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

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Abbreviations: AUC, area under the plasma concentration-time curve; CL, clearance; Clint, Intestinal Clearance, P-gp, p-glycoprotein, BCRP Breast Cancer Resistance Protein.

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Abstract

The role of the intestine in the elimination of DPC 333, [(2*R*)-2-{(3*R*)-3-Amino-3-[4-(2-methylquinolin-4-ylmethoxy)phenyl]-2-oxopyrrolidin-1-yl}-*N*-hydroxy-4-methylpentanamide] a potent inhibitor of tissue necrosis factor alpha converting enzyme, was investigated in mice and rats in vivo and in vitro. In MDCK cells stably transfected with p-glycoprotein (P-gp), DPC 333 the transport from B→A reservoirs exceeded the transport from A→B by ca 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 GF120918, confirming the contribution of P-gp/BCRP in B→A efflux of DPC 333. In QWBA studies with [^{14}C]-DPC 333 in mice and rats radioactivity was distributed throughout the small intestine in both species. In GF120918 pretreated bile cannulated rats radioactivity in feces was reduced 60%. Using the in situ perfused rat intestine model, ~20% of an IV dose of [^{14}C]-DPC 333 was measured in the intestinal lumen within 3h post dose; 12% as parent. Kinetic analysis of data suggested that excreted DPC 333 may be further metabolized in the gut. Intestinal clearance was 0.2-0.35 l/hr/kg. The above data suggests that, in the rodent, the intestine serves as an organ of DPC 333 excretion, mediated in part by the transporter p-glycoprotein.

Introduction

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, 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; Sparrenboom et al, 1997; Rabbaa et al, 1996; Smit et al, 1998a, 1998b; Dautrey, et al, 1999, van Asperen, et al, 2000, Lagas et al. 2006; Villanueva et al, 2006; Li et al, 2005; Leush et al, 2002).

P-glycoprotein (P-gp, MDR1) and breast cancer resistance protein (BCRP, ABCG2), are ATP-dependent multidrug efflux pumps belonging to the ATP-binding cassette (ABC) superfamily of proteins (Hyde et al., 1990; Allen et al, 1999), which 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 (Yeboah et al., 2006; Eisenblatter et al, 2003;) 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; Zhang et al, 2005, Merino et al, 2005). The acridonecarboxamide derivative GF120918 potently inhibits both of these transporters (Hyafil et al, 1993; deBruin et al. 1999) and has been shown to be a useful research tool in investigations into the role of these

transporters both in vivo and in vitro (Imbert et al, 2003; Rautio et al, 2006; Hassan et al. 2006; Breedveld et al, 2005)

Intestinal p-glycoprotein and/or BCRP may mediate the secretion of a large number of xenobiotics. Rabbaa et al (1996) demonstrated that the fluoroquinolone drug, ofloxacin was actively cleared via the rat intestine and that this process was inhibited by other fluoroquinones including ciprafloxacin. Co-administration of the P-gp inhibitor, verapamil, reduced intestinal clearance by ca 50%, suggesting a role of P-gp. Digoxin, has also been demonstrated to be actively secreted intestinally in rodents and this process 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 demonstrate positively that intestinal excretion of a number of amphiphilic cationic drugs is mediated in part by P-gp (Lagas et al. 2006; van Asperen, et al, 2000, Smit et al , 1998a; 1998b; Sparreboom et al, 1997). Intestinal p-glycoprotein has also been suggested as 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) demonstrated in humans that induction of intestinal p-glycoprotein by rifampin correlated directly with an increase in the systemic clearance of intravenously administered talinolol in the absence of changes in metabolic clearance. Intestinal excretion of the food derived heterocyclic amine carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-

b]pyridine (PhIP) was demonstrated to be markedly reduced in BCRP deficient mice (van Herwaarden et al, 2003).

DPC 333 [(2*R*)-2-{(3*R*)-3-Amino-3-[4-(2-methylquinolin-4-ylmethoxy)phenyl]-2-oxopyrrolidin-1-yl}-*N*-hydroxy-4-methylpentanamide] (Figure 1) is a potent, orally active, selective inhibitor of Tissue Necrosis Factor alpha Converting Enzyme (TACE) under evaluation for treatment of rheumatoid arthritis (Qian et al, in press). To facilitate the clinical development of this compound, in vitro/in vivo disposition and excretion studies were conducted. Preliminary results of intestinal permeability screening studies performed in Caco-2 cell culture suggested that DPC 333 is a substrate for cellular efflux mechanisms, perhaps partly via the transporters p-glycoprotein or BCRP. The studies in this report were conducted to investigate the potential contribution of the intestine and the transporter p-glycoprotein 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* ovarian (*Sf*9) insect 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 membranes, 20 µM Verapamil (positive control) or test drug, and 3-5 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 inhibits P-gp by trapping MgADP in the nucleotide binding site. Thus, 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 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 to a phosphate standard curve. The lamda 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 (Druekes et al. 1990).

Caco-2 Transport Studies

Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). Cell stocks were maintained in T-75 cm^2 flasks (Costar, Corning, NY) at 37^o C in a humidified atmosphere of 5% CO_2, 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 **m**g/ml streptomycin (GIBCO, Grand Island, NY). The culture media were replaced every other day. Monolayers were subcultured using 0.05% trypsin-0.02% EDTA when they reached 75-85 confluency at a split ratio 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, Corning, NY) at a density of 6 x 10^4 cells/cm^2). The transwell inserts were placed in 12-well culture plates with 0.5 ml media in the apical compartment and 1.5 ml media in the basolateral compartment. The media at both compartments were replaced every other day for 3-4 weeks before the cells were used for transport studies.

Prior to the transport experiments, the integrity of Caco-2 cell monolayers was assessed by determining transepithelial electronical resistances (TEER)

using an Evon epithelial voltohm meter (World Precision Instrument, Haven, CT). TEER values were in the range of 400 to 800 ohms.cm^2. The culture medium in Transwell^{TM} was aspirated and washed twice with transport buffer (HBSS containing 25 mM glucose and 10 mM HEPES, pH 7.4 except indicated). Then, the cells were incubated in the transport buffer at 37^{o}C for 30 min. The transport was initiated by replacing transport buffer in the donor compartment with fresh transport buffer containing [^{14}C]-DPC 333 with or without GF120918. After 2 hr incubation, total radioactivity in media of receptor compartment was determined using Parkard Tri-Carb liquid scintillation analyzer.

Efflux in MDR1 transfected MDCK Cells

MDCKII-MDR1 cells were obtained from The Netherlands Cancer Institute (Amsterdam, NL). Cell stocks were maintained in T-175cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2, 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 – 85% confluency.

Single-cell suspensions of MDCKII-MDR1 cells were plated onto the 0.1mm^2 Millicell® -96 polycarbonate membranes (1µM pore size, Millipore, Watford, UK) at a density of 4 x 10^5 cells/cm^2. The apical portion of the plate containing 150µL media was placed into the basolateral compartment containing 300µL media. The media was replaced 24 hours prior to transport experiments.

Prior to transport experiments, the integrity of the MDCKII-MDR1 monolayer was assessed by determining the transepithelial electrical resistances (TEER) using the REMS TEER measurement system (WPI, Stevenage, UK). TEER values were in the range of 500 – 700 ohms/cm^2. The culture medium in the Millicell® - 96 well plates was aspirated and washed three times with transport buffer (HBSS containing 10mM 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 60min incubation period aliquots were analyzed by LC-MS/MS.

