Overlapping Functions of ABC Transporters in Topotecan Disposition as Determined in Gene Knockout Mouse Models

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Abstract

It is established that efflux transporters of the ATP-binding cassette (ABC) superfamily can affect the pharmacokinetics of drugs through mechanisms pertaining to drug absorption, elimination, and distribution. To characterize the role of multiple transporters in topotecan’s pharmacokinetics, total (lactone + carboxylate) and lactone forms were measured by liquid chromatography/tandem mass spectrometry (LC/MS-MS) in plasma, bile, urine, and feces following intravenous administration at doses of 1 and 4 mg/kg to eight mouse strains: C57BL/6 [wild-type (WT)], Abcb1–/–, Abcc2–/–, Abcc4–/–, Abcg2–/–, Abcc2;Abcb1–/–, Abcc2;Abcg2–/–, and Abcc4;Abcg2–/–. Compared with WT mice and at both dose levels, the plasma areas under the curve for topotecan lactone were not significantly different in the Abcc2–/–, Abcc4–/–, and Abcb1–/– strains, whereas significant differences were found in Abcg2–/–, Abcc2;Abcb1–/– (only at the high dose), Abcc4; Abcg2–/–, and Abcc2;Abcg2–/– mice and ranged from 2.1- to 3.3-fold higher. Consistent with these changes, the fecal and biliary excretion of topotecan was reduced, whereas renal elimination was elevated in Abcg2–/–-based strains. Similarly, the Abcc2;Abcb1–/– strain also had elevated renal elimination and reduced fecal excretion of topotecan lactone. This was more pronounced at the 4 mg/kg dose level, suggesting possible saturation of Abcg2. The Abcc4 transporter was found not to be a major determinant of topotecan pharmacokinetics. It is concluded that Abcg2 has the most significant effect on topotecan elimination, whereas both Abcb1 and Abcc2 have overlapping functions with Abcg2. As such it is relevant to examine how polymorphisms in these transporters influence topotecan activity in patients and whether coadministration of transport modulators could positively affect efficacy without increasing toxicity. Mol Cancer Ther; 12(7); 1343–55. ©2013 AACR.

Introduction

Cancer chemotherapy involves concomitant administration of many drugs that could cause drug–drug interactions (DDI) and serious adverse effects in patients. The deleterious effects of DDIs are further complicated when drugs are substrates for membranous ATP-binding cassette (ABC) multidrug efflux transporters due to competition and the nonlinear aspect of active transport. Efflux transporters, especially ABCB1 (P-glycoprotein, P-gp), ABC2, ABC3, and ABC4 [also known as multidrug resistance proteins, multidrug resistance proteins (MRP), 2, 3, and 4], and ABCG2 (breast cancer resistance proteins, BCRP) can have an important impact on cancer chemotherapy, either at the systemic level where transporters, such as ABCB1, ABC2, and ABCG2, are localized at apical membranes of important epithelial barriers in the kidney, intestine, and at the canalicular membrane of hepatocytes that determine both drug absorption and elimination, or locally at the tumor where transporters may be resistance factors limiting intracellular drug concentrations. Local effects of transporters are also seen at sanctuary sites, such as blood–brain, blood–placenta, and blood–testis barriers, where they exert an important protective function by restricting penetration of potentially harmful chemicals to the brain, fetus, and testes, respectively. Evaluation of the effects of any single transporter has to be viewed in the context of their colocalization, overlapping substrate specificities, and function (1, 2). It should also be appreciated that the membrane orientation of the transporters could offset unidirectional effects, and, possibly maintain a “balance.” For example, ABC3 shares a similar substrate profile as that of ABCB1 and ABCC2 transporters; however, in contrast, it has a basolateral orientation in the hepatobiliary and intestinal axes and can have opposite effects on systemic availability. ABCG4 has a differential orientation, being apically localized in the renal epithelium and basolaterally localized in the hepatocytes (3, 4). The colocalization, redundancy, and differential orientation of ABC transporters provide not only functional flexibility, but also limit the ability to...
predict their impact from in vitro permeability and in situ/ ex vivo approaches. Therefore, the use of ABC transporter gene-disrupted mice offers a valuable means to characterize the role of each transporter on drug disposition, and was the approach used here.

Topotecan is a hydrophilic, semisynthetic analog of the camptothecins plant alkaloid. It disrupts DNA formation in the S-phase of the cell cycle by inhibiting topoisomerase I, which then prevents the religation of ssDNA, thus affecting transcription and replication processes, eventually leading to cell death (5). Topotecan is an established treatment for certain types of lung, ovarian, and cervical cancer and has shown favorable activity in the treatment of brain metastases (6, 7). Topotecan is a lactone species that coexists with an inactive open-ring or hydroxyacid form through a reversible pH-dependent equilibrium (Fig. 1). This chemical attribute has precluded the use of a single assay for measurement of topotecan in patient plasma samples and has allowed investigators the option to base their analyses on the lactone form or total topotecan, the sum of both the lactone and hydroxyacid forms (8, 9). The lactone form is rapidly and spontaneously converted to carboxylate form, which dominates at physiologic pH (Fig. 1), yet the lactone form is essential for topoisomerase I binding, antitumor activity, and toxicity (10, 11), and for that reason, we focused on the lactone form in our pharmacokinetic (PK) studies.

Topotecan is not metabolized to a significant degree, and thus, its elimination is dependent on transporters located in the hepatobiliary and renal axes. Definitive analyses of the role of ABC transporters on topotecan disposition are lacking at the systemic level, as well as those potentially related to tumor resistance. The magnitude of systemic and tumor cell ABC transport-mediated efflux could alter topotecan concentrations rendering it more or less effective within tumor cells. Studies report that topotecan is a substrate for ABCG2 and ABCB1, whereas conflicting reports are available for its interaction with ABCC4 transporters (12–16). Moreover, its interaction with other ABC transporters has not been reported in any detail, especially ABCC2, which like ABCG2, is localized in apical membranes in gut epithelium and in liver canaliculi, and due to its high expression in these critical organs could affect topotecan pharmacokinetic. The present study was undertaken to characterize the role of all the relevant ABC transporters on the systemic disposition of topotecan using gene knockout mice. These studies included both single knockouts (i.e., Abcg2−/−, Abcb1−/−, Abcc2−/−, and Abcc4−/−) and double knockout (i.e., Abcc2;Abcg2−/−, Abcc4;Abcg2−/−, and Abcc2; Abcb1−/−) strains to not only clarify the role of each but also assess their complimentary functions. Pharmacokinetic studies of topotecan were conducted in each strain at dose levels (1 and 4 mg/kg) that produce clinically relevant plasma concentrations (17) and could also indicate dose-dependent effects of the Abc-transporters given their capacity-limited function.

