATP1B3 where OATP1B3 demonstrated an approximately 2-fold greater uptake rate than OATP1B1 (Table 3).

**Hepatocyte Suspension: Active Uptake Assay**

The positive control hepatic uptake substrate rosuvastatin is transported by OATP1B1, OATP1B3, OATP2B1 and NTCP (Ho et al., 2006) and uptake into sandwich culture human hepatocytes is completely inhibited by rifamycin SV (100 µM), an inhibitor of OATPs and NTCP (Mita et al., 2006; Bi et al., 2012). This system enables the functional evaluation of active
uptake and passive diffusion mechanisms of drug entry into hepatocytes. Using suspended cryopreserved human hepatocytes, rosvastatin (1 µM) linear uptake was demonstrated with nearly complete inhibition by rifamycin SV (100 µM) (Figure 6). When axitinib was tested in the same system, compound accumulation into the hepatocytes reached a near maximum by 0.5 min and little additional uptake was observed by 1.5 min. Addition of rifamycin SV (100 µM) did not inhibit axitinib uptake into hepatocytes. The results for axitinib indicated passive diffusion was the primary hepatic uptake mechanism at 1 µM.

**GastroPlus™ Simulation of Axitinib Intestinal Concentrations and Efflux Transporter Drug Interaction Risk Assessment**

Both USFDA and EMA regulatory guidance recommend the assessment of drug interaction risk for efflux transporters systemically and at the level of the gastrointestinal tract (Anonymous, 2012b; Anonymous, 2012c). Both agencies recommend estimating the intestinal concentration by dividing the highest recommended dose by 250 mL. The risk assessment for intestinal drug interactions with efflux transporters is assessed by determining if the ratio of the intestinal concentration $[I_2]$ divided by the IC$_{50}$ or K$_i$ value is $>10$. However, this does not consider if the entire dose is in solution for drugs with low pH-dependent solubility. To investigate this possibility, Gastroplus™ software was utilized to simulate the intestinal concentration time profile of axitinib (data not shown) following the maximum recommended dose of 10 mg in the fed state (Inlyta®, 2012). GastroPlus™ was utilized to estimate the total mass of drug in solution achieved in each area of the GI tract, including the stomach, which was then summed to conservatively estimate total drug mass in solution. The GastroPlus™ estimate of total drug amount in solution was divided by 250 mL resulting in a gut concentration ($[I_{gut}]$) of 6.6 µM. This value was much lower than $[I_2]$ of 104 µM when the total 10 mg dose/250 mL was utilized.
The resulting $[I_{\text{gut}}]/IC_{50}$ vs $[I_2]/IC_{50}$ ratios were 2.2 vs 35 for P-gp and 1.5 vs 24 for BCRP. Regulatory guidance also requires a risk assessment for systemic interaction with efflux transporters. The USFDA and EMA guidance differ in the use of total maximum plasma concentration ($C_{\text{max}}$) or unbound plasma $C_{\text{max}}$ divided by $IC_{50}$ or $K_i$ the and a use of a safety factor of 10 or 50, respectively. The highest dose of axitinib administered clinically is 10 mg twice daily resulting in a mean total or unbound $C_{\text{max}}$ of 0.16 or 0.0008 $\mu$M, respectively (Rugo et al., 2005; Anonymous, 2012a). The total or unbound $[I_1]/IC_{50}$ was less than 0.1 or 0.02, the respective thresholds for the USFDA and EMA DDI guidance, for efflux transporter inhibition.

**Whole-Body Autoradiography in Mice**

The tissue distribution of $[^{14}\text{C}]$axitinib was determined in male pigmented mice by whole-body autoradiography at 1, 4, 8, 24 and 48 hours after oral administration. $[^{14}\text{C}]$Axitinib was administered orally by gavage at a dose level of 50 mg/kg (105 $\mu$Ci/kg). Maximum concentrations of radioactivity in mice generally occurred at 1 hour in all tissues (Figure 7) indicating rapid absorption and radioactivity was widely distributed in all tissues. The highest level of axitinib-derived radioactive equivalents was present in the gall bladder consistent with hepatic elimination of the drug (metabolism and transport). The radioactivity ratio in the gall bladder to blood at 1 hour post-dose was 24 while the next highest levels in the liver and stomach mucosa were 3.8 and 3.6, respectively. By 24 hours most tissues measured contained concentrations of radioactivity below the limit of quantification (BLQ) with the exception pigment containing tissues (skin and uveal tract). Low concentrations of radioactivity were detected in the brain. The brain to blood ratio was $\sim$0.09 at 1 hour post dose and brain concentrations were BLQ at 4 hour post-dose.
Discussion