Rat Intestinal membrane permeability experiments

Sprague-Dawley rats were supplied by Charles River Canada Inc. (Quebec, Canada). Ussing chambers and mounts were supplied by NaviCyte Co. (NV, USA).

Male Sprague-Dawley rats (250g) 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 on ice which was continuously bubbled with a O_2/CO_2 (95/5%) gas mixture. For these experiments ileal segments were used. Segments were cut along their mesenteric border and

the serosa 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 Tyrodes Buffer which was bubbled continuously. The stripped intestinal mucosae 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 cm^2. All 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 5mL 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-glycoprotein/BCRP inhibitor GF 120918 (0.2 µM) was added 10 minutes prior to test article addition to determine the effect of p-glycoprotein/BCRP 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 membrane was calculated. Analysis of representative medium samples for parent drug by HPLC

(vide infra) coupled with flow-through radiochemical detection to demonstrated stability under the experimental conditions above.

Quantitative Whole Body Autotradioagraphy Studies of [^{14}C]-DPC 333 in Intact Mice and Bile-Duct Cannulated Rats

Materials

[^{14}C]-DPC 333 was prepared by the Radiochemistry Department at DuPont Pharmaceuticals, Newark, DE. Specific activity was ca 1 µCi/mg DPC 333. The radiolabeled dose, undiluted with cold material, was reconstituted on the day of dosing with sterile water for injection, USP.

Animals

Female BALB mice (ca 25g at study initiation) were obtained from Charles River Laboratories Inc., Wilmington, MA.

Male Sprague-Dawley rats, which were bile-duct & jugular vein cannulated by the vendor, were obtained from Charles River Laboratories Inc., Wilmington, MA.

Whole-Body Autoradiography

Animals were prepared for QWBA based on the methods of Ullberg (1954) as follows.

Mice were individually housed with access to bottled water and were fasted overnight before dosing and during the in-life phase of the study. Each mouse received a single intravenous injection (tail vein) of 15 mg/kg, (10 µCi/mouse). One mouse/timepoint was euthanized by carbon dioxide inhalation at 0.03, 0.25, 0.5, 1, 2, 4, 6, 12 and 24 hr postdose. Each mouse carcass was frozen in a hexane-dry ice bath (approx. -70°C). Carcasses were then shipped to Quintiles, Inc., Kansas City, MO for processing, imaging and quantitation of the amount of [^{14}C]-DPC 333-derived radioactivity. Sections were exposed to phosphor imaging plates for seven days and then scanned into an Analytical Imaging System (AIS) workstation (Imaging Research, Inc., St. Catharine's, Ontario, Canada) via a Molecular Dynamics phosphor image scanner. Glossy, black and white images of the autoradiographs were produced immediately after scanning. Annotated, composite images, were produced using AIS were made to illustrate comparisons of tissue distribution. The lower limit of quantitation (LLOQ) was determined by determining the lowest concentration of radioactivity in ^{14}C-spiked blood, which could be measured to an accuracy of < ±10% of the radioactivity concentration, which was determined by liquid scintillation spectroscopy and had an inter-IP precision of < 10%. This was determined for each IP (n= 10, 1/rat) used for study as part of a system validation, which was performed before study initiation.

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Three bile duct- and jugular vein-cannulated rats were administered a single I.V. dose of [^{14}C]-DPC 333 (15 mg/kg ~60 μCi/rat) via jugular vein cannula, and one rat each was euthanized by CO_2 inhalation at 2, and 5 min and at 1h post-dose. Rat carcasses were immediately frozen after death was confirmed by immersion in a hexane/dry ice bath. Carcasses were drained, blotted dry and placed on dry ice for at least 2 h to complete the freezing process. Frozen rat carcasses were individually embedded along with section thickness quality control standards (^{14}C-spiked rat blood) in carboxymethylcellulose (Chay and Poland, 1994), (frozen @ ~ -70ºC). Appropriate sections (~30 microns thick) were collected on adhesive tape (Nakagawa NA-70 MAG, Japan) using a Leica CM3600 cryomicrotome (Leica Microsystems, Deerfield, IL) with temperature controlled at approximately -20°C. Sections were collected at five levels of interest in the sagittal plane. All major tissues, organs, and fluids were included in these levels. Sections were lyophilized, mounted on a black cardboard supports along with ^{14}C-autoradiographic calibration standards (Code RPA 511, Amersham Life Sciences, Buckinghamshire, England), wrapped with Mylar film and exposed to phosphor imaging plates (IPs) (BASIII, Fuji Photo Film Co., LTD., Japan) for 4 days. Exposed IPs were scanned into the WBA imaging system via a FLA 3000 BioImaging Analyzer (Fuji Biomedical Products, Fuji Photo Film Co., LTD., Japan) and digital images of the radioactivity in each section were obtained using M5+ MCID software (Imaging Research Inc., St. Catharine's, Ontario). Tissue concentrations were interpolated from each standard curve as nanocuries per gram and converted to μg equivalents ^{14}C-labeled test article/g of

tissue. The concentrations of radioactivity in the calibration standards used ranged from 0 to approximately 9400 nCi/g tissue (r^2 = 0.9994-0.9999). Tissue concentrations were obtained from tissues that could be visually identified on the autoradiograph. The limit of quantitation was determined as the mean background radioactivity concentration value for background plus three times the standard deviation (mean of 10 measurements/IP using sampling tools provided by the image analysis software, where small tool area = 1mm x 1mm; large sampling tool area = 5mm x 5mm). This was determined for small and large sampling tool areas on each IP (n= 7) used for study. Small tissues included the pituitary gland, adrenal gland, thyroid gland, skin and bone marrow and remaining tissues were considered as large tissues.

Disposition of [^{14}C]-DPC 333 in Bile Cannulated Rats

Materials

Unlabelled DPC 333 was prepared by DuPont Pharmaceuticals, Wilmington, DE. [^{14}C]-DPC 333 was prepared by the Radiochemistry Department at DuPont Pharmaceuticals, Newark, DE. Specific activity was ca 4 uCi/mg DPC 333 free base. The radiolabeled dose, diluted with cold material, was reconstituted on the day of dosing with sterile water for injection, USP. GF 120918 was prepared by the Radiochemistry Department at DuPont Pharmaceuticals, Newark, DE. GF120918 doses were formulated in Diethylene glycol:H_2O (1:9)

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Animal Mass Balance Studies

Male Sprague-Dawley rats (ca 250 g at study initiation), fitted with indwelling bile duct cannulas for the collection of bile, were obtained from Charles River Laboratories Inc., Wilmington, MA. Prior to administration of radiolabelled test compound, 2 rats were pretreated (20 mg/kg PO) with the potent p-glycoprotein and BCRP inhibitor GF120918 at 24h and 1h predose. Each rat received a single intravenous injection (tail vein) of 15 mg/kg, (5 mL/kg, 4 μCi/rat). of the dosing solution. Following administration of test material, each animal was placed into individual metabolism cages that allowed for separate collection of urine, feces, and bile. Bile was collected at 0.15, 0.5, 1,2,4,8,12,24,36, and 48h post dose. Urine and feces was collected at 4, 12, 24, 48h post dose. At 48h post dose, the animals were sacrificed by CO_2 asphyxiation and the intestinal contents collected into preweighed containers.

Sample Analysis.

