Role of Breast Cancer Resistance Protein in the Bioavailability and Fetal Penetration of Topotecan


Background and Methods: Breast cancer resistance protein (BCRP/MXR/ABCP) is a multidrug-resistance protein that is a member of the adenosine triphosphate-binding cassette family of drug transporters. BCRP can render tumor cells resistant to the anticancer drugs topotecan, mitoxantrone, doxorubicin, and daunorubicin. To investigate the physiologic role of BCRP, we used polarized mammalian cell lines to determine the direction of BCRP drug transport. We also used the BCRP inhibitor GF120918 to assess the role of BCRP in protecting mice against xenobiotic drugs. Bcrp1, the murine homologue of BCRP, was expressed in the polarized mammalian cell lines LLC-PK1 and MDCK-II, and the direction of Bcrp1-mediated transport of topotecan and mitoxantrone was determined. To avoid the confounding drug transport provided by P-glycoprotein (P-gp), the roles of Bcrp1 in the bioavailability of topotecan and the effect of GF120918 were studied in both wild-type and P-gp-deficient mice and their fetuses. Results: Bcrp1 mediated apically directed transport of drugs in polarized cell lines. When both topotecan and GF120918 were administered orally, the bioavailability (i.e., the extent to which a drug becomes available to a target tissue after administration) of topotecan in plasma was dramatically increased in P-gp-deficient mice (greater than sixfold) and wild-type mice (greater than ninefold), compared with the control (i.e., vehicle-treated) mice. Furthermore, treatment with GF120918 decreased plasma clearance and hepatobiliary excretion of topotecan and increased (re-)uptake by the small intestine. In pregnant GF120918-treated, P-gp-deficient mice, relative fetal penetration of topotecan was twofold higher than that in pregnant vehicle-treated mice, suggesting a function for BCRP in the maternal-fetal barrier of the placenta. Conclusions: Bcrp1 mediates apically directed drug transport, appears to reduce drug bioavailability, and protects fetuses against drugs. We propose that strategic application of BCRP inhibitors may thus lead to more effective oral chemotherapy with topotecan or other BCRP substrate drugs. [J Natl Cancer Inst 2000;92:1651–6]

After a period of treatment with a single cytotoxic drug, cancer cells can become resistant to multiple drugs, a phenomenon known as multidrug resistance. Several mechanisms of multidrug resistance have been identified, including the overexpression of P-glycoprotein (P-gp) and MRP1, which are members of the adenosine triphosphate-binding cassette (ABC) superfamily of transport proteins that are present in the plasma membrane and can actively transport drugs out of the cell (1–3). Recently, breast cancer resistance protein (BCRP) (also known as mitoxantrone resistance protein [MXR] and placenta-specific ABC transporter [ABCP]), a new member of this superfamily involved in multidrug resistance, was identified in an MCF-7 breast cancer cell subline that was selected for resistance to doxorubicin. This BCRP-overexpressing cell line was markedly cross-resistant to mitoxantrone and daunorubicin (4,5). Subsequently, several other groups (6–8) have shown overexpression of BCRP or its murine homologue, Bcrp1, in cell lines selected for resistance to the anticancer agents mitoxantrone, doxorubicin, and topotecan. BCRP-mediated drug resistance could be effectively reversed by GF120918 (a P-gp inhibitor) in human (9) and murine (7) cell lines.

In this study, we investigate the direction of BCRP-mediated drug transport in various polarized cell lines and determine the role of BCRP in protecting mice against xenobiotic drugs (10), by using the efficient BCRP inhibitor GF120918.

Materials and Methods

Animals

The mice were housed and handled according to institutional guidelines and Dutch laws. For all experiments, the animals used were male mdr1a1b(−/−) or wild-type mice of a 99% FVB genetic background; they were 9–14 weeks of age. The mice were kept in a temperature-controlled environment with a 12-hour light–12-hour dark cycle and were given a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

Reagents

Topotecan (Hycamtin®) and [14C]topotecan (56 Ci/mol) were from SmithKline Beecham Pharmaceuticals (King of Prussia, PA). GF120918 was from Glaxo Wellcome (Research Triangle Park, NC). Ketamine (Ketalar®) was from Parke-Davis (Hoofddorp, The Netherlands). Xylazine was from Sigma Chemical Co. (St. Louis, MO). Methoxyflurane (Metofane®) was from Mallinckrodt Veterinary, Inc. (Mundelein, IL). All other compounds used were reagent grade.