Materials and Methods

Reagents, antibodies, and cell culture

Monoclonal antibodies (mAb) C-219 (to detect ABCB1/ Abcb1) were acquired from Calbiochem, EMD Chemicals Inc. Mouse mAb (IU2H10) to detect ABC1/Abcc1 was obtained from Novus Biologicals. Rat mAb M41-80 to detect ABCC4/Abcc4 was bought from Alexis Biochemi- cals (Enzo Life Sciences). A rabbit polyclonal antibody (pAb) H300/sc-20766 against ABCC2/Abcc2, mouse mAb M3II-21/sc-59612 against ABCC3/Abcc3, rabbit pAb against ABC10/Abcc10 (H-300/sc-67241), mouse mAb BXP-21/sc-58222 to detect ABCG2/Abcg2 were obtained from Santa Cruz Biotechnology, Inc. Facitaxel was obtained from LC Laboratories. Mouse mAb β-actin, IR secondary antibodies IRDye 800 and 680-labeled anti-rabbit, anti-mouse, anti-rabbit, and anti-rat, NewBlot polyvinylidene difluoride (PVDF) stripping buffer and PVDF-blocking buffer were purchased from Li-Cor Biosciences. Mitoxantrone (MX), topotecan, ammonium for- mate, camptothecin, verapamil, and MTT were purchased from Sigma-Aldrich. High performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Fisher Scientific. Deionized water (Nanopure deionization system; Barnstead/Thermolyne) was used to prepare all aqueous solutions. All other reagents and solvents were purchased from VWR. HEK293/pcDNA3.1, HEK293/ABC1, HEK293/ABCC1, HEK293/ABCG2-R2, and HEK293/MPR3 cells were

![Chemical structure of the lactone and open-ring carboxylate forms of topotecan.](image-url)
previously established by transfecting HEK293 with either the empty pcDNA3.1 vector or pcDNA3.1 vector containing the full-length ABCB1 (HEK293/ABCBI), ABCC1 (HEK293/ABCC1), or ABCC3 (HEK293/ABCP3), and were cultured in a medium with 2 mg/mL of G418 (18–20). MRP2–transfected LLC/PK1 cells (LLC/PK1-hMRP2) and parental plasmid-transfected cells (LLC/PK1-CMV) were described previously (21). The MRP4–transfected NIH-3T3 cell line (NIH-3T3/MRP4-3) and NIH-3T3 cells transfected with parental vector (NIH-3T3/pSRα) were described previously (22). In addition, non–small cell lung carcinoma (NSCLC) cells H460 were cultured with mitoxantrone up to 20 μmol/L to produce ABCG2-overexpressing H460/MX-20 cells. Similarly, NSCLC H460 cells were cultured with paclitaxel 50 nmol/L for up to 3 months to produce ABCB1-producing H460/TX-50 cells (18, 23). All the cell lines were grown as adherent monolayers in flasks with Dulbecco’s modified Eagle medium (DMEM) culture medium (Hyclone Co.) supplemented with 10% FBS in a humidified incubator containing of 5% CO2 at 37°C. The resistance-fold (RF) values were calculated by dividing the IC50 values (concentration required to inhibit growth by 50%) measured in the ABC transporter overexpressing cells by that measured in the parental sensitive cells. The SoftMax Pro Data Acquisition & Analysis software (Molecular Devices) was used for these analyses.

Cell cytotoxicity by MTT assay

The MTT colorimetric assay with slight modifications from that previously described (24) was used to detect the sensitivity of cells to topotecan. Cells were harvested with trypsin and resuspended at a final concentration of 5 × 10^4 cells/mL for H460, 6 × 10^4 cells/mL for H460/MX-20 and H460/TX-50, 4 × 10^4 cells/mL for NIH-3T3/pSRα, 5 × 10^4 for NIH-3T3/MRP4-3, and 8 × 10^4 for all the other cell lines. Cells were seeded evenly into (180 μL/well) 96-well multivell plates. Different concentrations of topotecan up to 10 μmol/L (20 μL/well) in triplicate were added into designated wells. After 72 hours of incubation, 20 μL of MTT solution (4 mg/mL) was added to each well, and the plate was further incubated for 4 hours, allowing viable cells to convert the yellow-colored MTT into dark-blue formazan crystals. Subsequently, the medium was discarded, and 100 μL of dimethyl sulfoxide (DMSO) was added into each well to dissolve the formazan crystals. The absorbance was determined at 570 nm with a SpectraMax M3 multimode microplate reader (Molecular Devices). The resistance-fold (RF) values were calculated by dividing the IC50 values (concentration required to inhibit growth by 50%) measured in the ABC transporter overexpressing cells by that measured in the parental sensitive cells. The SoftMax Pro Data Acquisition & Analysis software (Molecular Devices) was used for these analyses.

