Role of P-Glycoprotein and the Intestine in the Excretion of DPC 333 [(2R)-2-[(3R)-3-Amino-3-[4-(2-methylquinolin-4-ylmethoxy)phenyl]-2-oxopyrrolidin-1-yl]-N-hydroxy-4-methylpentanamide] in Rodents


Infection and Cancer Discovery, AstraZeneca PLC, Waltham, Massachusetts (C.E.G.); Preclinical Development Drug Metabolism and Pharmacokinetics, AstraZeneca Plc, Wilmington, Delaware (J.L.); Infection and Cancer Discovery, AstraZeneca Plc, Macclesfield, United Kingdom (K.J.); Quest Pharmaceutical Services Inc, Newark, Delaware (E.S., C.-M.L., H.-S.S.); Bristol-Myers Squibb Company, Pennington, New Jersey (G.L., J.D., C.P.D., S.E.M., S.P.); Boehringer Ingelheim Pharmaceuticals, Ridgefield, Connecticut (L.-S.G.); Pharmacokinetics, Pharmacodynamics and Bioanalytical Sciences, Genentech Inc., San Francisco, California (M.O.); Incyte Pharmaceuticals, Newark, Delaware (T.M.); and Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts (F.W.L.)

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ABSTRACT:

The role of the intestine in the elimination of (2R)-2-[(3R)-3-amino-3-[4-(2-methylquinolin-4-ylmethoxy)phenyl]-2-oxopyrrolidin-1-yl]-N-hydroxy-4-methylpentanamide (DPC 333), a potent inhibitor of tissue necrosis factor α-converting enzyme, was investigated in mice and rats in vivo and in vitro. In Madine-Darby canine kidney cells stably transfected with P-glycoprotein (P-gp) and DPC 333, the transport from B→A reservoirs exceeded the transport from A→B by approximately 7-fold. In Caco-2 monolayers and isolated rat ileal mucosa, DPC 333 was transported from basolateral to apical reservoirs in a concentration-dependent, saturable manner, and transport was blocked by N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918), confirming the contribution of P-gp/breast cancer resistance protein in B→A efflux of DPC 333. In quantitative whole body autoradiography studies with [14C]DPC 333 in mice and rats, radioactivity was distributed throughout the small intestine in both species. In GF120918-pretreated bile duct–cannulated rats, radioactivity in feces was reduced 60%. Using the in situ perfused rat intestine model, ~20% of an i.v. dose of [14C]DPC 333 was measured in the intestinal lumen within 3 h postdose, 12% as parent. Kinetic analysis of data suggested that excreted DPC 333 may be further metabolized in the gut. Intestinal clearance was 0.2 to 0.35 l/h/kg. The above data suggest that in the rodent the intestine serves as an organ of DPC 333 excretion, mediated in part by the transporter P-gp.

The literature contains evidence for direct intestinal secretion of a number of structurally diverse xenobiotics ranging from inorganic metals (Zalups, 1998) to large organic molecules such as digoxin (Caldwell et al., 1980). Evidence has accumulated to suggest that active intestinal secretion may play a major part in the elimination of several drugs (Mayer et al., 1996; Rabbas et al., 1996; Sparrenboom et al., 1997; Smit et al., 1998a,b; Dautrey et al., 1999; van Aspen et al., 2000; Leusch et al., 2002; Li et al., 2005; Lagas et al., 2006; Villanueva et al., 2006).

P-glycoprotein (P-gp, MDR1) and breast cancer resistance protein (BCRP, ABCG2) are ATP-dependent multidrug efflux pumps belonging to the ATP-binding cassette superfamily of proteins (Hyde et al., 1990; Allen et al., 1999) that protect cells from xenobiotics by transporting them out of cells and reducing their intracellular levels. Physiologically, these transporters are widely expressed in the epithelial cells of intestine, liver, and kidney and in the endothelial cells of brain and placenta (Eisenblatter et al., 2003; Yeboah et al., 2006). The broad substrate specificity and distinctive expression locations suggest that P-gp/BCRP may have a direct role in modulating the absorption and disposition of drugs or xenobiotics (Hall et al., 1999; Merino et al., 2005; Zhang et al., 2005). The acridonecarboxamide derivative GF120918 potently inhibits both of these transporters.

ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistance; BCRP, breast cancer resistance protein; GF120918, N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; DPC 333, (2R)-2-[(3R)-3-amino-3-[4-(2-methylquinolin-4-ylmethoxy)phenyl]-2-oxopyrrolidin-1-yl]-N-hydroxy-4-methylpentanamide; TEER, transepithelial electronic resistance; MDCK, Madine-Darby canine kidney; HPLC, high-performance liquid chromatography; QWBA, quantitative whole body autoradiography; IP, imaging plate; GI, gastrointestinal.
In intestinal P-gp and/or BCRP may mediate the secretion of a large number of xenobiotics. Rabbaa et al. (1996) showed that the fluoroquinolone drug ofloxacin was actively cleared via the rat intestine and that this process was inhibited by other fluoroquinolones, including ciprofloxacin. Co-administration of the P-gp inhibitor verapamil reduced intestinal clearance by approximately 50%, suggesting a role of P-gp. Digoxin has also been shown to be actively secreted into the intestine in rodents, and this process was also interrupted by known P-gp inhibitors (Mayer et al., 1996; Salphati and Benet, 1998). Transgenic mice, deficient in P-gp (mdr1(-/-)), have been used to show positively that intestinal excretion of a number of amphipatic cationic drugs is mediated in part by P-gp (Sparreboom et al., 1997; Smit et al., 1998a,b; van Asperen et al., 2000; Lagas et al., 2006). Intestinal P-gp has also been suggested as a contributor to the clearance of digoxin, talinolol, and chemotherapeutic agents in humans (Greiner et al., 1999; Perdaems et al., 1999; Westphal et al., 2000). In elegant clinical experiments Westphal et al. (2000) showed in humans that induction of intestinal P-gp by rifampin correlated directly with an increase in the systemic clearance of i.v. administered talinolol in rodents, and this process also was interrupted by known P-gp inhibitors. The studies in this report were conducted to investigate the potential contribution of the intestine and the transporter P-gp to the elimination of DPC 333 in rodents in vivo.

**Materials and Methods**

**In Vitro Transporter Studies.** ATPase activity assay. In the present study, we expressed MDR1 in baculovirus-infected Spodoptera frugiperda (Sf9) cells and measured the ATP-dependent, vanadate-sensitive transport of known and potential MDR1 substrates using the method of Sarkadi et al. (1992). Briefly, a 60-μl reaction mixture containing 40 μg of membranes, 20 μM verapamil (positive control) or test drug, and 3 to 5 mM MgATP, 50 mM Tris-MES, pH 6.8, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide was incubated at 37°C for 20 min. An identical reaction mixture containing 100 μM sodium orthovanadate was assayed in parallel. Orthovanadate ATPase activity measured in the presence of orthovanadate represents non–P-gp ATPase activity and can be subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity. The reaction was stopped by the addition of 30 μl of 10% SDS + antifoam A. Two additional reaction mixtures (+ and − orthovanadate but without MgATP) were also prepared and incubated with the others and then supplemented with SDS and MgATP to represent time = 0 min of reaction. The incubations were followed with addition of 200 μl of 35 mM ammonium molybdate in 15 mM zinc acetate/10% ascorbic acid (1:4) and incubated for an additional 20 min at 37°C. The liberation of inorganic phosphate was detected by its absorbance at 800 nm and quantitated by comparing the absorbance with a phosphate standard curve. The A maximum for the measured phosphomolybdate chromophore is 850 nm; however, absorbance detection between 630 and 850 nm has been reported to be useful for this method (Druke et al., 1990).

**Caco-2 transport studies.** Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell stocks were maintained in 75-cm² flasks (Costar, Corning, NY) at 37°C in a humidified atmosphere of 5% CO2/95% air. The culture media consisted of high glucose (4.5 g/l) Dulbecco’s modified Eagle’s medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT), 1% nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO). The culture media were replaced every other day. Monolayers were subcultured using 0.05% trypsin/0.02% EDTA when they reached 75 to 85% confluency at a split ratio of approximately 1:5.

Single-cell suspensions of Caco-2 cells were plated onto the 12-mm diameter Transwell polycarbonate membranes (0.4-μm pore size, Costar) at a density of 6 × 10⁴ cells/cm². The Transwell inserts were placed in 12-well culture plates with 0.5 ml of media in the apical compartment and 1.5 ml of media in the basolateral compartment. The media at both compartments were replaced every other day for 3 to 4 weeks before the cells were used for transport studies.