Drug Preparation, Administration, and Analysis

GF120918 was suspended at 5 mg/mL in a mixture of hydroxypropyl methylcellulose (10 g/L)/2% (vol/vol) Tween 80/H2O (0.5 : 1 : 98.5 [vol/vol]/vol) for oral administration. Animals, lightly anesthetized with methoxyflurane, were administered GF120918 (50 mg/kg; 10 μL of drug solution/g body weight) or a corresponding amount of vehicle by gavage into the stomach. Topotecan (0.2 mg/mL) (freshly prepared in 5% [wt/vol] d-glucose; 5 μL/g body weight) was administered orally at a dose of 1.0 mg/kg body weight. For intravenous administration, topotecan or, where indicated, [14C]topotecan at 5 μL of drug solution/g body weight was injected into the tail vein of mice lightly anesthetized with methoxyflurane.

Animals were killed by cardiac puncture or axillary bleeding after being anesthetized with methoxyflurane, and their blood was collected. Heparinized
plasma was mixed with three volumes of ice-cold methanol (−20°C). Their organs were removed and subsequently homogenized in 4% (wt/vol) bovine serum albumin. Where applicable, the intestinal content was separated from the intestinal tissue before homogenization. Radioactivity in homogenates was determined as described previously (11). Because topotecan is hardly metabolized in vivo, the amounts of 14C reflect total unchanged levels of topotecan (12). The total topotecan levels (lactone plus carboxylate form) in plasma were determined by high-pressure liquid chromatography as described earlier (13). The area under the plasma concentration–time curve (AUC) was calculated (from 0 to 4 hours for oral administration and from 1 minute to 4 hours for intravenous administration) by use of the linear trapezoidal rule. Plasma bioavailability (i.e., the extent to which a drug becomes available in plasma after administration) of administered drug was determined as the ratio of the AUC after oral and intravenous administrations. For gallbladder cannulation experiments, mice were anesthetized and cannulated as described previously (14). Anesthetics, a combination of ketamine (100 mg/kg) and xylazine (6.7 mg/kg), were injected intraperitoneally into the mice at 2.33 μL/g body weight.

**Ribo nuclease Protection Analysis**

Total RNA was isolated from mouse tissues by use of the TRizol® reagent (Life Technologies, Inc. [GBBCO BRL], Rockville, MD), according to the manufacturer’s instructions. Ribo nuclease (RNase) protection assays were performed, as described previously (15), with 10 μg of total RNA per sample. A mouse probe for bcrp1 was made by cloning a 405-nucleotide (nt) polymerase chain reaction fragment (positions 1554–1959 relative to the translation start) into the pGEM-T vector (Promega Corp., Madison, WI). After the vector was linearized with restriction endonuclease EcoRI, a 280-nt antisense RNA probe was generated by transcription with SP6 RNA polymerase, yielding a protected probe fragment of 205 nt.

**Expression of Full-Length Mouse bcrp1 Complementary DNA in LLC-PK1 and MDCK-II Cells**

The full-length mouse bcrp1 complementary DNA (cDNA) (7) was excised from pBluescript KS with Smal and NotI and was cloned into the LZRS-MS-IRES-GFP expression vector between the SnoBI and NotI sites (16). The resulting vector was a monocistronic construct containing bcrp1 followed by sequences for an internal ribosome entry site and the enhanced green fluorescent protein. This construct was transfected to the amphotropic Phoenix producer cell line (17) by use of the calcium phosphate precipitation method. Viral supernants from these transfected cells were used to transduce LLC-PK1 or MDCK-II cells. Transduced clones were selected first for expression of the enhanced green fluorescent protein and then for the reduced accumulation of mitoxantrone by flow cytometry. The expression of bcrp1 cDNA in selected clones was determined by northern blot analysis.