Western blot analysis

Crude membrane fractions from homogenized tissues were prepared and Western blot analyses were conducted as previously described with minor modifications as noted (25, 26). Briefly, the samples were prepared by homogenizing approximately 40 mg liver or kidney in 0.5 mL of 10 mmol/L Tris–Cl (pH 7.4), 150 mmol/L NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (w/v) NP-40, 100 μg/mL phenylmethylsulfonylfluoride (PMF) and complete protease inhibitor cocktail mix (Roche Applied Sciences). Homogenized samples were incubated on ice for 30 minutes with occasional rocking for 15 minutes, which was followed by centrifugation at 15,000 × g for 20 minutes. The supernatant was collected, which was used to determine the protein concentrations of the samples, using the BCA Protein Assay Kit (Pierce Biotechnology, Inc.). Approximately 50 μg of total protein per lane was resolved by electrophoresis on a NuPAGE 4% to 12% Bis-Tris gel (Invitrogen) and electrophoretically transferred onto PVDF membranes. Equal protein loading was confirmed by comparing β-actin expression. Samples were treated with one or more of the following primary antibodies to detect Abcc1 (IU2H10; 1:100), Abcc2 (H-300; 1:75), Abcc3 (M3II-21; 1:150); Abcc4 (M41-80; 1:100), Abcc10 (H300; 1:200), Abcb1 (C-219; 1:200); Abcg2 (BXP-21; 1:200), and β-actin (Lcor2642212; 1:2,000). The following secondary antibodies were used: IRDye 680-labeled anti-rabbit (1:10,000), IRDye 800–labeled anti-mouse, anti-rabbit, and anti-rat (1:5,000) and IRDye 680–labeled anti-mouse and anti-rat (1:5,000). Blotting and imaging were carried out using the Odyssey imaging system according to the manufacturer’s protocols (LI-COR). Band intensity measurements were measured using NIH ImageJ software (http://rsweb.nih.gov/ij/).

Animal handling, genotyping, blood chemistries, and hematology

Eight strains of male mice, C57BL/6 [wild-type (WT)], Abcb1(a/b); Abcg2/Abcc2; Abcb1(a/b); Abcc2/Abcc4; Abcb1(a/b); Abcc2/Abcc4; Abcb1(a/b); Abcc2/Abcc4; and Abcb1(a/b); Abcc2/Abcc4–/– mice with a C57BL/6 background were generated by cross-breeding 2 single knockout strains at Charles River Laboratories. All animals were maintained on an alternating 12 hours light/dark cycle with free access to water and rodent chow ad libitum. The Institutional Animal Care and Use Committee at Mount Sinai Medical Center (New York, NY) approved all animal procedures. Genotyping, blood chemistry, and hematologic variables were determined at Charles River Research Animal Diagnostic Services.

Pharmacokinetic, fecal, and renal excretion studies

Each mouse under anesthesia had a cannula implanted in the left carotid artery that was exteriorized at the back of the neck to permit the collection of serial blood samples. The pharmacokinetic experiments were carried out on conscious, freely mobile mice placed in metabolic cages with an ample water supply (Nalgene; Braintree Scientific Inc.) that allowed for the separate collection of urine and...
Topotecan dissolved in saline was administered as an intravenous bolus via a tail vein to groups of mice at doses of 1 and 4 mg/kg. Serial (n = 8–10) blood samples of 20 μL were collected through the left carotid artery cannula for up to 6 hours. The blood samples were centrifuged at 4°C and the resultant plasma was separated into 2 parts (5 μL each). One part for measurement of total topotecan was stored at −80°C immediately, whereas the other 5 μL aliquot designated for measurement of topotecan lactone had 4 parts of acetonitrile containing 80 ng/mL of the internal standard (camptothecin) added immediately followed by a 10 second vortex and centrifugation for 10 minutes at 16,000 × g. The resultant supernatant was stored at −80°C to detect lactone form of topotecan by liquid chromatography/tandem mass spectrometry (LC/MS-MS). To avoid blood volume depletion, 20 μL of saline was replaced after each blood collection. Urine and feces of each animal were collected for 24 hours in preweighed tubes and were stored at −80°C until analysis by LC/MS-MS.

Biliary topotecan excretion

A procedure to collect bile by gall bladder cannulation and bile duct ligation in male C57BL/6 WT, Abcb1(a/b)−/−, Abcg2−/−, Abcc2−/−, Abcc4−/−, Abcb1(a/b);Abcc2−/−, Abcg2;Abcc2−/−, and Abcg2/Abcc4−/− mice (n = 3–5) was slightly modified from that previously described (29). Briefly, mice were anesthetized with isoflurane anesthesia (VetEquip Inc.) throughout the experiment. The common bile duct was ligated distally after opening the abdominal cavity and a durometer vinyl catheter (Scientific Commodities Inc.) with an inner diameter of 0.28 mm was inserted into the incised gall bladder and fixed with 2 additional ligatures. After successful cannulation of the gall bladder, 4 mg/kg of topotecan was administered as an intravenous bolus through the lateral tail vein injection. Bile was collected at 10-minute intervals for 1 hour. At the end of the 1 hours, mice were sacrificed by cervical dislocation. Several tissues were removed and homogenized in 4% bovine serum albumin; intestinal contents were separated from intestinal tissues before homogenization and the plasma and organs were harvested on dry ice and stored at −80°C until analyzed by LC/MS-MS.

Total and lactone form of topotecan determination by LC/MS-MS

Total topotecan (lactone and carboxylate forms) and topotecan lactone in plasma were quantified using an electrospray ionization interface LC/MS-MS system (API QTrap 5500; Applied Biosystems) operated in positive ion mode. Chromatography was conducted using a Phenomenex Luna C18 (50 mm × 2.0 mm) column with a gradient mobile phase of double-distilled water (pH ~ 6.5–7.0) and acetone at a flow rate of 0.2 mL/min for topotecan lactone. For the determination of the lactone form, the previously treated aliquots were vortexed and injected into the LC/MS-MS. To measure total (lactone + carboxylate) topotecan and ensure that topotecan was all in the lactone form (pH ~ 3–3.5), 5 μL plasma samples were acidified with 50% formic acid (0.5 μL) followed by vortexing for 30 seconds with 4-part of acetonitrile containing the internal standard camptothecin (80 ng/mL) and then processed as for the lactone fraction. Chromatography was conducted using Phenomenex Luna C18 (50 mm × 2.0 mm) column with a gradient mobile phase of 5 mmol/L ammonium formate (0.1% formic acid) and acetone at a flow rate of 0.2 mL/min for topotecan. Mass transitions (m/z) monitored were 422.2 → 377.2 for topotecan and 349 → 305 for the internal standard, respectively. The lowest limit of quantitation was 1.0 ng/mL. Total topotecan and topotecan lactone concentrations in bile, urine, and feces were quantitated directly from the frozen samples using similar methods described earlier.