Before the transport experiments, the integrity of Caco-2 cell monolayers was assessed by measuring transepithelial electrical resistance (TEER) using an Evon epithelial volt-ohm meter (World Precision Instruments, Inc., Sarasota, FL). TEER values were in the range of 400 to 800 Ω·cm². The culture medium in Transwell was aspirated and washed twice with transport buffer (Hanks’ balanced salt solution containing 25 mM glucose and 10 mM HEPES, pH 7.4, except indicated). The cells then were incubated in the transport buffer at 37°C for 30 min. The transport was initiated by replacing transport buffer in the donor compartment with fresh transport buffer containing 14CJDPC 333 with or without GF120918. After 2-h incubation, total radioactivity in media of receptor compartment was determined using Tri-Carb liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Boston, MA).

**Efflux in MDR1-transfected Madine-Darby canine kidney cells.** Madine-Darby canine kidney (MDCK) II-MDR1 cells were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands). Cell stocks were maintained in T-175-cm² flasks at 37°C in a humidified atmosphere of 5% CO2/95% air. The culture media consisted of high glucose (4.5 g/l) Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The culture media were replaced every other day. Monolayers were subcultured using 0.05% trypsin/0.02% EDTA when they reached 75 to 85% confluency.

Single-cell suspensions of MDCKII-MDR1 cells were plated onto the 0.1-mm² Millicell-96 polycarbonate membranes (1-μm pore size, Millipore, Watford, UK) at a density of 4 × 10⁴ cells/cm². The apical portion of the plate containing 150 μl of media was placed into the basolateral compartment containing 300 μl of media. The media were replaced 24 h before transport experiments.

Before transport experiments, the integrity of the MDCKII-MDR1 monolayer was assessed by determining the TEER using the REMS TEER measurement system (WPI, Stevenage, UK). TEER values were in the range of 500 to 700 Ω·cm². The culture medium in the Millicell-96-well plates was aspirated and washed three times with transport buffer (Hanks’ balanced salt solution containing 10 mM HEPES, pH 7.4). Transport was initiated by replacing transport buffer in the donor compartment with transport buffer containing DPC 333, verapamil, or vinblastine. For inhibition experiments, cyclosporin A was added to both apical and basolateral chambers. Following a 60-min
incubation period, aliquots were analyzed by liquid chromatography/tandem mass spectrometry.

**Rat Intestinal Membrane Permeability Experiments.** Sprague-Dawley rats were supplied by Charles River Canada Inc. (Senneville, QC, Canada). Ussing chambers and mounts were supplied by NaviCyte Co. (Reno, NV).

Male Sprague-Dawley rats (250 g) were anesthetized with ether, the abdomen opened, and the intestinal segments of interest were quickly removed and rinsed twice with chilled normal saline (0.9%). Smaller segments were cut (approximately 2.5 cm) and placed in cold Tyrode’s buffer, which was continuously bubbled with an O2/CO2 (95:5%) gas mixture. For these experiments ileal segments were used. Segments were cut along their mesenteric border, and the serosa was removed using blunt dissection. The mucosal side was rinsed with cold saline, and the segment was gently placed into position on the Ussing chambers (NaviCyte). During the preparation, the segments were submerged in Tyrode’s buffer, which was bubbled continuously. The stripped intestinal mucosa from the rat ilium were then mounted in modified Ussing chambers with stirring conditions as described by Ungell et al. (1998). The effective exposed area of the tissues was 1.8 cm². All the experiments were carried out unidirectionally at 37°C. The serosal and the mucosal reservoirs were filled with Tyrode’s buffer, and oxygen was provided to both chambers. To the donor compartment 5 ml of buffer containing test article (−0.3 μCi/ml) was added, and to the receiving compartment 5 ml of drug-free buffer was added. In parallel experiments, the potent P-gp/BCRP inhibitor GF120918 (0.2 μM) was added 10 min before the test article addition to determine the extent of GF120918 blockade on basolateral to apical transfer. Receiving chamber samples (0.2 ml) were removed at 15, 30, 45, 60, 75, 90, 105, and 120 min and replaced with equal amount of drug-free buffer. For the investigation of apical to basal transport, drug was placed in the mucosal side. For the investigation of basal to apical transport, drug was placed in the serosal side.