Radioactivity in urine, cage washes, and bile was quantitated by directly assaying aliquots by liquid scintillation spectrometry (LSC). 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 [^{14}C]-CO_2, and then analyzing samples by LSC.

Samples were counted for 10 minutes or until 160,000 disintegrations (0.5% 2σ) were accumulated, whichever came first. Low-activity samples were counted for up to 75 minutes or until 6400 disintegrations (2.5% 2σ) were accumulated.

Effect of p-glycoprotein blockade on DPC 333 Pharmacokinetics

Male Sprague-Dawley rats (ca. 250 g at study initiation), fitted with indwelling jugular cannulas for the collection of blood, were obtained from Charles River Laboratories Inc., Wilmington, MA. Prior to conduct of dosing studies, animals were anesthetized with pentabarbital and duodenal catheters implanted for intestinal delivery of solutions. Approximately 10 minutes prior to administration of test compound, one group of 3 rats were pretreated with verapamil (1 mg/kg) a known p-glycoprotein inhibitor (Chang et al, 2006) and another group of 3 rats were administered saline. Following pretreatment each rat then received a single oral 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 hours following administration of test material. Plasma was prepared and the concentration of DPC 333 determined via LC-MS/MS. Plasma concentration vs time data were then analyzed by non compartmental 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. (Quebec, Canada). Ketamine and Xylazine were purchased through VWR from Fort Dodge Lab Inc. (IA, USA).

Krebs-Ringer-Bicarbonate buffer components (KH_2PO_4, $MgSO_4$, NaCl, , KCl, $CaCl_2 \cdot 2H_2O$, $NaHCO_3$, Glucose) were purchased from Sigma Chemical Co. (MO, USA).

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 (~200g), prefitted with indwelling jugular vein cannulas prior to surgery, were anesthetized with ketamine/xylazine (100mg/kg:7mg/kg). The fur from the abdominal region was removed with clippers, the skin cleaned with alcohol, and the rat 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 housing with thermostatically controlled electric heaters to maintain the rat and all perfusion solutions at 37ºC. 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 perfused with Krebs-Ringer at a flow rate of 250µL/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 opened body cavities to prevent loss of fluids. Once

the intestinal effluent flow had been established, [^{14}C]-DPC 333 (15mg/kg, 14.4μCi) was then administered IV via the indwelling jugular vein cannula. Following administration of test substance, intestinal effluent samples were collected into pre-weighed 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 prior to 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. Prior to HPLC analysis, perfusate or plasma samples were mixed with an equal volume of Acetonitrile (EM Science, NJ) and vortexed for ca 30 seconds. The mixtures were centrifuged at ca 3000Xg for 10 minutes and the supernatants filtered with a 0.45 μM syringe filter (Whatman Inc., NJ). Aliquots (100μL) were then analyzed for parent compound by HPLC/flow through radiochemical detection. Briefly, the chromatographic system consisted of Hewlett-Packard model 1100 chromatography system (pumps, controllers, and injectors), a Metachem Polaris

C_{18}-A 2x150mm column and a linear gradient mobile phase at a flow rate of 0.4 mL/min. Mobile phase A was 10mM Ammonium Formate pH 7.4 (Fisher Scientific Co., MO) and mobile phase B was Acetonitrile. The gradient conditions started at 6%mobile phase B, ramping linearly to 11% B over 10 minutes, then to 45%B over 6 minutes, then to 61%B over 2 minutes, then held at 61%B over 2 minutes and finally ramping to 71%B over the final 2 minutes. Column eluent was monitored via IN/US ®-RAM flow-through radioactivity detector outfitted with a 1 mL liquid/liquid flow-through cell (Boca Raton, Fl). Column eluent was mixed with Flow-Scint (Packard Instrument company, Meriden, CT) at a 1:4 ratio prior to detection.

Results

In Vitro Experiments

The role of DPC 333 as a p-glycoprotein substrate was suggested in Verapamil-activated Vanadate-sensitive human P-gp ATPase activity assays (Figure 2). DPC 333 activity in this assay was similar to that of verapamil and vinblastine, two known p-glycoprotein substrates. In MDCK cells stably transfected with MDR1, DPC 333 the transport from B→A reservoirs exceeded the transport from A→B by ca 7 fold (Figure 3). This ratio was reduced to 1 by addition of the p-glycoprotein 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 0-200 µM (0.5 µCi/incubation). After 2h incubation radiochemical content in receptor reservoirs determined. In Caco-2 monolayers, DPC 333 was transported from A to B or B to A reservoirs in a concentration dependent manner. (Figure 4). DPC 333 transport from B→A reservoirs was concentration dependent and saturable, suggesting an active transport mechanism (Figure 4). Transport from the basolateral to apical reservoir was partially blocked with GF120918, suggesting that P-gp or BCRP played a role in B→A efflux of DPC 333.

When DPC 333 was incubated with isolated rat ileum in Ussing chambers, B→A efflux greatly exceeded A→B (Figure 5a). Further, B→A efflux was nearly

completely blocked by GF120918. (Figure 5b). This in vitro data taken together suggests that DPC 333 is a p-glycoprotein 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 are presented in Figure 6a and Table 1, respectively. Concentrations of [^{14}C]-DPC 333-derived radioactivity were generally well distributed to all tissues in mice at early timepoints (2 min – 1h), 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 tract and intestinal contents. After 2hr, high concentrations of radioactivity were observed in the liver and contents of the gall bladder and GI tract only. By 24h post-dose, low levels of radioactivity were present in the liver, heart, kidney, blood, and lung and remaining tissue showed none or only trace levels. These WBA data suggest that [^{14}C]-DPC 333 is well distributed throughout the bodies of normal mice. However penetration into brain, spinal cord and bone were not demonstrated in this experiment. The study showed that [^{14}C]-DPC 333 is cleared via renal and biliary excretion and possibly intestinal secretion due to radioactivity observed in the GI tract.

Figure 6b is a whole body autoradiograph of a bile duct-cannulated rat 2 minutes after IV dosing. Rat tissue concentrations are presented in Table 2. In

general, concentrations of [^{14}C]-DPC 333-derived radioactivity were well distributed to all tissues in rats at all timepoints (2 min – 1h), except brain and spinal nerve cord, which showed little or no radioactivity. Highest concentrations were present in liver, kidney, heart, pituitary gland, salivary gland, lacrimal gland, lung, gastrointestinal tract and intestinal contents. These WBA results suggest that [^{14}C]-DPC 333 is well distributed throughout the body of rats in a manner similar to the mouse. The radioactivity observed in the contents of the GI tract was strong evidence to support the hypothesis of intestinal excretion as all bile was diverted away from the bile duct and thus from entering the intestine.

Effect of **P-gp/BCRP Blockade on DPC 333 mass balance and pharmacokinetics** ***In Vivo***

To further investigate this hypothesis, [^{14}C]-DPC 333 was administered IV to bile duct cannulated rats and the excretion of radioactivity determined in bile, urine and feces. Bile cannulation removes the contribution of biliary excretion of radioactivity to gut contents. Of the 4 animals treated with test compound, 2 rats were pretreated with 25 mg/kg GF120918 24 and 1hr prior to administration of [^{14}C]-DPC 333 to determine the effect of P-gp/BCRP blockade on ^{14}C excretion. The majority of radioactivity administered IV to control rats was distributed between urine (40%) and bile (56%) 48 h post –dose (Table 3). However, ca 5% of an IV dose was recovered in the feces within 48 hours, suggesting that the

intestine served as an organ of excretion for DPC 333 and/or its equivalents. In the GF120918 pretreated animals, fecal radioactivity was reduced to ca 2% of total dose. Biliary equivalents (~54%) were reduced relative to untreated animals. Urinary equivalents (~45%) were increased by an amount roughly concomitant to the combined reduction in fecal and biliary routes of elimination.