In brief, each clean urine sample obtained by centrifugation was diluted 300 times and that of bile 500 times with double-distilled water followed by transfer of an aliquot (20 μL) that was deproteinized by a 2-fold volume of acetonitrile containing the internal standard camptothecin (80 ng/mL). After centrifugation (16,000 × g, 10 minutes), the supernatant was injected into the LC/MS-MS system. Each sample of feces was first homogenized in a 2% (w/v) solution of double-distilled water. An aliquot (20 μL) of the resultant homogenate was deproteinized with a 2-fold volume of acetone containing the internal standard camptothecin (80 ng/mL). After centrifugation (16,000 × g, 10 minutes), the supernatant was injected into the LC/MS-MS system. Total (lactone + carboxylate) topotecan in urine, bile, and feces were measured as described earlier after acidification with 50% formic acid (0.5 μL) before deproteinization to convert to the lactone form (pH ~ 3–3.5).

Data analysis

Each individual subject’s topotecan (total and lactone) plasma concentrations were analyzed by noncompartmental analysis using WinNonlin Professional Version 5.2 (Pharsight Co.) to obtain estimates of the pharmacokinetic parameters that included the volume of distribution (Vd), total systemic clearance (CL), the elimination half-life (t1/2), and the area under the plasma concentration–time curve from 0 to infinity (AUC0–∞). Whereas, topotecan (total and lactone) elimination parameters, i.e., biliary excretion rate (μg/h), percentage dose in urine (fTu, %) and percentage dose in feces (fFu, %) were estimated separately. Comparison of pharmacokinetic variables and other elimination parameters between different strains at each dose level were completed using a one-way ANOVA, followed by Tukey multiple comparisons with significant differences based on *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001. In addition, intrastrain comparisons for CL, fTu, %, and fFu, % were conducted on the basis of the mean and SD values from 2 independent groups (1 and 4 mg/kg) using paired Student t tests, where P < 0.05 was considered statistically significant. Comparisons of RF values for parental and drug resistance cells were conducted on the basis of the mean and
SD values from 2 independent groups using unpaired Student t tests, where \( P < 0.05 \) was considered statistically significant. All in vitro experiments were repeated at least 3 times. All statistical analyses were completed using GraphPad Prism version 5.04.

Results

Topotecan interaction with ABC transporters using in vitro systems

To generate an ABC transport profile for topotecan, in vitro cytotoxicity assays were completed in pairs of sensitive and drug-resistant or ABC transporter–overexpressing cells (Fig. 2). The IC\(_{50}\) value ratio for each pair reported as a RF was 3.7 to 12.1, and in low-to-high rank order was ABCC4 (Fig. 2F) = ABCB1 (transfected cells; Fig. 2A) < ABCC2 (Fig. 2E) < ABCB1 (paclitaxel-selected cells; Fig 2B) < ABCG2 (Fig. 2C and D). The findings that indicate topotecan may be a substrate for ABCB1 and ABCG2 are consistent with previous studies (12, 16). The interaction of topotecan with ABCC transporters family, whose role has only been recently implicated in drug resistance and drug disposition (4) indicated that ABCC2 (RF = 4.4; Fig. 2E) and ABCC4 (RF = 3.7; Fig. 2F) transporters conferred significant resistance to topotecan. RF values for other MRP members in the ABCC family, specifically, human ABCC1/MRP1, ABCC3/MRP3, ABCC5/MRP5, ABCC10/MRP7, and ABCC12/MRP9 were not significantly different for topotecan (Supplementary Table S1). In summary, based on cytotoxicity assays, ABCB1, ABCG2, ABCC2, and ABCC4 conferred significant topotecan resistance in vitro and may be involved in its pharmacokinetic characteristics.

Overlapping functions of transporters in altering pharmacokinetic of topotecan

The in vitro findings guided us to select the relevant single and double ABC transporters knockout mouse models to study topotecan disposition. Each of the following strains received 1 and 4 mg/kg of topotecan as an intravenous bolus, WT, Abcc2/−/−, Abcc4/−/−, Abcb1/−/−, Abcg2/−/−, Abcc2;Abcg2/−/−, Abcc4;Abcg2/−/−, and Abcc2;Abcb1/−/−, and both total topotecan (carboxylate + lactone) and topotecan lactone were measured by LC/MS-MS in plasma, urine, bile, and feces (Supplementary Figure 2).

Figure 2. Cell survival curves of multidrug resistant (MDR) cells and the corresponding parental cells toward topotecan are shown in A: HEK293 and transfected HEK293/ABCB1 cells (A); NSCLC-H460 and paclitaxel selected ABCB1 overexpressing H460/TX-50 cells (B); HEK293 and transfected HEK293/ABCG2-R2 cells (C); NSCLC-H460 and mitoxantrone-selected ABCG2-overexpressing H460/MX-20 cells (D); parental (LLC/PK1-CMV) and ABCG2-transfected LLC/PK1-hMRP2 cells (E); and parental (NIH-3T3/pSRa) and ABCG4-transfected NIH-3T3/MRP4-3 cells (F). Cell survival was determined by MTT assay as described in Materials and Methods. Data points represent the mean ± SD of triplicate determinations carried out at least 3 independent times. The resistance-fold (RF) was calculated by dividing the IC\(_{50}\) for the MDR cells by that of the parental sensitive cells. The statistical differences were determined using the Student t test with significant differences based on \(^* P < 0.05\) compared with parental cells. OD, optical density.
Table 1. Selected topotecan lactone pharmacokinetic parameters in ABC transporter knockout strains at 2 intravenous dose levels