Axitinib inhibits cellular VEGF receptor tyrosine kinase phosphorylation and target-mediated functional activity (Hu-Lowe et al., 2008). It is a more selective inhibitor of VEGF receptor-1, -2 and -3, PDGFR and c-KIT than both sunitinib and sorafenib and has greater affinity for these targets (Goldstein et al., 2010). Axitinib is a low molecular weight, lipophilic and weakly basic molecule with high permeability. Tyrosine kinase inhibitors, such as dasatinib, imatinib, gefitinib, sunitinib and lapatinib, possess similar molecular characteristics including multiple aromatic rings, similar molecular weight (ranging from 398-494 AMU), moderate hydrophobicity (logP values >2), and most are primarily cleared via metabolism by CYP3A4. These chemical features are also typical for substrates of ABC transporters (Polli et al., 2008; Chen et al., 2009; Oostendorp et al., 2009; Shukla et al., 2009; Carcaboso et al., 2010). Herein, we found that axitinib showed efflux in Caco-2 cells, was a weak substrate for human P-gp and was not a BCRP substrate using transfected cell lines. Axitinib is however an inhibitor of P-gp and BCRP. Axitinib inhibits VEGFR by forming a binding complex to the ATP catalytic binding site of the kinase domain (Bender, 2004; Solowiej et al., 2009). It is not known if axitinib inhibits P-gp and BCRP by binding to the substrate or ATP binding site. Axitinib appeared to be a substrate for uptake by OATP1B1 and OATP1B3 in transfected cell systems. However, additional hepatocyte uptake studies showed that the OATP inhibitor rifamycin SV did not have any impact on the uptake of axitinib under the same conditions where rosuvastatin uptake was markedly inhibited. Considering the physicochemical properties of axitinib, the high permeability and passive uptake into the hepatocyte, the results suggest that OATPs do not play a significant role in its hepatic disposition. While only OATPs were evaluated in this study and it appeared that passive uptake of axitinib into hepatocytes predominated the potential role of other...
uptake transporters was not evaluated in this study. Thus, considering the major uptake and efflux transporters evaluated it appears that transporters play a minor role in the absorption, distribution and excretion of axitinib in humans.

Our finding that axitinib is at most only a weak substrate for MDR1 and not a BCRP substrate is in contrast to the recent publication by Poller et al. (2011), that identified axitinib as a good and moderate substrate for MDR1 and BCRP, respectively. We found that, over a concentration range of axitinib from 1 to 5 μM, the P-gp mediated efflux ratio in the MDR1-MDCK was ≤1.45 while our positive control substrate, digoxin, exhibited an efflux ratio of 24. BCRP efflux ratios of axitinib were near unity while the efflux ratio of our positive control, topotecan, was greater than 14. Recently, Di et al., reported that FACS selected low efflux MDCKII cells (MDCK-LE) showed much lower mRNA expression for canine P-gp than the parental wild type MDCK cell line (dog Mdr1 > 62-fold higher in MDCK cell line). It is possible that axitinib is a substrate for canine P-gp and that the MDR1-MDCK and BCRP-MDCK cell lines utilized by Poller et al. (2011), may contain higher levels of canine P-gp than our cell lines resulting in the contrasting results between the two laboratories. Additionally, it has been demonstrated, previously, that P-gp activity differs between species (Tang-Wai et al., 1995). This species specific activity may account for differences in axitinib efflux in the various systems. Interestingly, in the BCRP-transfected MDCK cells, higher levels of canine Mdr1 were present than in wild type MDCK cells based on western blot analysis while expression was absent in the BCRP-MDCK-LE cell lines (Figure 2). This emphasizes the importance of cross laboratory evaluation of drug transporter substrate and inhibition properties.

Our in vivo QWBA studies in mice provided an opportunity to compare the brain distribution of [14C]axitinib radioactive equivalents (Figure 7) with the results obtained by Poller
et al. (2011) where parent drug was specifically measured. We confirmed their finding that axitinib has restricted mouse brain distribution since the level of axitinib radioactive equivalents present in the brain was markedly lower than whole blood or other tissues. Poller et al. (2011) went on to study the brain disposition of axitinib in abcg2–/–, abcb1a/b–/– and in triple knockout mice. These results showed no effect of abcg2–/–, a marked increase in brain exposure in abcb1a/b–/– and an even greater increase in the triple knockout. The ability of P-gp and bcrp to work together to limit brain disposition has also been described for lapatinib and dasatinib (Chen et al., 2009; Polli et al., 2009). The results of Poller et al. (2011) suggested that the mouse mdr1 limits the brain distribution of axitinib with the role of bcrp being unclear. While axitinib, like several other TKIs, exhibits poor CNS penetration in rodents, there is currently no information available for human CNS exposure following longer duration therapy with axitinib.