To investigate the role of p-glycoprotein as the principal efflux transporter of DPC 333, rats were pretreated with verapamil, which inhibits p-glycoprotein. Pretreatment of rats with verapamil resulted in a nearly 6 fold increase in DPC 333 Cmax (Table 4). Overall exposure (AUC) increased 1.6 fold following verapamil pretreatment relative to saline controls.

Together, these data suggest that intestine plays a role in the elimination of DPC 333 in rats, and that p-glycoprotein may contribute predominantly to this phenomenon.

In Situ Perfused Rat Intestine Studies

To estimate the contribution of the intestine to the total clearance of DPC 333, [^{14}C]-DPC 333 was administered IV to rats in the In situ perfused small intestine model. Within 3 hours post IV dose 17% of the total radioactivity administered was collected in the intestinal effluent. Approximately 53% and 6% of the dose was recovered in the bile and urine respectively. Total recovery was 76.6% of total dose by 3h. Distribution and recovery was similar to that measured in the intact animals by 3h in separate distribution studies conducted in bile duct cannulated rats. At early timepoints, intestinal effluent radioactivity was predominantly parent molecule. (Data not shown.) By 2h post administration, a

single unidentified metabolite began to contribute to the profile but through 4h post dose parent compound in the effluent comprised 50- 75% of total radioactivity (data not shown). Parent compound was not detected in urine. Plasma radioactivity was predominantly parent compound at 15 minutes post dose but the contribution of parent dropped to less than 20% by 3h post administration. Profiles of plasma and intestinal effluent were qualitatively similar, with parent molecule and a single, unidentified metabolite the only detectable peaks. Systemic clearance Cl_{SYS} of 2.1 L/h/kg, estimated from this plasma data (Table 4), closely matched that measured in similar studies in intact animals (2.7 L/h/kg) (Qian et al., in press). Intestinal clearance, Cl_{SI}, was estimated at 0.2 L/h/kg, approximately 10% of measured total systemic clearance.

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 abc protein p-glycoprotein or BCRP mediates this excretion in part or wholly. In mouse and rat QWBA distribution studies, significant portions of [^{14}C]-DPC 333 derived radioactivity administered IV were detected along the gut lumen within 2-5 min post-dose, directly suggesting a role of the intestine in elimination DPC 333 or its equivalents *in vivo*. By 1 hr approximately, 15 and 17% of the administered radioactivity was detected in the intestinal contents of rats and mice respectively. In mice, by 2 minutes post administration radioactivity was distributed throughout the small intestine and after 2 hr, 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 cannulated rat QWBA studies was strong evidence to support the hypothesis of intestinal excretion because all bile was diverted away from the bile duct and thus prevented from entering the intestine.

In separate studies, [^{14}C]-DPC 333 derived radioactivity was detected in the feces of bile duct cannulated rats following IV administration of [^{14}C]-DPC 333, supporting the WBA results. In this study 5% of the dose was recovered in the feces at 48 hr compared with 15-17% measured in small intestine by 1hr 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-glycoprotein/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 abc transporters p-glycoprotein or BCRP. At the intestinal level the efflux of DPC 333 was demonstrated to be modulated significantly by verapamil, which inhibits p-glycoprotein (Chang et al, 2006) but does not inhibit BCRP (Zhang et al 2005). Verapamil pretreatment increased DPC 333 Cmax almost 6-fold, and AUC by 60% in rats. Though BCRP involvement cannot be ruled out completely, these data suggest strongly that p-glycoprotein plays a role in DPC 333 efflux.

Results of in vitro studies conducted with human mdr1, caco-2 monolayers and isolated rat intestinal mucosa agree with in vivo data suggesting that DPC 333 was a p-glycoprotein 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 nearly completely blocked with GF120918 and cyclosporin. Kinetic analysis of the caco-2 and ileal section data gave Kms of 25

and 46 µM, respectively. This 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 3h post IV administration; 12% of the dose as parent. Intestinal clearance of ca. 0.21 L/hr/kg suggested that the intestine contributed to approximately 10% of total systemic clearance (Table 4). 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 (IPIM). That is, the data supports the possibility that nearly all of the DPC 333 derived radioactivity measured was initially excreted as parent by p-glycoprotein or BCRP.

A plot of plasma DPC 333 concentration vs rate of luminal excretion of DPC 333 and equivalents yielded a hyperbolic curve, suggesting a saturable excretion mechanism (Figure 7A). This would be expected, given the strong data supporting the transporter mediated flux of DPC 333. This suggests that the rate of all equivalents appearing in the lumen are dependent solely on plasma parent concentration. If metabolite was 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 (Figure 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 (Figure 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 (PABA), 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 demonstrated that acetominophen, 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 demonstrated 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-glycoprotein or BCRP. In rat, DPC 333 intestinal clearance is estimated to be a minimum of 12% of systemic clearance (0.35 L/hr/kg, based on luminal parent only) with a maximum of ca 18% (0.52 L/hr/kg, based on total equivalents) of

systemic clearance. If indeed this elimination pathway exists in humans and the relative contribution of the intestine to total clearance is roughly similar to that of rodents, then this suggests a potential impact on total clearance due to intestinal interactions with other P-gp or BCRP substrates.

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Figure Legends

Figure 1. Chemical Structure of DPC 333

Figure 2. Stimulation of Human P-Glycoprotein-ATPase Activity by DPC 333, Verapamil and Other Molecules (20 mM)

Figure 3. Permeability of DPC 333 and Other Molecules in Monolayers of MDCK II Cells Transfected with MDR1.

Figure 4. Distribution of [^{14}C]-DPC 333-Derived Radioactivity in Caco-2 monolayers

Figure 5. Permeability of DCP 333 in Rat Ileal Mucosal sections

Figure 6. Whole Body Autoradiographs of An Intact Mouse (a) and A Bile Duct-Cannulated Rat (b) Sacrificed at 2 Minutes After an IV Dose of [^{14}C]-DPC 333.

Figure 7. Effect of Plasma Concentration of DPC 333 (a) or Metabolite (b) on the Elimination of DPC 333 Equivalents into Intestinal Lumen Following IV Administration of DPC 333 in the In Situ Perfused Rat Intestine Model

Figure 8. Predicted vs. measured luminal DPC 333 and equivalents following IV administration of [^{14}C]-DPC 333 (15 mg/kg) in the In Situ Perfused Rat Intestine Model. Luminal excretion predicted by the following expression:

$$nmoles = \left(\frac{V_{\max} \times C_{plasma}}{EC_{50} + C_{plasma}} \right) \times \Delta t \text{ where } C_{plasma} = \left(\frac{Dose}{15} \right) \left(A\, e^{-\alpha t} + B\, e^{-\beta t} \right)$$

and V_{max} =11.8 nmol/min; EC_{50} =2266 nM; A=163399; α=0.21 h^{-1}; B =18824; β=0.020h^{-1}; and Dose =15 mg/kg.