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C57/BL6 (n = 7)</th>
<th>Abcc2&lt;sup&gt;−/−&lt;/sup&gt; (n = 6)</th>
<th>Abcc4&lt;sup&gt;−/−&lt;/sup&gt; (n = 6)</th>
<th>Abcb1&lt;sup&gt;−/−&lt;/sup&gt; (n = 5)</th>
<th>Abcg2&lt;sup&gt;−/−&lt;/sup&gt; (n = 7)</th>
<th>Abcc2;Abcg2&lt;sup&gt;−/−&lt;/sup&gt; (n = 5)</th>
<th>Abcc4; Abcg2&lt;sup&gt;−/−&lt;/sup&gt; (n = 5)</th>
<th>Abcc2; Abcb1&lt;sup&gt;−/−&lt;/sup&gt; (n = 5)</th>
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<td><strong>1 mg</strong></td>
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<td>V&lt;sub&gt;d&lt;/sub&gt; (L/kg)</td>
<td>19.5 ± 5.6</td>
<td>17.9 ± 8.1</td>
<td>20.4 ± 5.5</td>
<td>14.0 ± 2.5</td>
<td>9.9 ± 4.3</td>
<td>71 ± 1.9</td>
<td>9.1 ± 2.5</td>
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<td>CL (L/h/kg)</td>
<td>12.8 ± 3.0</td>
<td>9.2 ± 3.9</td>
<td>14.2 ± 7.8</td>
<td>7.0 ± 2.2</td>
<td>4.9 ± 2.9</td>
<td>3.6 ± 1.0</td>
<td>5.1 ± 0.7</td>
<td>5.8 ± 0.8</td>
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<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.1 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 0.8</td>
<td>1.4 ± 0.2</td>
<td>1.5 ± 0.4</td>
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<td>1.2 ± 0.3</td>
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<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (h&lt;sup&gt;2&lt;/sup&gt;/ng/mL)</td>
<td>81.9 ± 17.4</td>
<td>130.1 ± 65.0</td>
<td>91.2 ± 51.2</td>
<td>154.0 ± 46.7</td>
<td>242.4 ± 75.8</td>
<td>291.5 ± 76.2</td>
<td>200.0 ± 25.5</td>
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<td><strong>4 mg</strong></td>
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<td>V&lt;sub&gt;d&lt;/sub&gt; (L/kg)</td>
<td>23.6 ± 6.7</td>
<td>33.3 ± 10.2</td>
<td>21.7 ± 3.8</td>
<td>14.9 ± 5.4</td>
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<td>CL (L/h/kg)</td>
<td>14.0 ± 5.3</td>
<td>19.1 ± 7.9</td>
<td>15.9 ± 4.7</td>
<td>11.4 ± 2.8</td>
<td>15.9 ± 1.6</td>
<td>4.0 ± 0.6</td>
<td>6.5 ± 2.2</td>
<td>5.9 ± 0.5</td>
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<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.3 ± 0.6</td>
<td>1.4 ± 0.9</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 1.2</td>
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<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (h&lt;sup&gt;2&lt;/sup&gt;/ng/mL)</td>
<td>314.3 ± 101.3</td>
<td>240.6 ± 94.3</td>
<td>271.3 ± 81.5</td>
<td>372.3 ± 105.4</td>
<td>735.6 ± 270.7</td>
<td>1,021.9 ± 151.2</td>
<td>684.0 ± 244.3</td>
<td>676.4 ± 55.8</td>
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<td><strong>Fecal (f&lt;sub&gt;f&lt;/sub&gt;, %)</strong></td>
<td>18.0 ± 11.8</td>
<td>16.5 ± 9.9</td>
<td>18.8 ± 11.9</td>
<td>14.7 ± 7.8</td>
<td>32.6 ± 18.1</td>
<td>34.7 ± 5.1</td>
<td>30.7 ± 11.9</td>
<td>35.7 ± 11.3</td>
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<tr>
<td>Renal (f&lt;sub&gt;r&lt;/sub&gt;, %)</td>
<td>25.5 ± 10.4</td>
<td>18.0 ± 9.7</td>
<td>24.2 ± 16.5</td>
<td>12.1 ± 8.8</td>
<td>12.0 ± 9.8</td>
<td>4.9 ± 2.7</td>
<td>12.5 ± 6.9</td>
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| NOTE: Topotecan lactone pharmacokinetic parameters — V<sub>d</sub>, CL, t<sub>1/2</sub>, AUC<sub>inf</sub>, f<sub>r</sub>, % and f<sub>f</sub>, % in male C57BL/6 (WT), Abcc2<sup>−/−</sup>, Abcc4<sup>−/−</sup>, Abcb1<sup>−/−</sup>, Abcg2<sup>−/−</sup>, Abcc2;Abcg2<sup>−/−</sup>, Abcc4; Abcg2<sup>−/−</sup>, Abcc2; Abcb1<sup>−/−</sup> mice, after intravenous administration of 1 and 4 mg/kg topotecan. The pharmacokinetic parameters were analyzed by noncompartmental analysis using WinNonlin Professional Version 5.2. The statistical differences were determined using the one-way ANOVA with Tukey multiple comparison (GraphPad Prism version 5.04) tests to compare interstrain differences. Data are expressed as mean values of the SD (n = 5–7); * P < 0.05; ** P < 0.01; *** P < 0.001 compared with WT; * P < 0.05; ** P < 0.01; *** P < 0.001 compared with WT; * P < 0.01; ** P < 0.001 compared with Abcc2<sup>−/−</sup>; * P < 0.05; ** P < 0.01; *** P < 0.001 compared with Abcc2<sup>−/−</sup>; ** P < 0.01; *** P < 0.001 compared with Abcc2<sup>−/−</sup>; ** P < 0.01; *** P < 0.001 compared with Abcc2<sup>−/−</sup>; ** P < 0.01; *** P < 0.001 compared with Abcc2<sup>−/−</sup>.
In the order of 3.6-fold in Abcc2;Abcg2, however, higher topotecan lactone AUCs were observed at 1 mg/kg dose, and suggests saturation of Abcg2 transporter at the higher dose. Comparisons within the different gene knockout strains allowed the overlapping function of the transporters to be discerned. The mean topotecan AUC in the Abcc2; Abcg2–/– strain was 2.2-fold (1 mg/kg) and 4.2-fold (4 mg/kg) greater than the single knockout Abcg2–/– strain, and 1.2-fold (1 mg/kg) and 1.5-fold (4 mg/kg) greater than the Abcg2–/– strains, and attests to the dominant effect of Abcg2, as well as to a contributing effect of Abcc2 on topotecan elimination. Abcc4 did not have a significant effect on topotecan elimination based on small changes in the AUC obtained in the Abcc4;Abcg2–/– mouse relative to the Abcg2–/– mouse. To decipher the role of Abcb1, AUC comparisons between the Abcc2;Abcb1–/– and both.