The radioactivity in each receiving chamber was determined by liquid scintillation spectrometry, and the cumulative radioactivity permeating the side. 1104 GARNER ET AL.

**Materials.**

Radioactivity in urine, cage washes, and bile was quantified by directly assaying aliquots by liquid scintillation spectroscopy. Total radioactive residues in solid samples (feces, intestinal contents) were determined by combusting aliquots of homogenized samples in an oxidizer (Packard Instruments, Meriden, CT), trapping the liberated 14CO2, and then analyzing samples by liquid scintillation spectrometry. Samples were counted for 10 min or until 160,000 disintegrations (0.5% 2σ) were accumulated, whichever came first. Low-activity samples were counted for up to 75 min or until 6400 disintegrations (2.5% 2σ) were accumulated.

**Effluent.** Male Sprague-Dawley rats (approximately 250 g at study initiation), fitted with indwelling jugular cannulas for the collection of blood, were obtained from Charles River Laboratories Inc. Before conduct of dosing studies, animals were anesthetized.
with pentobarbital, and duodenal catheters were implanted for intestinal delivery of solutions. Approximately 10 min before administration of test compound, one group of three rats was pretreated with verapamil (1 mg/kg), a known P-gp inhibitor (Chang et al., 2006), and another group of three rats was administered saline. Following pretreatment each rat then received a single p.o. gavage dose of DPC 333 of 15 mg/kg. Blood was collected from each animal at 0.083, 0.17, 0.25, 0.5, 1.5, and 2 h following administration of test material.

Plasma was prepared, and the concentration of DPC 333 was determined via liquid chromatography/tandem mass spectrometry. Plasma concentration versus time data were then analyzed by noncompartmental methods using WinNonlin (Mountain View, CA).

**In Situ Intestinal Perfusion.** Sprague-Dawley rats previously fitted with portal vein and bile duct cannulas were supplied by Charles River Canada Inc. Ketamine and xylazine were purchased from Fort Dodge Lab Inc. (Fort Dodge, IA). Krebs-Ringer bicarbonate buffer components (KH₂PO₄, MgSO₄, NaCl, KCl, CaCl₂, H₂O, NaHCO₃, glucose) were purchased from Sigma Chemical Co. (St. Louis, MO).

A single-pass intestinal perfusion technique was used. Surgery was adapted from that described by Wang et al. (1997, 1999). Briefly, male Sprague-Dawley rats (~200 g) prefilterd with indwelling jugular vein cannulas before surgery were anesthetized with ketamine/xylazine (100:7 mg/kg). The fur from the abdominal region was removed with clippers; the skin was cleaned with alcohol; and the rat was then placed in a supine position on a heating pad within a chamber designed to maintain body temperature throughout the experiment. The apparatus, which was constructed in-house, consisted of a Lucite (Lucite International, Southampton, UK) housing with thermostatically controlled electric heaters to maintain the rat and all the perfusion solutions at 37°C. Once the rat was positioned, laparotomy was performed, and the small intestine was fitted at inlet and outlet with polished glass tubing to allow for perfusion of the interior with Krebs-Ringer buffer (pH 7.4, 37°C). The intestinal lumen was then gently flushed to remove intestinal contents. The proximal end of the intestine was connected to a perfusion syringe on a variable speed compact infusion pump, and the interior of the intestine was perfused with Krebs-Ringer buffer at a flow rate of 250 µl/min. Effluent was collected from the glass tubing at the distal end of the intestine. The intestine was carefully arranged and continuously monitored to avoid kinks and ensure a consistent flow. Saline-soaked cotton gauze was used to cover open body cavities to prevent loss of fluids. Once the intestinal effluent flow had been established, [³¹⁴C]DPC 333 (15 mg/kg, 14.4 µCi) was then administered i.v. via the indwelling jugular vein cannula. Following administration of test substance, intestinal effluent samples were collected into preweighed glass vials every 15 min for up to 180 min. Blood samples were sampled at times of effluent collection time points for plasma analysis of parent compound. Blood volume was maintained by immediately transfusing back into the cannula an equal volume of blood taken from donor rats. At the end of the experiment, the remaining intestinal perfusate solution was expelled by infusing air and then flushing with normal saline through the intestinal segment. Aliquots of the collected material were analyzed for total radiochemical content by liquid scintillation spectrometry before characterization of radiochemical profile by HPLC.