Axitinib is an inhibitor of MDR1 and BCRP in vitro with IC50 values of 3 and 4.4 µM, respectively. The assessment of drug interaction liability was based on the white paper on transporters in drug development (Giacomini et al., 2010) and most recent FDA and EMA drug interaction guidance documents (Anonymous, 2012b; Anonymous, 2012c). Axitinib was below the guidance cutoff values for defining systemic drug interaction risk with efflux transporters. However, [I2]/IC50 values were 35 and 24 for P-gp and BCRP, respectively, which exceeded the cutoff value of 10 when total administered dose was used to estimate the intestinal concentration. As shown in Figure 2, axitinib is highly soluble at low pH but solubility declines rapidly as pH increases above 2.0 which suggested that in the intestinal regions where efflux transporters are located and pH is more neutral, the solution concentration of axitinib achieved might be limited. Therefore, the intestinal concentration estimate of 104 µM calculated from a 10 mg dose/250 mL is likely to overpredict the actual intestinal solution concentrations.
were undertaken to account for the extensive pH dependent solubility of axitinib which conservatively estimated a lower intestinal solution concentration of 6.6 μM. By incorporating the GastroPlus™ simulated intestinal concentration of 6.6 μM, the results suggested that a clinical drug interaction study was not needed since [I_{gut}]/IC_{50} values were 2.2 and 1.5 for P-gp and BCRP, respectively. This approach is consistent with the EMA guidance which indicates that for low solubility compounds, the maximum concentration at the pH range of the GI tract can be used. Thus, the potential for axitinib to alter the pharmacokinetics of digoxin, a narrow therapeutic index chronic heart failure medicine, or drugs that are substrates for BCRP appears by this assessment to be low. This work demonstrated the utility of GastroPlus™ to simulate gastro-intestinal concentrations to better predict the amount of the inhibitor in solution at the efflux transporter site considering regional differences in pH and pH-dependent inhibitor solubility. A conservative approach was used to sum the axitinib simulated to be in solution in each GI region including the stomach where P-gp and BCRP are not expressed. If only the regions where efflux transporters are expressed were considered the [I_{gut}]/IC_{50} ratio would have been much lower for axitinib because the solubility is very low as pH becomes neutral. Further study would be necessary to determine which approach is most predictive. Finally, this application of GastroPlus™ appears well suited for consideration as an alternative in the DDI assessment of efflux transporters and enzymes expressed in the intestine for other drugs where the total dose/250 mL exceeds the aqueous solubility at pH values observed in the intestine.

Axitinib was shown to be a substrate of OATP1B1 and OATP1B3 in transfected HEK293 cells. However, in a functional assay with suspended human hepatocytes axitinib uptake was not altered by the uptake transport inhibitor rifamycin SV. Taken together, these results suggested that axitinib is a weak substrate for OATP1B1 and OATP1B3 and that passive
diffusion is the main mechanism of entry into the hepatocyte when investigated at 1 µM. In early clinical trials with axitinib, variability in pharmacokinetics or drug exposure was observed. Inter-subject variability has been associated with OATP1B1 genetic polymorphisms in patients taking various statins and irinotecan (Nozawa et al., 2005; Romaine et al., 2010). Since axitinib showed some OATP1B1 substrate activity this observation led us to the hypothesis that an inactive phenotypic variant could be a factor in clinical PK variability. However, no significant association between the SLCO1B1 (T521C) genetic polymorphism and variability in axitinib plasma exposure was noted in a pooled analysis of 11 healthy volunteer studies during early clinical trials (Brennan et al., 2012). Thus, it is likely that the observed high permeability of axitinib overrides any potential differences in active hepatic uptake that could be exhibited by phenotypic variants of these transporters. This observation highlights that, for a transporter to alter the disposition of a drug, the active transporter process must play a significant role relative to the passive contribution.
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DMD #51193

Authorship Contributions.

Participated in research design: Reyner, Sevidal, West, Fenner, Ullah, Lee
Conducted experiments: Reyner, Sevidal, West, Ullah
Contributed new reagents or analytic tools:
Performed data analysis: Clouser-Roche, Freiwald, Ullah, Reyner, Sevidal, West.
Wrote or contributed to the writing of the manuscript: Reyner, Lee, Ullah, Smith
References


interactions with the tyrosine kinase inhibitors imatinib, nilotinib, and dasatinib. *Drug Metab Dispos* **38**:1371-1380.