Figure 3. Topotecan lactone pharmacokinetic variables AUC0–∞ (A); CL (B); and Vd (C) in male C57BL/6 WT, Abcc2–/–, Abcc4–/–, Abcb1–/–, Abcc2;Abcg2–/–, Abcc2;Abcb1–/– mice after intravenous administration of 1 and 4 mg/kg topotecan. The pharmacokinetic parameters were estimated by noncompartmental analysis using WinNonlin Professional Version 5.2. The statistical differences were determined using the one-way ANOVA with Tukey multiple comparison (GraphPad Prism version 5.04) tests to compare interstrain differences. Data are expressed as mean values of the AUC, CL, or Vd ± SD (n = 5–9); *, P < 0.05; **, P < 0.01; ***; P < 0.001 compared with WT; #, P < 0.05; ##, P < 0.01; ###, P < 0.001 compared with Abcc2–/–).
Role of transporters in renal, hepatic, and biliary elimination of topotecan

Measurement of topotecan in urine, bile, and feces can delineate the contribution of biliary and renal elimination pathways to total clearance. In the preclinical setting, the use of “metabolism” cages permits the separate collection of urine, needed for renal clearance, and feces; however, drug contained in feces represents that eliminated in bile and by intestinal extrusion following intravenous administration. A direct means to determine biliary excretion can be achieved through the combined use of bile duct ligation and gall bladder cannulation, yet due to the intricacies of this approach and the difficulty to maintain the patency of the exteriorized cannula limit the duration of the experiment to 1 hour. Therefore, 2 experimental approaches were used: one in which individual mice in each strain were placed in metabolism cages to obtain serial blood samples, urine, and fecal samples, and second, separate groups of mice at the 4 mg/kg dose only underwent the bile duct surgical procedure to provide bile samples to assess biliary excretion. In this manner, a thorough analysis of the routes of topotecan elimination and contribution of each transporter could be ascertained.

Figure 4A and B shows the topotecan lactone excreted by renal and fecal excretion, respectively, at both dose levels. At 1 mg/kg (Fig. 4A), topotecan lactone was

The total clearance values of topotecan lactone at both dose levels (Fig. 3B) follow the same pattern as the AUC values, yet in the opposite direction as required, as the CL is the inverse of the total AUC (i.e., CL = dose/AUC). The apparent \( V_d \) is dependent on drug-plasma protein and drug-tissue binding, yet in the absence of changes in these parameters in each strain, the \( V_d \) values reflect changes in total clearance and the terminal elimination rate constant (i.e., \( V_d = CL/\lambda n \)), and therefore the significant differences in \( V_d \) between strains (Table 1) are likely due to primary changes in clearance, transport-mediated effects on biliary and renal excretion. For completeness, the pharmacokinetic variables obtained from total topotecan measurements are provided (Supplementary Table S1 and Supplementary Figs. S2 and S3) and were found to follow the same trends among different strains as those obtained for topotecan lactone.

Figure 4. Urinary (A) and fecal (B) excretion of topotecan lactone in male C57BL/6 (WT), Abcc2/−/−, Abcc2/−/−;Abcc4/−/−, Abcc2/−/−;Abcg2/−/−, and Abcc2/Abcc1/−/− mice over 24 hours after intravenous administration of 1 and 4 mg/kg topotecan. The statistical differences were determined using the one-way ANOVA with Tukey multiple comparison (GraphPad Prism version 5.04) tests to compare interstrain differences. Data are expressed as percentage of dose ± SD (n = 5–9; *P < 0.05; **, P < 0.01; compared with WT).
Abcb1 topotecan lactone was also increased close to 2-fold in order of over 2-fold. Of some interest, renal excretion of greater than the WT strain with the differences on the 3 strains based on Abcg2 deletion being significantly greater than the WT strain with the differences on the order of over 2-fold. Of some interest, renal excretion—supported by increased protein expression—of topotecan lactone was also increased close to 2-fold in Abcc2;Abcg2 (Fig. 4A). A quite similar pattern in renal excretion was seen at the higher 4 mg/kg topotecan dose, yet none of the differences reached significance, that could either reflect experimental variability or saturation of compensatory renal clearance mechanisms. The compensatory increases in renal excretion of topotecan in the knockout strains, particularly in the Abcg2–/– strains, are partially attributed to enhanced Abcc4-mediated renal excretion supported by increased protein expression—yet may be considered paradoxical as even the Abcc4;Abcg2–/– strain had elevated renal excretion. Similar patterns of renal excretion were observed for total topotecan at both dose levels in WT and knockout mice (Supplementary Fig. S3). Overall, it can be concluded that the absence of Abcg2 has the most profound effect on topotecan biliary excretion that is compensated by elevated renal excretion, to a lesser extent Abcb1 and Abcc2 function in a similar manner.

The patterns of changes in fecal elimination expressed as the percentage dose are essentially inverse to those for renal excretion for each strain. The mean percentage dose excreted in the feces at both 1 and 4 mg/kg ranged from about 5% to a high of 45%, with the Abcg2–/–-based strains having the lowest fractional fecal excretion at about 5%. Fecal excretion of topotecan lactone was also decreased in the absence of both Abcc2 and Abcb1 at both dose levels, but not to the extent observed in the absence of Abcg2 (Fig. 4B). Intrastrain dose comparisons showed a significant reduction of fecal elimination in the Abcc2; Abcb1–/– strain at 4 mg/kg compared with 1 mg/kg that is consistent with saturation of Abcg2 transporter at high dose levels. Fecal elimination of total topotecan followed a similar trend in all the mouse strains as that of topotecan lactone (Supplementary Fig. S3B and Supplementary Table S2).