**Transport Assay**

Transport assays were carried out as described earlier (14), with minor modifications. M199 medium containing t-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μg/mL), and fetal calf serum (10%) was used throughout. Cells were seeded on microporous polycarbonate membrane filters (3.0-μm pore size, 24.5-mm diameter, Transwell™, 3414, Costar®) at a density of 2 × 10⁶ cells/well. The cells were grown for 4 days in M199 medium with one change of medium. Ninety minutes before the start of the experiment, medium on both the apical and the basal sides of the monolayer was replaced with 2 mL of OptiMEM (Life Technologies Ltd., Paisley, Scotland) containing t-glutamine (2 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL) without fetal calf serum, at pH 6.5 (adjusted with HCl). The experiment was started by replacing the medium on either the apical or the basal side of the cell layer with 2 mL of serum, at pH 6.5 (adjusted with HCl). The experiment was started by replacing of total radioactivity added at the beginning of the experiment. The tightness of the monolayer was measured by monitoring the paracellular flux of [3H]ulin to the opposite compartment. This flux was always lower than 1% of the total radioactivity per hour.

**Statistical Analysis**

The two-sided unpaired Student’s t test was used throughout to assess the statistical significance of difference between the two sets of data. Results are presented as the means ± standard deviation. Differences were considered to be statistically significant when P<0.05.

**RESULTS**

**Polarized Transport of [14C]Topotecan by Mouse Bcrp1 in Epithelial Cell Lines**

To determine the direction of Bcrp1-mediated drug transport in polarized epithelia, we transduced the pig kidney cell line LLC-PK1 with a retroviral expression vector containing mouse bcrp1 cDNA. By northern blot analysis, expression of bcrp1 cDNA in two independent transductants (L-Bcrp1.1 and L-Bcrp1.2) was 25%–50% of that found in D320 cells, a doxorubicin-selected cell line that highly overexpresses bcrp1 ([7]; data not shown). The parental and transduced cell lines were grown to confluent polarized monolayers on porous membrane filters, and vectorial transport of [14C]topotecan (10 μM) across the monolayers was determined. Background transport of topotecan by the endogenous P-gp (18) present in the LLC-PK1-derived lines was abolished by the addition of the P-gp inhibitor PSC 833 (10 μM). This compound hardly affects Bcrp1-mediated drug transport (data not shown). Although topotecan was translocated equally well in both apical and basolateral directions in the parental line LLC-PK1, in the bcrp1-transduced lines, it was translocated more in the apical direction and less in the basolateral direction (Fig. 1, a and b; data for L-Bcrp1.2 [not shown] were similar to those for L-Bcrp1.1). When the Bcrp1-P-gp inhibitor GF120918 (7,9) was used, polarized topotecan transport was abolished in parental and bcrp1-transduced lines, resulting in equal levels of (passive) translocation of topotecan in both directions (Fig. 1, c and d). Similar results were obtained for [14C]topotecan and [3H]mitoxantrone when mouse bcrp1 was expressed in the polarized canine kidney cell line MDCK-II (data not shown). Thus, Bcrp1-mediated drug transport is apically directed in various polarized cells, which suggests that Bcrp1 is located apically in polarized epithelia, and can be effectively inhibited by GF120918.

**Expression of bcrlp1 Messenger RNA in Mouse Tissues**

P-gp mediates apically directed drug transport in polarized cultured cells and the elimination of drugs by organs such as liver and intestine. P-gp also reduces the uptake of drugs from the intestine and prevents the accumulation of drugs in certain critical tissues and the fetus (10,19–21). To assess a possible pharmacologic role of Bcrp1, we first determined the tissue distribution of mouse bcrp1 by using RNase protection assays. Fig. 2, a, shows that mouse bcrp1 is highly expressed in kidney and expressed more moderately in liver, colon, heart, spleen, and placenta. The moderate levels of bcrp1 expression in the mouse placenta contrast with the very high levels of BCRP expression previously observed in human placenta (4,22).