**Analysis of intestinal perfusate and plasma.** DPC 333 was determined in plasma and intestinal perfusate by HPLC radiochemical analysis. Before HPLC analysis, perfusate or plasma samples were mixed with an equal volume of acetonitrile (EM Science, Gibbstown, NJ) and vortexed for approximately 30 s. The mixtures were centrifuged at approximately 3000g for 10 min, and the supernatants were filtered with a 0.45 µM syringe filter (Whatman Inc., Florham Park, NJ). Aliquots (100 µl) were then analyzed for parent compound by HPLC/flow-through radiochemical detection. Briefly, the chromatographic system consisted of a Hewlett Packard (Palo Alto, CA) model 1100 chromatography system (pumps, controllers, and injectors), a Metachem Polaris (Ventura, CA) C₁₈-A 2 × 150-mm column, and a linear gradient mobile phase of acetonitrile (EM Science, Gibbstown, NJ) and water for 2 h following administration of test material.

**Results**

**In Vitro Experiments.** The role of DPC 333 as a P-gp substrate was suggested in verapamil-activated vanadate-sensitive human P-gp

**Intestinal Excretion of DPC 333**

**Fig. 2.** Stimulation of human P-glycoprotein-ATPase activity by DPC 333, verapamil, and other molecules (20 mM).

**Fig. 3.** Permeability of DPC 333 and other molecules in monolayers of MDCK II cells transfected with MDR1.
ATPase activity assays (Fig. 2). DPC 333 activity in this assay was similar to that of verapamil and vinblastine, two known P-gp substrates. In MDCK cells stably transfected with MDR1 and DPC 333, the transport from B\(\rightarrow\)A reservoirs exceeded the transport from A\(\rightarrow\)B by approximately 7-fold (Fig. 3). This ratio was reduced to 1 by addition of the P-gp inhibitor cyclosporin A.

To investigate the potential for intestinal efflux or elimination of DPC 333, \([^{14}C]DPC\) 333 was incubated against confluent Caco-2 monolayers. Initial concentrations of \([^{14}C]DPC\) 333 in either apical (A) or basolateral (B) donor reservoirs was 0 to 200 \(\mu\)M (0.5 \(\mu\)Ci/incubation). After 2-h incubation, radiochemical content in receptor reservoirs was determined. In Caco-2 monolayers, DPC 333 was transported from A to B or B to A reservoirs in a concentration-dependent manner (Fig. 4). DPC 333 transport from B\(\rightarrow\)A reservoirs was concentration-dependent and saturable, suggesting an active transport mechanism (Fig. 4). Transport from the basolateral to apical reservoir was partially blocked with GF120918, suggesting that P-gp or BCRP played a role in B\(\rightarrow\)A efflux of DPC 333.

When DPC 333 was incubated with isolated rat ileum in Ussing chambers, B\(\rightarrow\)A efflux greatly exceeded A\(\rightarrow\)B (Fig. 5A). Furthermore, B\(\rightarrow\)A efflux was nearly completely blocked by GF120918 (Fig. 5B). These in vitro data taken together suggest that DPC 333 is a P-gp and/or BCRP substrate and that these transporters may contribute to the basolateral to apical flux in the intestine.

**Rat and Mouse Whole-Body Autoradiography.** An autoradiograph (mouse at 2 min postdose) and mouse tissue concentration data showed...
are presented in Fig. 6A and Table 1, respectively. Concentrations of $^{14}$C/DPC 333-derived radioactivity were generally well distributed to all the tissues in mice at early time points (2 min to 1 h) except brain and spinal nerve cord, which showed little or no radioactivity. Highest concentrations were present in liver, gall bladder contents, kidney, heart, salivary gland, lacrimal gland, lung, gastrointestinal (GI) tract, and intestinal contents. After 2 h, high concentrations of radioactivity were observed in the liver and contents of the gall bladder and GI tract only. By 24 h postdose, low levels of radioactivity were present in the liver, heart, kidney, blood, and lung, and remaining tissue showed none or only trace levels. These QWBA data suggest that $^{14}$C/DPC 333 is well distributed throughout the body of normal mice. However, penetration into brain, spinal cord, and bone was not shown in any experiments.