Topotecan biliary excretion rates were obtained in bile duct ligated, gall bladder cannulated mice at the 4 mg/kg i.v. dose (Fig. 5). Compared with the WT strain, significantly decreased biliary excretion of topotecan lactone was found for Abcc2; Abcb1–/– and for all the Abcg2–/–-based strains with a high of a 19-fold reduction in the Abcc2; Abcg2–/– strain. Within strain comparisons indicated significantly decreased biliary elimination of topotecan lactone in the Abcc2; Abcb1–/– strain compared with Abcc2–/– and Abcb1–/– strains, and in Abcc2; Abcg2–/– strain compared with both the Abcc2–/– and Abcg2–/– strains. These excretion rate data (amount excreted/hour) take into account the changes in bile flow rate that relative to WT mice decreased in the order of Abcc2–/– = Abcc2; Abcg2–/– < Abcc2; Abcb1–/– < Abcg2–/– = Abcc4; Abcg2–/– (Supplementary Fig. S4A), which except for the Abcc2–/– strain coincided with the excretion rate. This suggests that bile flow may partially depend on the functional capacity of the biliary pumps for topotecan. Total topotecan biliary excretion pattern were similar to that of topotecan lactone in all strains (Supplementary Fig. S4B). In summary, the changes in fecal excretion of topotecan are primarily attributed to changes in its biliary excretion afforded by Abcg2, Abcb1, and Abcc2.

**Transporters expression analysis and clinical chemistry in the ABC knockout models**

It is possible that elimination of one or more transporters could result in altered expression of the remaining transporters to compensate for the loss of function. Examination of protein expression of the ABC transporters in kidney and liver, before any drug treatment, indicated (Fig. 6A and B) there were insignificant differences in the expression in the different gene knockout strains compared with the WT strain except in a few organ-specific cases. For example, in liver Abcc4 is basolaterally localized and was significantly elevated in the Abcc2–/–, Abcb1–/–, Abcc2; Abcg2–/–, and Abcc2; Abcb1–/– strains compared with WT (Fig. 6A). This elevated expression of Abcc4 could have contributed to higher topotecan plasma concentrations in addition to the loss in biliary excretion potential in the absence of Abcc2, Abcb1, and Abcg2. In kidney, the most pronounced change occurred with Abcc3, a basolateral transporter was increased about
1.6-fold, in Abcc2;Abcb1−/− mouse, whereas Abcc4, an apical transporter was significantly upregulated in the Abcc2−/−, Abcc2;Abcg2−/−, and Abcc2;Abcb1−/− mouse strains compared with WT (Fig. 6B) and certainly may have had a role in increased renal excretion in these strains.

All the mouse strains used in this study were healthy and had no obvious phenotypic abnormalities. The mice gained normal weight and had no abnormality in their fertility or survival (data not shown). Consistent with previous findings, total bilirubin levels were increased in the Abcc2−/− and Abcc2;Abcg2−/− strains, whereas higher levels of triglycerides and cholesterol were seen in Abcc2−/− mice. There were no other significant changes in the clinical chemistry panels in any of the strains compared with WT mice (Supplementary Table S3).

Discussion

The analysis of how ABC transporters function in drug disposition has become more complex as their overlapping roles and substrate profiles are delineated. The pivotal locations of ABC transporters in the gastrointestinal tract, liver, and kidneys make their contribution to drug absorption and elimination register as changes in macro-pharmacokinetic parameters such as oral bioavailability and clearance. Their presence in regional sites, such as the blood-brain barrier, can also have profound influences on drug distribution, but may not be realized without tissue analyses of drug concentrations. The net effects, whether they emanate on a systemic or regional tissue scale can curtail or enhance the effectiveness of drugs depending on target site drug concentrations, and further, contribute to toxicity in nontarget organs.
Coupled to the ABC transporter, somewhat ubiquitous presence, overlapping function, and their capacity to efflux diverse chemical structures is their ability to operate in a nonlinear and saturable manner, subject to competitive inhibition, making predictions of their influence difficult without detailed pharmacokinetic investigations. The current study was designed to characterize the role of ABC transporters on the systemic pharmacokinetics of topotecan, a highly used anticancer drug.

Topotecan is a known substrate for the ABCG2 transporter and confirmed here, whereas its interaction with ABCB1 and ABCC4 uncertain, and details of other ABC family members lacking. Moreover, there have been few studies to address the overlapping function of different transporters that limit an understanding of the role of single ABC transporters in topotecan pharmacokinetics. The benefits of using double knockout mice are known (30, 31), and can resolve conflicting interpretations of pharmacokinetic data obtained in single ABC transporter knockout mouse models, when there is functional redundancy of at least 2 transporters. In the present study, we have dissected the overlapping function of ABC transporters as it pertains to topotecan systemic pharmacokinetic properties through the use of gene knockout mouse models that provide a realistic means to predict the consequences of the transporters in humans.

The pharmacokinetics of topotecan in patients with cancer seem to be dose-proportional and multicompartmental in nature with an elimination $t_1/2$ of 2 to 3 hours (10, 32). Renal elimination and biliary excretion are the major routes of elimination for topotecan, with 26% to 80% of the dose recovered in urine, 17.9% ± 3.6% in feces, and 18% of the dose eliminated in the bile (33). Comparing these general characteristics to the WT mice in our study, a similar dose-proportional relationship in topotecan lactone AUC was observed at 1 to 4 mg/kg doses (Table 1). The percentage dose eliminated by each route for the 2 doses was a mean of 15% to 18% for renal, 26% to 36% for fecal (Table 1), and about 20% by biliary excretion based on the 1-hour collection (data not shown). Thus, compared with normal mice, patients seem to have a slightly greater propensity to eliminate topotecan by renal excretion, yet the variability is high and could represent disease states and polymorphic changes in transporter genes (34, 35).