**Effect of GF120918-Mediated Inhibition of Bcrp1 on the Pharmacokinetics of Topotecan in Mice**

We studied the pharmacologic role of Bcrp1 in vivo in liver, intestine, kidney, and placenta by analyzing the effects of the Bcrp1 inhibitor GF120918, which is well tolerated by both
mice and humans [(23); unpublished data], on the pharmacokinetics of topotecan. Because GF120918 inhibits both P-gp and Bcrp1, we used P-gp-deficient mdr1a/1b(−/−) mice (10) to exclude any confounding effects of P-gp inhibition. Comparison of the expression of bcrp1 in several organs between wild-type and mdr1a/1b(−/−) mice established that expression of bcrp1 was not increased by the loss of P-gp (Fig. 2, b). To study topotecan bioavailability, we administered GF120918 or vehicle orally to mdr1a/1b(−/−) mice 15 minutes before oral or intravenous administration of topotecan, and we determined the plasma concentration of topotecan as a function of time (Fig. 3, a and c). In GF120918-treated animals, the bioavailability of topotecan given orally, as measured by the AUC, was more than sixfold higher than that in vehicle-treated animals (596 ± 62 versus 96 ± 18 hours · mg/L; P < .001; Fig. 3, a). In GF120918-treated animals, the bioavailability of topotecan given intravenously increased about twofold (406 ± 25 versus 200 ± 29 hours · mg/L; P < .001; Fig. 3, c). Taking the bioavailability of intravenously administered topotecan in vehicle-treated mice as 100%, the bioavailability of topotecan administered orally was 48% ± 9% in vehicle-treated mice and 299% ± 31% in GF120918-treated mice. Thus, Bcrp1 appears to be a major determinant for the bioavailability of topotecan that is administered orally.

Topotecan is a weak to moderate substrate for P-gp (24). Because the bioavailability of topotecan administered orally to

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**Fig. 1.** Transepithelial transport of [14C]topotecan (10 μM) in LLC-PK1 (a and c) and L-Bcrp1.1 (a representative clone) (b and d) monolayers. The experiment was started with the addition of [14C]topotecan to one compartment (basal or apical) of the culture dish. After 0.5, 2, 4, and 6 hours, the percentage of radioactivity appearing in the opposite compartment was measured and plotted. The P-glycoprotein (P-gp) inhibitor PSC 833 (a and b) or the breast cancer resistance protein (BCRP)/P-gp inhibitor GF120918 (c and d) was present as indicated. Results are the means; the error bars indicate the standard deviations (n = 3). Transport from the apical to the basal sides and from the basal to the apical sides (at 6 hours) was only significantly different for L-Bcrp1.1 cells treated with 10 μM PSC 833 (P = .002; two-sided Student’s t test).

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**Fig. 2.** Expression of the murine breast cancer resistance protein (bcrp1) messenger RNA in mouse tissues. Ribonuclease protection analysis was performed with 10 μg of total RNA/sample. a) bcrp1 expression in mdr1a/1b(−/−) mouse tissues: colon (lane 1), cecum (lane 2), heart (lane 3), lung (lane 4), skeletal muscle (lane 5), thymus (lane 6), stomach (lane 7), epididymis (lane 8), brain (lane 9), liver (lane 10), kidney (lane 11), spleen (lane 12), placenta (lane 13), and uterus (lane 14). b) Comparison of bcrp1 expression between wild-type (wt) and mdr1a/1b(−/−) (ko) mice: wt brain (lane 15), ko brain (lane 16), wt liver (lane 17), ko liver (lane 18), wt kidney (lane 19), ko kidney (lane 20), wt spleen (lane 21), ko spleen (lane 22), wt small intestine (lane 23), and ko small intestine (lane 24). Positions of bcrp1- and Gapdh (i.e., glyceraldehyde-3-phosphate dehydrogenase)-protected RNA fragments are indicated. The Gapdh expression differs between tissues and is used to compare amounts of RNA from the same tissues from mice with different genotypes.
vehicle-treated wild-type mice (41 ± 7 hours · mg/L) is two-fold lower than that in vehicle-treated P-gp-deficient mdr1a/1b(−/−) mice (96 ± 18 hours · mg/L) (P < 0.001; compare lower curves in Fig. 3, a and b). P-gp also appears to have a role in the bioavailability of topotecan. When wild-type mice, clinically the most relevant model, were treated with GI20918, the bioavailability of topotecan given orally increased ninefold (381 ± 41 versus 41 ± 7 hours · mg/L; P < 0.001). This result indicates that inhibition of both Bcrp1 and P-gp by GI20918 has a strong effect on uptake of topotecan administered orally, although the resulting availability did not quite reach the level observed in GI20918-treated mdr1a/1b(−/−) mice (596 ± 62 hours · mg/L).