FIG. 6. Whole-body autoradiographs of an intact mouse (A) and a bile duct–cannulated rat (B) sacrificed at 2 min after an i.v. dose of $^{14}$C/DPC 333.

Discussion

The results of these studies suggest that the intestine is an organ of DPC 333 excretion in rodents. Additionally, our data suggest that the ATP-binding cassette protein P-gp or BCRP mediates this excretion in part of all. In mice and rat QWBA distribution studies, significant portions of $^{14}$C/DPC 333-derived radioactivity administered i.v. were detected along the gut lumen within 2 to 5 min postdose, directly suggesting a role of the intestine in elimination of DPC 333 or its equivalents in vivo. By approximately 1 h, 15 and 17% of administered radioactivity was detected in the intestinal contents of rats and mice, respectively.

In mice, by 2 min postadministration, radioactivity was distributed throughout the small intestine, and after 2 h, high concentrations of radioactivity were observed in the liver, gall bladder, and GI tract only. Because of the fasted state of the mice and the rapid appearance of radioactivity throughout the entire length of the intestine, the contribution of biliary excretion to the intestinal radioactivity measured initially was probably minimal. However, the presence of intact bile ducts in these animals suggests that the contribution of biliary excretion to intestinal radioactivity could not be entirely ruled out. Repetition of these results in the bile duct–cannulated rat QWBA studies was strong evidence to support the hypothesis of intestinal excretion because all the bile was diverted away from the bile duct and thus prevented from entering the intestine.
Concentration of radioactivity in blood and tissues at specified times postdose determined by whole-body autoradiography for female mice following i.v. administration of \( ^{14} \text{C} \text{DPC 333} \)

Results are in nanocuries per gram tissue.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Animal Sacrifice Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 min</td>
</tr>
<tr>
<td>Blood</td>
<td>864.6</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>529.4</td>
</tr>
<tr>
<td>Brain</td>
<td>14.40</td>
</tr>
<tr>
<td>Brown fat</td>
<td>497.1</td>
</tr>
<tr>
<td>Gall bladder contents</td>
<td>N.R.</td>
</tr>
<tr>
<td>Heart</td>
<td>1061</td>
</tr>
<tr>
<td>Kidney</td>
<td>1732</td>
</tr>
<tr>
<td>Lacrimal gland</td>
<td>1023</td>
</tr>
<tr>
<td>Liver</td>
<td>3135</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>144.4</td>
</tr>
<tr>
<td>Lung</td>
<td>853.7</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>1287</td>
</tr>
<tr>
<td>Spleen</td>
<td>343.0</td>
</tr>
<tr>
<td>White fat</td>
<td>147.0</td>
</tr>
</tbody>
</table>

N.R., not represented (tissue or organ not present in original section).

LLOQ = 1.263 nCi/g.

a Intestinal contents exceeded upper linear range and therefore were not quantitated.

In separate studies, \( ^{14} \text{C} \text{DPC 333} \)-derived radioactivity was detected in the feces of bile duct–cannulated rats following i.v. administration of \( ^{14} \text{C} \text{DPC 333} \), supporting the WBA results. In this study, 5% of the dose was recovered in the feces at 48 h compared with 15 to 17% measured in small intestine by 1 h in WBA and in situ perfused intestine studies. This suggests that there may be reabsorption processes operative, perhaps in the cecum or colon. That the potent P-gp/BCRP inhibitor GF120918 was capable of partially blocking the appearance of fecal radioactivity suggested that the intestinal excretion of equivalents in vivo was mediated at least in part by ATP-binding cassette transporters P-gp or BCRP.

In perfused intestine studies, DPC 333 was a P-gp substrate. DPC 333 was subject to basolateral to apical efflux in both Caco-2 monolayers, MDR1-transfected MDCK monolayers, and rat ileal mucosa, and this efflux was reduced by verapamil, which inhibits P-gp (Chang et al., 2006) but does not inhibit BCRP (Zhang et al., 2005). Verapamil pretreatment increased \( \text{C}_{\text{max}} \) almost 6-fold and area under the plasma concentration-time curve by 60% in rats. Although BCRP involvement cannot be ruled out completely, these data suggest strongly that P-gp plays a role in DPC 333 efflux.
was nearly completely blocked with GF120918 and cyclosporin. Kinetic analysis of the Caco-2 and ileal section data gave $K_{\text{m}}$ of 25 and 46 $\mu$M, respectively. These data suggested that the intestine may have a relatively high capacity for basolateral to apical clearance of DPC 333.