Table 1. Concentration of Radioactivity in Blood and Tissues at Specified Times Postdose Determined by Whole-Body Autoradiography for Female Mice Following Intravenous Administration of [^{14}C]-DPC 333 (results in nCi/g tissue)

Tissue[a]	Animal Sacrifice Time									
	(2 minutes)	(5 minutes)	(15 minutes)	(30 minutes)	(1 hour)	(2 hours)	(4 hours)	(6 hours)	(12 hours)	(24 hours)
Blood	864.6	654.2	204.6	118.6	37.30	28.10	19.70	23.20	14.60	11.40
Bone marrow	529.4	457.5	161.6	63.40	20.20	14.90	8.300	10.40	6.000	3.200
Brain	14.40	10.80	4.000	2.300	0.9000	0.7000	0.5000	0.6000	0.4000	0.3000
Brown fat	497.1	485.6	170.3	90.70	36.80	20.00	12.00	14.80	5.700	3.600
Gall bladder contents	NR	25420	15340	37810	32590	44500	17350	NR	NR	NR
Heart	1061	559.3	121.0	54.10	15.10	12.90	7.700	11.00	5.500	3.800
Kidney	1732	1915	692.0	324.3	56.80	42.30	31.80	37.80	14.00	7.400

Lacrimal gland	1023	620.0	187.4	NR	27.30	16.80	NR	10.20	4.800	2.200
Liver	3135	2793	1049	597.6	170.1	106.6	98.20	95.20	36.50	17.60
Lymph nodes	144.4	298.8	175.2	112.8	67.90	46.00	13.40	8.000	10.50	4.800
Lung	853.7	347.7	196.4	92.70	26.30	22.70	17.60	19.20	16.70	7.500
Salivary gland	1287	805.8	260.0	106.9	24.90	21.50	16.50	15.90	6.500	3.300
Skeletal muscle	229.3	171.3	80.70	28.20	5.500	2.90	2.100	2.400	1.500	0.9000
Skin	136.0	169.4	92.80	59.00	19.30	11.30	5.400	8.100	4.800	3.900
Spleen	343.0	433.3	266.4	128.4	NR	42.60	21.60	17.90	7.600	4.400
White fat	147.0	103.5	69.40	43.10	19.70	11.20	6.200	3.400	1.900	1.800

[a]Intestinal contents exceeded upper linear range and therefore not quantitated

LLOQ = 1.263 nCi/g

NR Not represented (tissue or organ not present in original section).

NS Not sampled (sample shape not discernable from background).

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Table 2. Concentration of Radioactivity in Blood and Tissues at Specified Times Post-dose Determined by Whole-Body Autoradiography for Bile Duct-Cannulated Male Rats Following Intravenous Administration of [^{14}C]-DPC 333 (results in nCi/g tissue)

	Animal Sacrifice Time		
Tissue	2 minutes	5 minutes	1 hour
Adrenal Gland	1449	1152	325.6
Blood	419.3	346.1	129.6
Bone marrow	461.1	320.1	262.3
Brown fat	NS	614.0	188.5
Brain	13.96	10.43	4.689
Brain ventricle	327.7	269.2	NS
Epididymis	46.80	55.82	78.88
Harderian Gland	232.6	205.2	383.9
Heart	677.2	462.9	160.7
Intestine	1110	879.9	2590
Kidney	1534	1010	1031
Liver	1253	1759	465.6
Lung	418.2	486.7	230.7

Lymph Node	NS	169.5	NS
Muscle	454.7	199.4	187.6
Pancreas	639.5	504.7	282.6
Pituitary Gland	796.3	432.5	292.3
Prostate	133.2	197.0	160.5
Salivary	626.9	401.2	260.8
Seminal vesicle	23.88	14.97	29.77
Skin	156.8	164.4	249.1
Spleen	389.4	634.9	536.4
Stomach (Glandular)	690.6	725.3	2309
Stomach (Non-glandular)	123.7	141.1	170.3
Testis	9.302	13.69	31.23
Thymus	195.6	115.7	159.7
Thyroid	640.7	338.2	209.6
White Fat	176.45	137.2	203.9
LLOQ (small tissues)	4.197	2.951	3.349
LLOQ (large tissues)	3.437	2.548	2.344

NR Not represented (tissue or organ not present in original section).

NS Not sampled (sample shape not discernable from background).

Table 3. Distribution of Radioactivity 48 h Following IV administration of [^{14}C]-DPC 333 (15 mg/kg) to Naive or GF 120918 Pretreated Rats.

	Naïve			Pretreated		
	Rat 1	Rat 2	Ave	Rat 1	Rat 2	Ave
Feces	5.9	4.4	5.1	2.5	1.1	1.8
Urine	47.8	32.2	40.0	47.8	42.7	45.3
Bile	48.0	63.9	56.0	49.3	58.3	53.8
Sum	101.7	100.4	101.1	99.6	102.0	100.8

Table 4. Pharmacokinetic Parameters of DPC 333 Following PO Administration of DPC 333 (15 mg/kg) to Rats Following Intraduodenal Infusion with Verapamil (1 mg/kg) or Saline.

Treatment	Cmax (nM)	Fold Increase In Cmax	AUC_{0-120} (nM*min)	Fold Increase in AUC_{0-120}
Saline	106		5388	
Verapamil	605	5.7	8704	1.6

Table 5. Pharmacokinetic Parameters derived following IV administration of [^{14}C]-DPC 333 (15 mg/kg) to Rats in the In-situ perfused intestine model.

Parameter	*Rat 1*	*Rat 2*	*Rat 3*	***Mean***	***S.D.***
AUC nM*h	10029	26060	15442	17177	8155
nmol Dosed	6565	5878	5290	5911	638
nmol Excreted	652	895	680	742	133
Rat Wt. Kg	0.20	0.21	0.19	0.20	0.01
Clsys, L/hr/kg	3.3	1.1	1.9	2.07	1.11
Clsi, L/hr/kg	0.3	0.2	0.2	0.24	0.08