We next determined how GI20918 given orally affected the levels of topotecan excreted in the small intestine. GI20918 was administered orally to mdr1a/1b(−/−) mice; 15 minutes later, [14C]topotecan was administered intravenously; then 15 and 60 minutes later, the amount of [14C]topotecan excreted into the small intestine was measured. Fifteen and 60 minutes after [14C]topotecan was administered to GI20918-treated animals, the percentage of total [14C]topotecan in the small intestinal lumen was about twofold and threefold lower, respectively, and the plasma levels were about 1.5-fold and 2.5-fold higher compared with vehicle-treated animals (Table 1). These observations could reflect diminished excretion of topotecan into the small intestine and/or increased (re-)uptake from the small intestine, both caused by GI20918. To analyze this effect further, we separately determined the hepatobiliary, direct intestinal, and renal excretion of [14C]topotecan. For the measurement of hepatobiliary excretion, anesthetized mdr1a/1b(−/−) mice with a cannulated gallbladder were given GI20918 or vehicle orally 15 minutes before they were given [14C]topotecan intravenously, and the amount of topotecan excreted was determined over the next hour. Hepatobiliary excretion of unchanged topotecan was substantially decreased in GI20918-treated animals for the first 10 minutes after topotecan administration (5.5% ± 2.6%) compared with that of vehicle-treated animals (14.7% ± 2.4%) (P = 0.011); however, after about 20 minutes, the excretion rate in both groups became similar (Fig. 3, d). This observation and the fact that biliary topotecan excretion was not completely blocked by GI20918 suggest that hepatic Bcrp1 was not completely blocked by GI20918 or that there are additional transporters for topotecan in the bile canalicular membrane. In contrast to the hepatobiliary excretion of topotecan, the effect of GI20918 treatment on direct intestinal (7.5% ± 2.5% with GI20918 and 11.6% ± 1.1% with vehicle) or renal (12.6% ± 7.9% with GI20918 and 18.0% ± 10.4% with vehicle) excretion of total radioactivity was not statistically significant. These data suggest that the GI20918-induced high bioavailability of topotecan administered orally results primarily from a combination of its increased intestinal (re-)uptake and decreased hepatobiliary excretion.
Pharmacologic Role of Bcrp1 in Placenta

P-gp has been shown to be functionally active in the pharmacologically important blood–brain, blood–testis, and maternal–fetal barriers (10,20). The high expression of BCRP messenger RNA (mRNA) in human placenta and (to a lesser extent) in mouse placenta suggested to us that BCRP might also play a role in protecting fetuses against xenobiotics. So that we could test this hypothesis, pregnant mdr1a/1b(−/−) dams at gestation day 15.5 were administered GF120918 or vehicle orally 2 hours before intravenous administration of [14C]topotecan; 30 minutes after receiving [14C]topotecan, fetuses and maternal plasma were collected. We found that levels of [14C]topotecan were about 3.2-fold higher in fetuses of GF120918-treated dams, whereas at the same time maternal plasma levels were only about 1.6-fold increased (Table 1). These results indicate that mouse Bcrp1 plays an important role in protecting the fetus from topotecan. Because BCRP mRNA expression is much higher in human placenta, the role of BCRP in humans could be even more pronounced. In contrast, for the blood–brain and blood–testis barriers, we found no indication that Bcrp1 has a role in limiting drug penetration, as determined by the distribution of intravenously administered [14C]topotecan or [3H]mitoxantrone in tissues of GF120918-treated and vehicle-treated mdr1a/1b(−/−) mice (data not shown).

**DISCUSSION**

Our data show that Bcrp1 mediates apically directed transport of its drug substrates and support the view that Bcrp1 is important in preventing intestinal (re-)uptake and in mediating hepatobiliary excretion of transported drugs. In these ways, Bcrp1 restricts the bioavailability of orally administered drugs. Moreover, it also protects fetuses through its presence in the maternal–fetal barrier. Our data strongly suggest that Bcrp1 is present and functional in the apical membrane of the intestinal epithelium, in the bile canalicular membrane, and in the membrane of placental trophoblasts that is in contact with the maternal circulation.