Topotecan is not extensively metabolized (36–38) with only low concentrations of 3 topotecan metabolites (N-desmethyl topotecan, TPT-O-glucuronide, and N-desmethyl TPT-O-glucuronide) identified in blood in patients. Other than the ABC transporters, the involvement of other membrane transporters to topotecan elimination seem limited, with a recent finding that OAT3, a member of the SLC22 family, may contribute to topotecan secretion in the renal tubules of the rats and humans (39). Thus, there is general agreement in the clearance mechanisms involved in topotecan elimination in mice and humans, and that these processes are intimately connected to active transporters of the ABC family.

Through an extensive set of pharmacokinetic investigations that used 8 mouse strains, 2 dose levels, and the collection of urine, feces, bile (one dose level), and serial blood samples, we determined the most important ABC transporters and their complimentary functions in the disposition of topotecan. These analyses were facilitated by a sensitive LC/MS-MS method to measure topotecan, both total and lactone forms, in as little as 5 μL of plasma that permitted a serial blood sampling protocol in mice. There were similar trends in the pharmacokinetic variables, renal, fecal, and hepatobiliary excretion at both dose levels for both lactone and total forms of topotecan suggesting that either form is sufficient to assess its disposition in mice.

Abcg2 had the most pronounced influence on topotecan elimination via biliary excretion, yet Abcc2 and Abcb1 also contributed to this process that is revealed at the high dose level in the double knockout strains (Table 1). Indeed, the Abcc2;Abcb1/–/– and Abcc2;Abcg2/–/– strains had elevated renal, reduced fecal and biliary excretion, particularly prominent at the 4 mg/kg dose level. It was through the use of the double knockout strains and 2 dose levels that a role of Abcc2 and its overlapping function with Abcg2 and Abcb1 could be ascertained. There is some conflicting data on the interaction between ABCC4 or Abcc4 and topotecan. Previously, Leggas and colleagues (14) have shown that topotecan is a substrate of ABCC4 and in the Abcc4/–/– mouse showed significant accumulation of topotecan in brain tissues and cerebrospinal fluid compared with WT mice. Somewhat counter findings were reported by de Vries and colleagues (12), where topotecan concentrations in brain homogenates of Abcc4/–/– were not significantly different from that of WT mice. Although our study was not designed to evaluate how Abcc4/–/– affected brain distribution of topotecan, we found an approximate 4-fold change in the RF in cells that overexpressed ABCC4 that supports a topotecan-Abcc4 interaction. However, sole deletion of Abcc4 did not significantly affect topotecan clearance even though it would be anticipated to contribute to renal elimination based on its apical orientation in renal tubules (40); apparently topotecan either has a low affinity for Abcc4 in renal tubules or other transporters compensate for this function as the percentage dose (Table 1) eliminated in urine was not significantly different than in WT mice. In fact, in the absence of both Abcc4 and Abcg2 renal excretion increased relative to WT mice, although paradoxical, could be indicative of another renal transport mechanism. Nonetheless, examination of the renal expression of Abcc4 among the different strains showed appreciable upregulation in the Abcc2/–/–; Abcc2; Abcg2/–/–, and Abcc2;Abcb1/–/– strains, which except for the Abcc2/–/– strain, had elevated renal excretion that supports a contributory role of Abcc4 in the renal excretion of topotecan. Therefore, although the role of Abcc4 in the renal elimination of topotecan is not compelling there is support for a partial role.
The therapeutic index of topotecan like many anticancer drugs is narrow, which presents a greater risk of toxicity and of therapeutic failure due to small variations in exposure (i.e., AUC; ref. 41). For such drugs that are also substrates for the ABC transporters, the task of maintaining therapeutic concentrations is compounded by the saturable nature of transport function, the potential for DDIs, and gene polymorphisms that may influence transporter activity. With respect to the latter, a pilot study indicated that the presence of single-nucleotide polymorphisms (SNP) in ABCG2 Q141K (ABCG2 421C>A) in a cohort of patients with cancer significantly altered topotecan bioavailability and increased plasma AUC by 1.35-fold (34). This mutation also significantly altered imatinib pharmacokinetic (35) and was associated with a significantly high risk of diarrhea in patients treated with oral gefitinib (42). Therefore, the pharmacokinetic information obtained in Abc gene knockout mouse models can anchor our assessment of their roles, and support examination of how pharmacogenetic variations in these genes in patients may alter the pharmacokinetics of a drug.

In summary, we found that either topotecan lactone form or total topotecan can reliably be used to determine topotecan disposition. It is clear that Abcg2 is the primary transporter involved in topotecan disposition; however, in its absence or under saturating conditions, Abcb1 and Abcc2 can partially assume its role via biliary excretion. Assuming these transporters function similarly in humans with regard to topotecan elimination, their induction, inhibition, or polymorphic changes that alter their activity warrant scrutiny as to the ultimate impact on topotecan’s pharmacokinetic characteristics. However, it is precisely this overlapping function that may minimize the deleterious impact on patients by maintaining therapeutic drug exposures and limiting toxicities.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.K. Tiwari, J.M. Gallo
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. Tiwari, R. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.K. Tiwari, J.M. Gallo
Writing, review, and/or revision of the manuscript: A.K. Tiwari, J.M. Gallo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.K. Tiwari
Study supervision: J.M. Gallo

Acknowledgments
The authors thank Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands) for providing the detailed protocol for gall bladder cannulation and bile duct ligation.

Grant Support
This work was supported by the NIH grant CA114574 (J.M. Gallo).

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Received February 7, 2013; revised April 22, 2013; accepted April 23, 2013; published OnlineFirst May 1, 2013.

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Compensatory Roles of ABC Transporters in Topotecan PK


Molecular Cancer Therapeutics

Overlapping Functions of ABC Transporters in Topotecan Disposition as Determined in Gene Knockout Mouse Models

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Mol Cancer Ther 2013;12:1343-1355. Published OnlineFirst May 1, 2013.

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