Figure 1

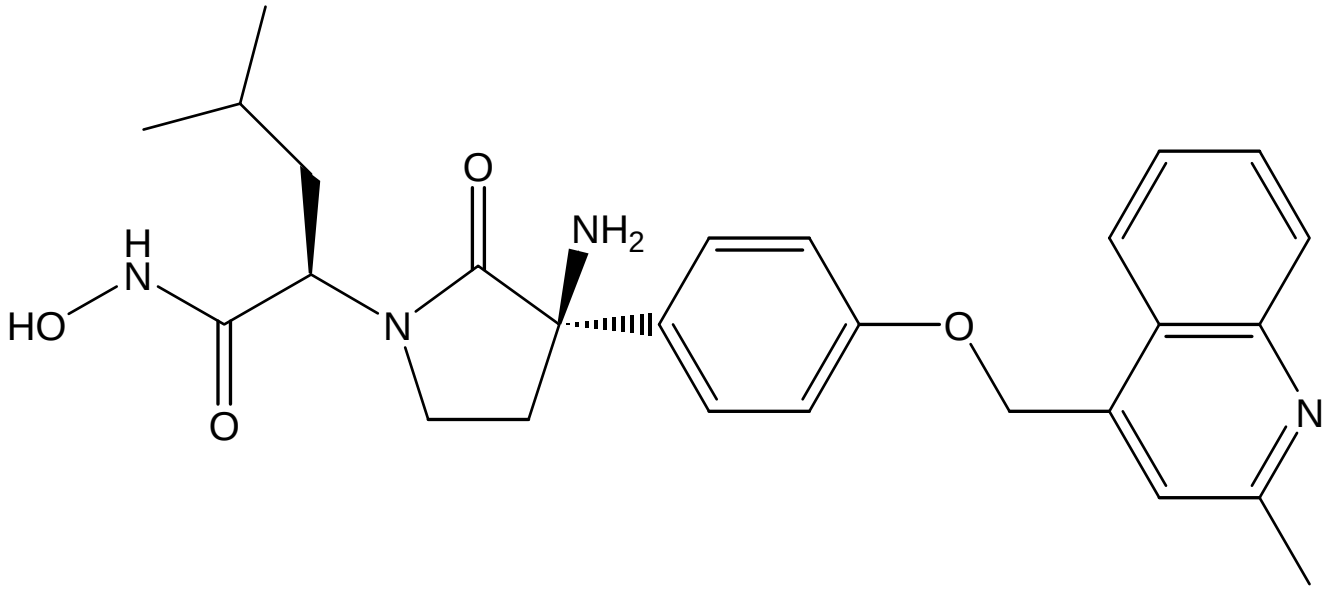

Figure 2

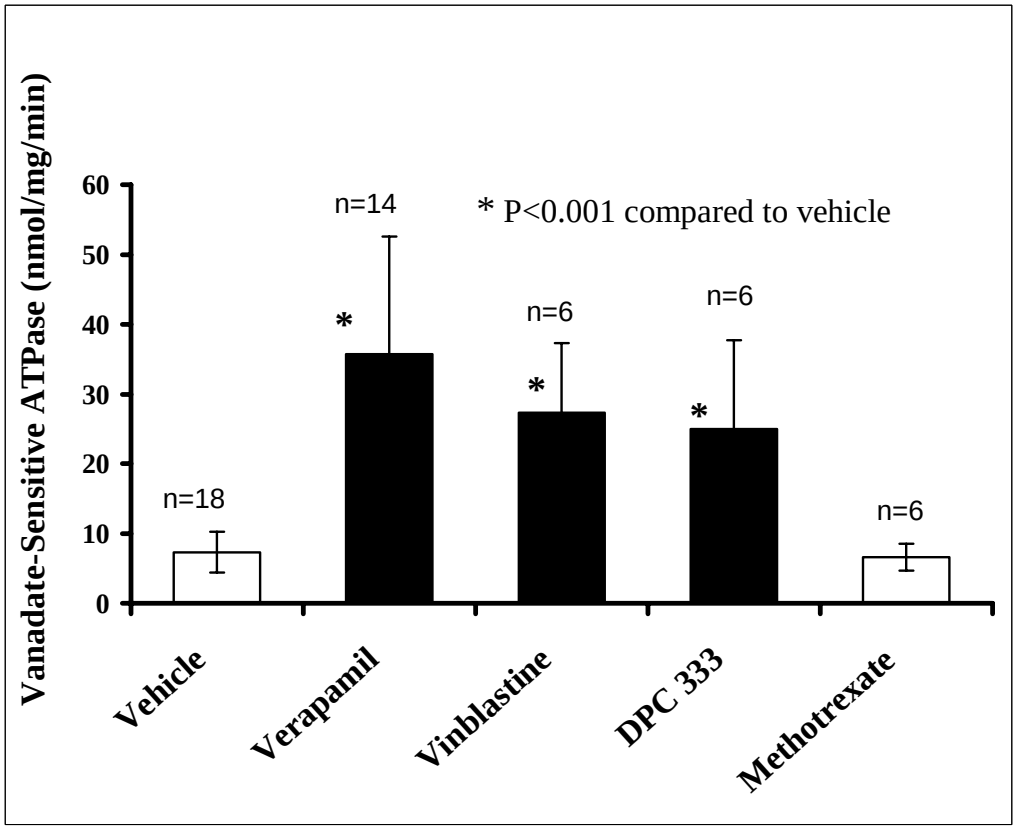

Figure 3

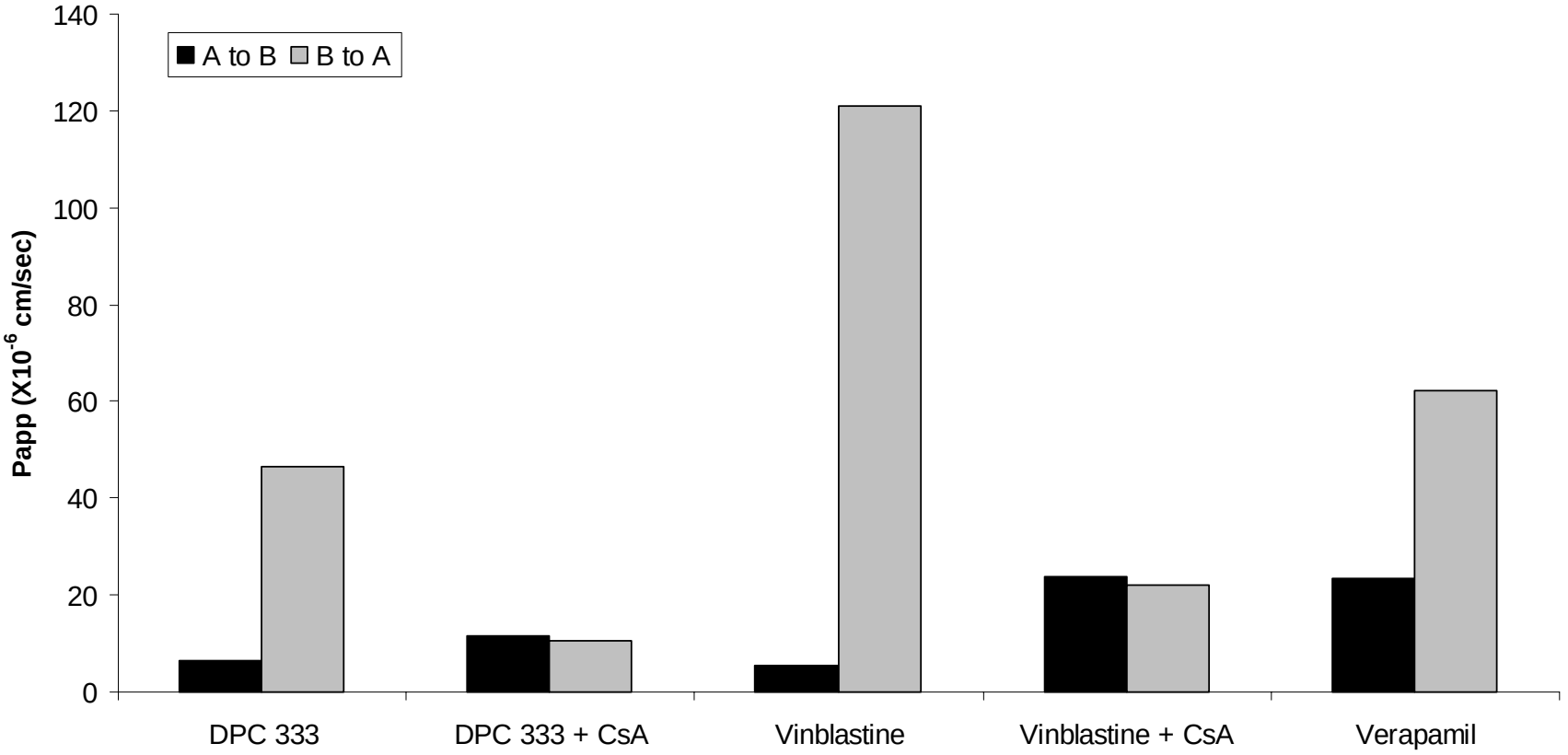