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Footnotes

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Legends for Figures

Figure 1. Molecular structure of axitinib.

Figure 2. pH dependent solubility of axitinib determined as described in Materials and Methods.

Figure 3. Western blot evaluation of MDCK and MDCK-LE and transfected cell lines. The left panel (A) shows specific detection of human MDR1 in MDR1-MDCK cells (lane 3) whereas, no signal was observed in MDCK (lane 2), BCRP-MDCK (lane 4) or MDCK-LE (lane 5) cell extracts. The right panel (B) shows canine/human Mdr1 detection in MDCK (lane 2) and elevated expression in BCRP-MDCK (lane 3) cells. No canine Mdr1 was detected in MDCK-LE (lane 4) or BCRP-MDCK-LE (lane 5) cells. BCRP-MDCK and BCRP-MDCK-LE cells were positive in western blots for BCRP (data not shown). Full experimental details are provided in Materials and Methods.

Figure 4. (A) Degree of digoxin flux with increasing axitinib concentrations (filled circle). IC$_{50}$ for axitinib as an inhibitor of digoxin efflux in Caco-2 cells was 3.0 µM when utilizing the maximal inhibition of ketoconazole data for curve fitting. Inhibition was maximal at 7 µM and did not increase from 10 to 75 µM presumably due to solubility limitations (open circle). (B) Degree of digoxin flux with increasing ketoconazole concentrations (filled circle). IC$_{50}$ for ketoconazole as inhibitor of digoxin efflux in Caco-2 cells was 4.1 µM. Full experimental details are provided in Materials and Methods.

Figure 5. (A) Degree of topotecan flux with increasing axitinib concentrations (filled circle). The IC$_{50}$ for axitinib as an inhibitor of topotecan efflux in MDCK-LE-BCRP cells was 4.4 µM assuming complete inhibition as observed with Ko143. Inhibition was maximal at 5.6 µM and
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did not increase at 10 and 20 µM presumably due to solubility limitations (open circle). (B) Degree of topotecan flux with increasing Ko143 concentrations (filled circle). The IC$_{50}$ for Ko143 as an inhibitor of topotecan efflux in Caco-2 cells was 0.08 µM. Full experimental details are provided in Materials and Methods.

Figure 6. Human hepatocyte uptake of axitinib. A) Uptake of axitinib (1 µM) and inhibition by rifamycin SV (no inhibition observed) and B) Uptake of the positive control rosuvastatin and inhibition by rifamycin SV (inhibition observed). Full experimental details are provided in Materials and Methods.

Figure 7. Whole-body autoradiograms of male mouse at 1, 4, 8, 24 and 48 hours after a single oral 50 mg/kg dose of [14C]axitinib. The sections were exposed for 7 days and evaluated with FujiX Bio-Imaging Analyzer BAS 1500 (Raytek, Germany) using the blood calibration standards for quantification. Electronic images were obtain by phosphorimaging. Two typical plates (C&D) are shown at each time point to allow identification of most organs. Abbreviations on the autoradiograms are ad: adrenal, bdr: bladder, bf: brown fat, bl: blood, bm: bone marrow, bug: bulbo-urethral gland cp: choroid plexus, ed: epididymis, fa: white fat, gb: gall bladder, Hd: Harderian gland, li: large intestine, lv: liver, mu: muscle, my: myocardium, nm: nasal mucosa, oe: oesphagus, , pb: pineal body, pg: preputial gland pr: prostate, re: rectum, sc: spinal cord, sg: salivary glands, si: small intestine, skp: pigmented skin, sv: seminal vesicles, th: thymus, to: tongue, ty: thyroid, to: tongue, tp: tooth pulp, ts: testis and uvt: uveal tract. Full experimental details are provided in Materials and Methods.
Tables

Table 1: Effect of increasing axitinib concentration on efflux transport in MDR1-MDCK monolayers.