The highest levels of bcrp1 mRNA were found in the kidney, suggesting that Bcrp1 might play an important pharmacologic role in the renal excretion of substrate drugs. Our experiments measuring this renal excretion gave highly variable results between individual mice and were essentially not influenced by GF120918 (12.6% ± 7.9% with GF120918 and 18.0% ± 10.4% with vehicle). Studies in patients also have found high variability in renal elimination of topotecan (mean = 40%; range = 26%–80%) (25). A possible explanation for the high variability in renal excretion is that human and murine kidneys could have several transport mechanisms for topotecan that vary extensively among individuals.

Oral administration of drugs is highly preferred for its convenience and potential use on an outpatient basis. However, the therapeutic use of orally administered drugs is frequently limited by the poor and (consequently) highly variable drug bioavailability, factors that are largely determined by the extent to which the drugs are absorbed from the gut, metabolized, and excreted. The narrow therapeutic index of most anticancer drugs implies that this variability will frequently result in excessive toxicity or, conversely, in inadequate efficacy. For instance, for topotecan administered orally, the bioavailability in humans is moderate, with a high inter-patient variation (30% ± 7.7%) (26), and current chemotherapeutic schedules for topotecan are, therefore, mainly based on intravenous administration (27). Our findings suggest that, by combining topotecan administered orally with an effective BCRP (and P-gp) inhibitor, such as GF120918, the bioavailability of topotecan and thus its clinical usefulness might be dramatically improved. We should note that, based on these data, no conclusions can be made about whether the therapeutic index of topotecan (i.e., toxicity of topotecan for a tumor as opposed to its overall toxicity to the organism) is improved by GF120918. However, the ability to inhibit placental Bcrp1 with orally administered GF120918 suggests that a BCRP component of multidrug resistance in clinical tumors could also be blocked with GF120918 administered orally because the systemic exposure to GF120918 is apparently high enough.

Although we cannot exclude the possibility that other, as yet unidentified, GF120918-sensitive topotecan transporters are also contributing to the in vivo pharmacologic effects that we observed, the potential clinical application of GF120918 to improve the bioavailability of topotecan administered orally to patients should be pursued. In fact, we have started clinical trials to test whether it is feasible to increase the bioavailability of topotecan administered orally to patients by blocking BCRP with GF120918. If this procedure is successful in patients as well, it may prove to be applicable to other drugs transported by BCRP.

**REFERENCES**


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**Table 1.** GF120918 and [14C]topotecan in mdr1a/1b(−/−) mice

<table>
<thead>
<tr>
<th>[14C]Topotecan</th>
<th>Vehicle treated</th>
<th>GF120918 treated</th>
<th>Ratio</th>
<th>P*</th>
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<tr>
<td><strong>Effect of GF120918 on intestinal content of [14C]topotecan-derived radioactivity†</strong></td>
<td></td>
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<tr>
<td>15 min</td>
<td>16.2 ± 1.7</td>
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<td>Intestinal, %</td>
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<td>60 min</td>
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<td>102 ± 4</td>
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<td>Intestinal, %</td>
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<td>Plasma, ng/mL</td>
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<td>57.9 ± 11.2</td>
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<tr>
<td>Fetus, ng/g</td>
<td>0.43 ± 0.07</td>
<td>0.87 ± 0.09</td>
<td>2.02</td>
<td>.001</td>
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</tbody>
</table>

*P values (from a two-sided Student’s t test) indicate statistical significance of the difference between vehicle-treated and GF120918-treated animals.

†GF120918 (50 mg/kg) was administered orally; 15 minutes later, [14C]topotecan (1 mg/kg) was administered intravenously; 15 or 60 minutes after administration of [14C]topotecan, the intestinal content of [14C]topotecan was determined. Results are the mean percentages of dose (intestinal content) or of [14C]topotecan concentrations (ng/mL, plasma) ± standard deviation (n = 3).

‡At gestation day 15.5, GF120918 (50 mg/kg) was administered orally to pregnant dams; 2 hours later, [14C]topotecan (0.2 mg/kg) was administered intravenously; 30 minutes later, the fetal distribution and maternal plasma concentration of [14C]topotecan were determined. Results are the mean [14C]topotecan concentrations ± standard deviation (n = 3 or 4 dams, n = 30 fetuses for vehicle, and n = 41 fetuses for GF120918.)

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NOTES

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