Figure 4

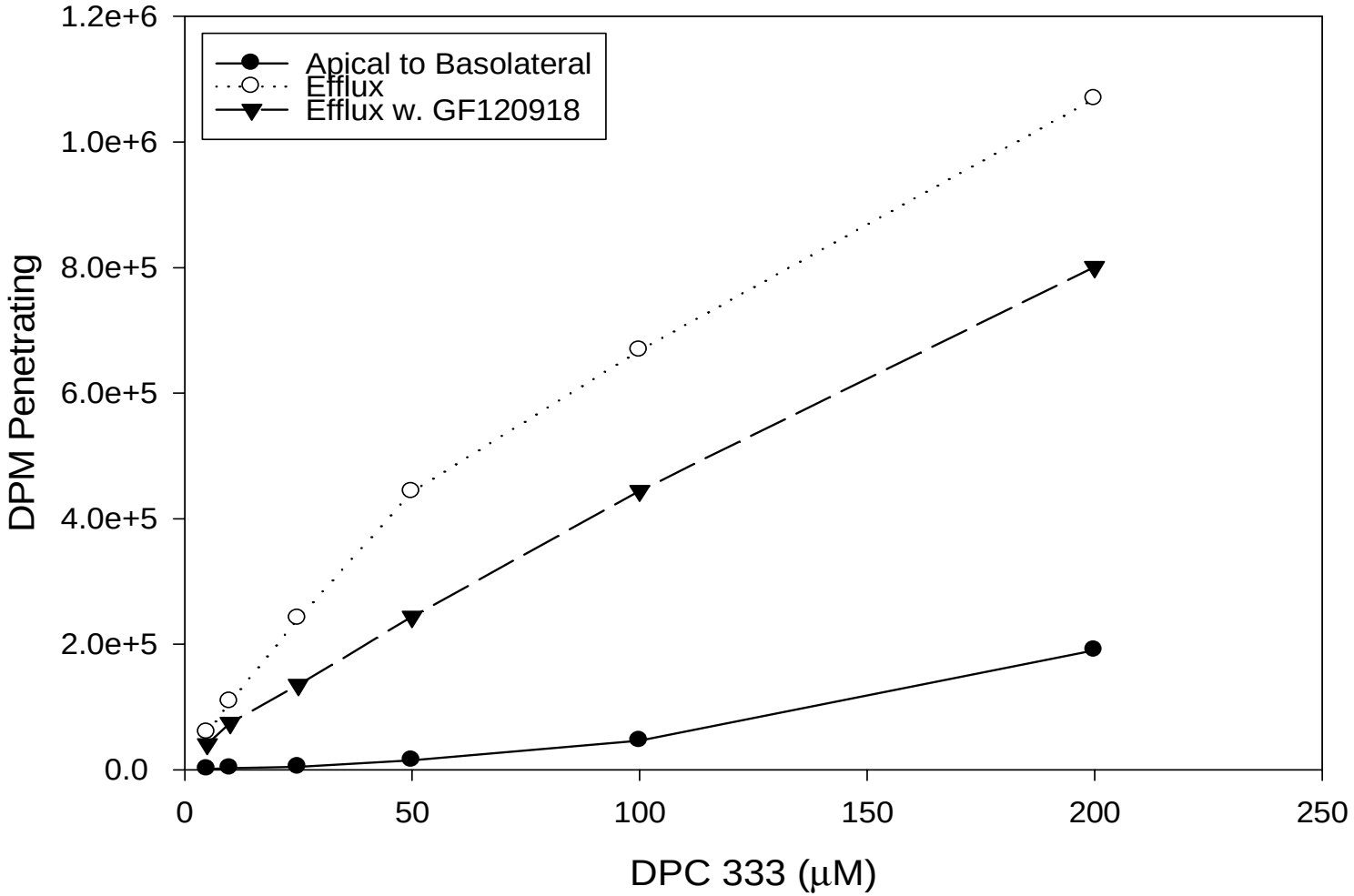

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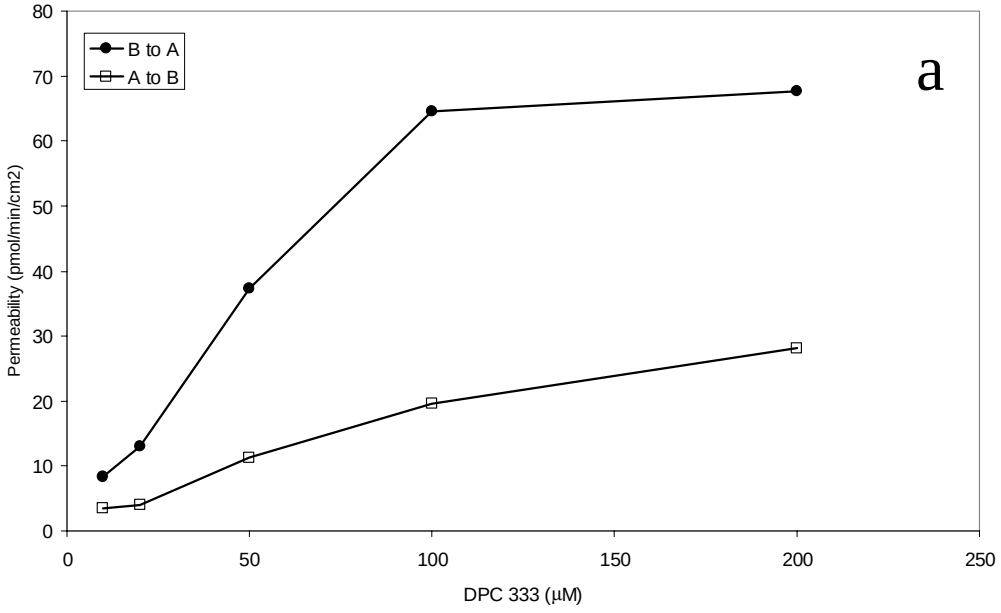
Figure 5