The contribution of the intestine to the total clearance of DPC 333 was estimated from data generated in the rat in situ perfused small intestine model. Approximately 17% of total radioactivity administered was excreted into the intestinal lumen within 3 h post-i.v. administration and 12% of the dose as parent. Intestinal clearance of approximately 0.21 l/h/kg suggested that the intestine contributed to approximately 10% of total systemic clearance (Table 5). However, a careful kinetic analysis of the data generated in this model suggests that DPC 333 clearance may exceed that measured in the in situ perfused intestine model. That is, the data support the possibility that nearly all the DPC 333-derived radioactivity measured was initially excreted as parent by P-gp or BCRP.

A plot of plasma DPC 333 concentration versus rate of luminal excretion of DPC 333 and equivalents yielded a hyperbolic curve, suggesting a saturable excretion mechanism (Fig. 7A). This would be expected, given the strong data supporting the transporter-mediated flux of DPC 333. This suggests that the rate of all the equivalents appearing in the lumen is dependent solely on plasma parent concentration. If metabolite were actively excreted into the lumen, then the rate of its appearance in the lumen would also be a function of plasma metabolite concentration, saturable, and therefore hyperbolic across a large concentration range. However, luminal appearance of “metabolite” was independent of plasma concentration (Fig. 7B).

The intestinal elimination rate data and plasma concentration data were fit to a combined pharmacokinetic/hyperbolic elimination model. This model accurately predicted intestinal excretion of parent and total equivalents (Fig. 8).

The above analysis suggests that DPC 333 equivalents in the lumen were initially excreted as parent via a transporter and then subsequently metabolized in the lumen, presumably by brush-border enzymes. This phenomenon is seen with $p$-aminobenzoic acid, $p$-aminosalicylic acid, and sulfanilic acid (Yasuhara et al., 1984), which are acetylated by intestinal acetyltransferases following luminal secretion. Pang et al. (1986) have also shown that acetaminophen is excreted into the intestine and then glucuronidated luminally. The data cannot rule out the possibility that excreted DPC 333 is reabsorbed into mucosal cells, metabolized, and then the metabolite re-excreted. This phenomenon has been shown with $p$-nitrophenol in the rat (Fischer et al., 1995; Rafiei et al., 1996).

To summarize, the above data taken in whole would suggest that in rodents DPC 333 is cleared via the intestine in part or in whole via the transporter P-gp or BCRP. In rats, DPC 333 intestinal clearance is estimated to be a minimum of 12% of systemic clearance (0.35 l/h/kg, based on luminal parent only) with a maximum of approximately 18%

### Table 5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
<th>Mean</th>
<th>S.D.</th>
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<tbody>
<tr>
<td>AUC, nM · h</td>
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<td>nmol Dosed</td>
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<td>5,878</td>
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<tr>
<td>nmol Excreted</td>
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<td>895</td>
<td>680</td>
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<tr>
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<td>$C_{\text{ss}}$, l/h/kg</td>
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<td>0.2</td>
<td>0.2</td>
<td>0.24</td>
<td>0.08</td>
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</table>

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**Fig. 7.** Effect of plasma concentration of DPC 333 (A) or metabolite (B) on the elimination of DPC 333 equivalents into intestinal lumen following i.v. administration of DPC 333 in the in situ perfused rat intestine model.

**Fig. 8.** Predicted versus measured luminal DPC 333 and equivalents following i.v. administration of [14C]DPC 333 (15 mg/kg) in the in situ perfused rat intestine model. Luminal excretion predicted by the following expression:

$$\text{nmol} = \frac{V_{\text{max}} \times C_{\text{plasma}}}{EC_{50} + C_{\text{plasma}}} \times \Delta t \text{ where } C_{\text{plasma}} = \frac{\text{dose}}{15} \left(\text{Ae}^{-\alpha t} + \text{Be}^{-\beta t}\right)$$

and $V_{\text{max}} = 11.8 \text{ nmol/min}$, $EC_{50} = 2266 \text{ nM}$, $A = 163,399$, $\alpha = 0.21 \text{ h}^{-1}$, $B = 18,824$, $\beta = 0.020 \text{ h}^{-1}$, and dose = 15 mg/kg.