<table>
<thead>
<tr>
<th>Axitinib (µM)</th>
<th>AB (µM)</th>
<th>BA (µM)</th>
<th>BA/AB Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>7.87 ± 1.31</td>
<td>9.83 ± 0.70</td>
<td>1.25</td>
</tr>
<tr>
<td>2.0</td>
<td>6.55 ± 0.46</td>
<td>9.47 ± 0.29</td>
<td>1.45</td>
</tr>
<tr>
<td>5.0</td>
<td>5.91 ± 1.18</td>
<td>8.40 ± 0.67</td>
<td>1.42</td>
</tr>
<tr>
<td>1.0 + CsA (10 µM) b</td>
<td>20.1 ± 1.00</td>
<td>17.6 ± 0.50</td>
<td>0.876</td>
</tr>
<tr>
<td>Digoxin (10 µM)</td>
<td>0.51 ± 0.17</td>
<td>12.3 ± 0.90</td>
<td>24.3</td>
</tr>
<tr>
<td>Digoxin (10 µM) + CsA (10 µM)</td>
<td>1.34 ± 0.25</td>
<td>2.25 ± 0.03</td>
<td>1.67</td>
</tr>
</tbody>
</table>

a S.D.=Standard Deviation.

b Permeability values for axitinib alone are distinctly lower than when in the presence of the MDR1 inhibitor, cyclosporine A (CsA). Recovery (data not shown) is roughly half, at all concentrations, for axitinib alone than when coincubated with CsA. This observation is likely responsible for the permeability differences.
Table 2: Effect of increasing axitinib concentration on transport studies in BCRP-MDCK-LE cell monolayers.

<table>
<thead>
<tr>
<th>Axitinib (μM)</th>
<th>AB</th>
<th>BA</th>
<th>BA/AB Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>10.2 ± 0.90</td>
<td>10.2 ± 0.70</td>
<td>1.00</td>
</tr>
<tr>
<td>2.0</td>
<td>11.5 ± 1.50</td>
<td>10.0 ± 1.10</td>
<td>0.871</td>
</tr>
<tr>
<td>5.0</td>
<td>12.0 ± 1.70</td>
<td>10.1 ± 1.00</td>
<td>0.841</td>
</tr>
<tr>
<td>1.0 + Ko143 (10 μM)b</td>
<td>20.3 ± 1.00</td>
<td>20.7 ± 1.50</td>
<td>1.02</td>
</tr>
<tr>
<td>Topotecan (2 μM)</td>
<td>0.54 ± 0.12</td>
<td>7.73 ± 0.77</td>
<td>14.4</td>
</tr>
<tr>
<td>Topotecan (2 μM) + Ko143 (10 μM)</td>
<td>1.32 ± 0.38</td>
<td>1.56 ± 0.25</td>
<td>1.18</td>
</tr>
<tr>
<td>Talinolol</td>
<td>0.97 ± 0.11</td>
<td>1.52 ± 0.19</td>
<td>1.56</td>
</tr>
</tbody>
</table>

a S.D.=Standard Deviation.

b Permeability values for axitinib alone are distinctly lower than when in the presence of the BCRP inhibitor, Ko143. Recovery (data not shown) is roughly half, at all concentrations, for axitinib alone than when coincubated with CsA. This observation is likely responsible for the permeability differences.
Table 3. Uptake rate for axitinib in OATP-transfected HEK293 cells.

<table>
<thead>
<tr>
<th>Axitinib (μM)</th>
<th>OATP2B1-HEK293 (n=4)</th>
<th>OATP1B1-HEK293 (n=4)</th>
<th>OATP1B3-HEK293 (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60 ± 1.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.14 ± 0.32</td>
</tr>
<tr>
<td>2.0</td>
<td>NA</td>
<td>5.94 ± 1.58</td>
<td>10.13 ± 2.75</td>
</tr>
<tr>
<td>4.0</td>
<td>NA</td>
<td>6.63 ± 4.64</td>
<td>13.50 ± 3.09</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>4 μM</td>
<td>2.69 ± 1.57</td>
<td>8.54 ± 1.41</td>
</tr>
</tbody>
</table>

<sup>a</sup> NA indicates no activity observed.

<sup>b</sup> Rates in pmoles/min (Mean ± SD) after subtraction of average background uptake (HEK293 cells).
Figure 2

A graph showing the solubility versus pH. The y-axis represents solubility in μM, and the x-axis represents pH. The graph displays a decreasing trend as pH increases.
Figure 3

Human MDR1 antibody (Mdr(G-1))  MDR1 antibody (canine/human)

170 kDa>

1 MDR1 (+) control (MES-SA/Dx5)  1 MDR1 (+) control (MES-SA/Dx5)
2 MDCK  2 MDCK
3 MDR1-MDCK  3 BCRP-MDCK
4 BCRP-MDCK  4 MDCK-LE
5 MDCK-LE  5 BCRP-MDCK-LE
Figure 5

(A) Plot showing the degree of topotecan flux (%) against log Axitinib concentration.

(B) Plot showing the degree of topotecan flux (%) against log Ko143 concentration.
Figure 7

Plate C

1 hr

4 hr

8 hr

24 hr

48 hr

Plate D