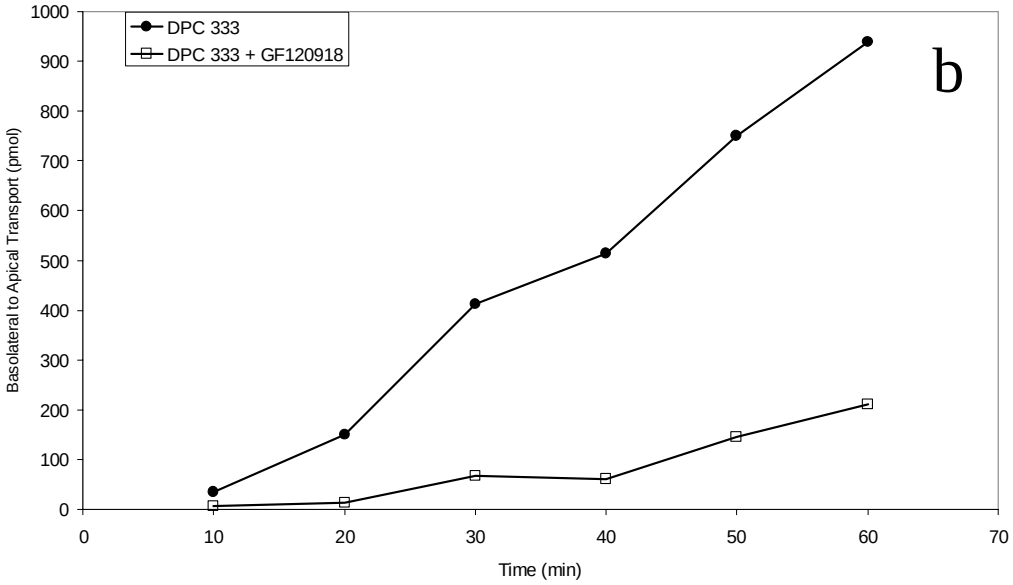
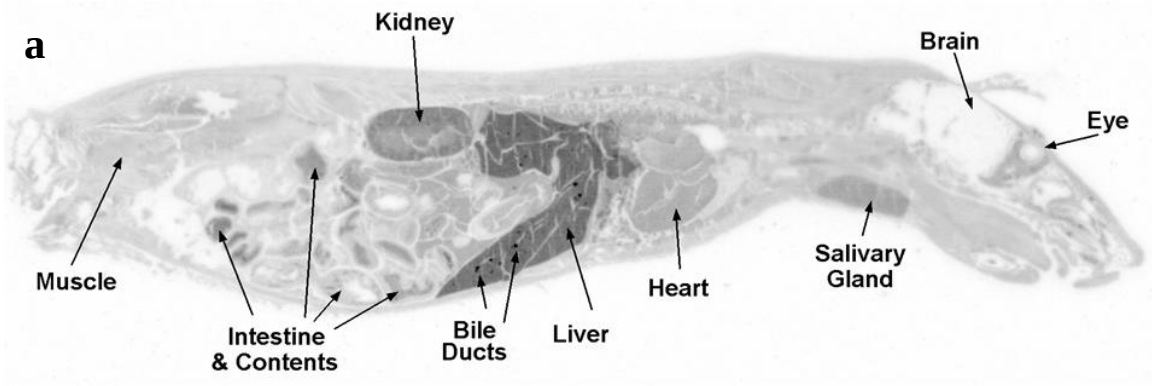


Figure 6



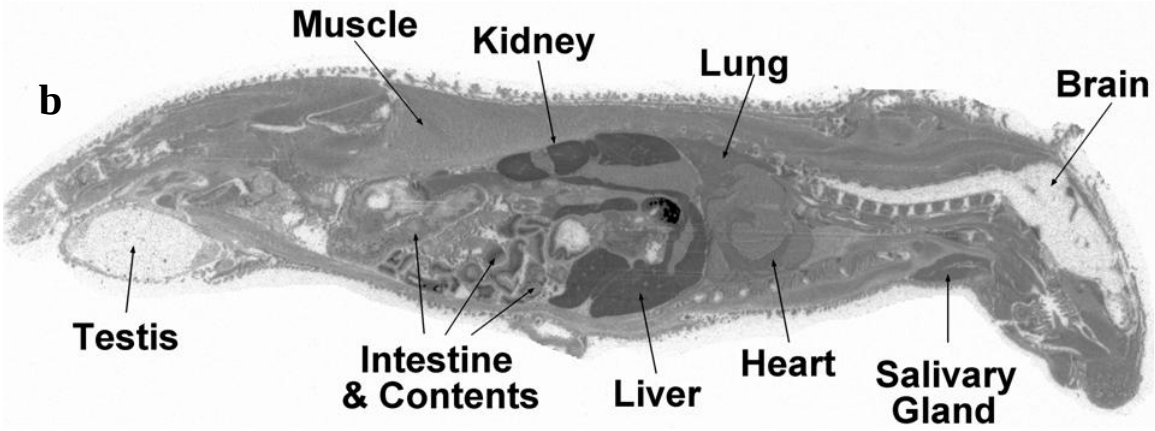
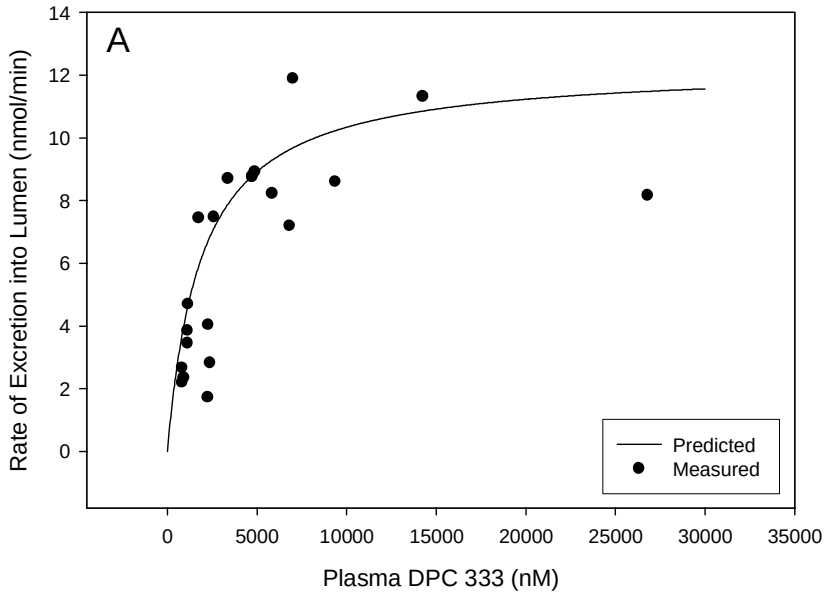


Figure 7



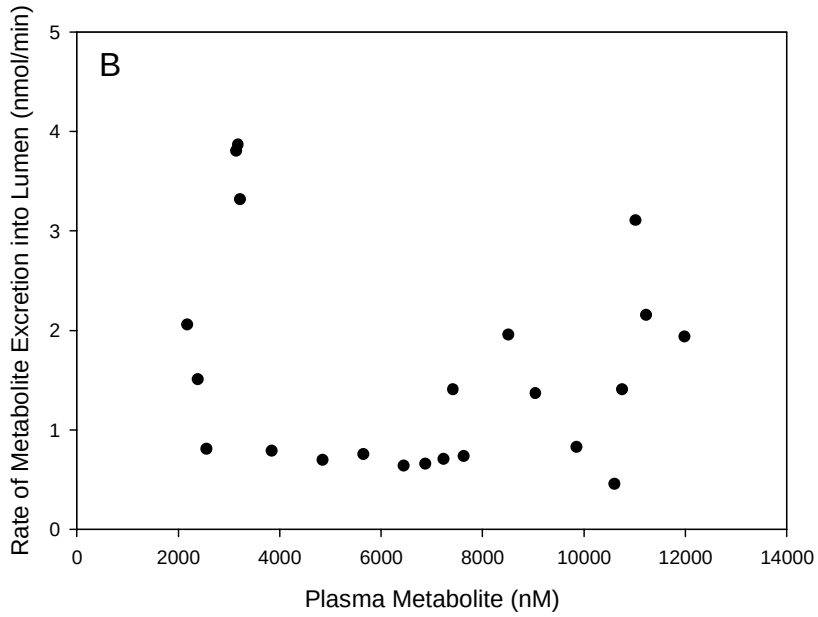


Figure 8