The Role of Efflux and Uptake Transporters in Lapatinib (Tykerb, GW572016) Disposition and Drug Interactions.

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Lapatinib = GW572016, N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino}methyl)-2-furyl]-4-quinazolinamine; TKI = tyrosine kinase inhibitor; Pgp = P-glycoprotein, BCRP = Breast cancer resistance protein; A→B = Apical to basolateral; B→A = Basolateral to apical; MDCK = Madin Darby canine kidney cells; P_{app} = apparent permeability; IC_{50} = concentration required for 50% inhibition; ABCG2 = genomic nomenclature for BCRP protein; ABCB1 = genomic nomenclature for the Pgp protein.

Abstract

Lapatinib is a tyrosine kinase inhibitor approved for use in combination with capecitabine to treat advanced or metastatic breast cancers over expressing HER2 (ErbB2). This work investigated the role of efflux and uptake transporters in lapatinib disposition and drug interactions. In vitro studies evaluated whether lapatinib is a substrate for efflux transporters, or an inhibitor of efflux/uptake transporters. In vivo studies included whole-body autoradiography (WBA) and an evaluation of the role of efflux transporters on the intestinal absorption and brain penetration of lapatinib using chemical or genetic knock-out animals. Lapatinib is a substrate for the efflux transporters P-glycoprotein (Pgp) and Breast Cancer Resistance Protein (BCRP). Further, lapatinib is an inhibitor (IC_{50} values 0.025 to 5 μM) of Pgp, BCRP and OATP1B1 (a hepatic uptake transporter). In contrast, lapatinib yielded little inhibition of renal transporters (OATs, OCTs, URAT). In vivo studies demonstrated that brain concentrations of lapatinib were low and influenced by efflux transporters at the blood-brain barrier. In contrast, lapatinib's systemic exposure after oral dosing was unchanged when efflux by Pgp and BCRP was absent from the gastrointestinal tract. These in vitro and in vivo preclinical investigations provide a mechanistic basis for elucidating clinical drug interactions.

Drug transporters can have a significant impact on the absorption, distribution, metabolism, excretion and toxicity of a drug (Borst et al., 2002). Transporters can be effective barriers to drug exposure, be the rate determining step in the uptake and/or excretion of a compound or metabolite, and be a cause of drug-drug interactions. Drug efflux transport systems are the most extensively studied family of drug transporters due to the phenomenon of multi-drug resistance, where cancer cells become cross-resistant to multiple cytotoxic anticancer agents after treatment with only a single drug (Borst et al., 2002; Lepper et al., 2005). Drugs that are substrates for efflux transporters are pumped out of the cell resulting in decreased intracellular accumulation, permitting cancer cells to survive lethal doses of cytotoxic drugs. This phenomenon has been characterized in a number of cancers, including breast cancer.

Breast cancer is the most prevalent cancer in the world, affecting over four million women (Parkin et al., 2005). Each year, over one million women are diagnosed and 450,000 die from this disease. Breast cancer is frequently associated with increased expression and activation of the epidermal growth factor receptor (EGFR; ErbB), a family of transmembrane tyrosine kinase receptors (Rowinsky, 2004; Johnston et al., 2006). These signaling proteins regulate cellular growth, proliferation, survival and differentiation. The EGRF-ErbB family includes four members: EGRF1 (HER1, ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Upon activation by ligand, these receptors undergo dimerization resulting in autophosphorylation and initiation of intracellular signal transduction pathways. EGRF1 and HER2 over expression occur in 27% to 30% and 20% to 25% of breast cancers, respectively (Moy and Goss, 2006). It has been proposed that increased expression and activation of these receptors is associated with a higher risk for recurrence of breast cancer and a poorer clinical outcome (Rowinsky, 2004;

Johnston et al., 2006; Moy and Goss, 2006). Indeed, inhibition of EGFRs has been demonstrated

to have important anti-tumorigenic effects, which has led to the development of several antibody

and small molecule treatments (Parkin et al., 2005; Rowinsky, 2004; Johnston et al., 2006).

Lapatinib (Tykerb, GW572016) is a novel member of the 4-anilinoquinazoline class of tyrosine

kinase inhibitors (TKI) (Moy and Goss, 2006; Boyd et al., 2005; Johnston and Leary, 2006). It is

a dual inhibitor of both EGFR1 and HER2 with IC_{50} values of approximately 10 nM against the

purified receptors in vitro, and potently inhibits growth of EGFR and/or HER2 over expressing

tumors both in vitro and in vivo. Evidence of clinical efficacy has been reported, especially in

HER2-positive breast cancers, for lapatinib used either alone or in combination with other anti-

cancer agents (Bence et al., 2005; Geyer et al., 2006). An extensive phase II and III program in

advanced breast cancer for both refractory disease and as first-line therapy in combination with

chemotherapy is in progress (www.clinicaltrials.gov).

The objective of this work was to investigate the role of efflux and uptake transporters in the

disposition and drug interactions of lapatinib given that this drug is being used to treat breast and

potentially other cancers where drug transporters can influence therapeutic outcome, disposition

and toxicity. From these in vitro and in vivo investigations, a mechanistic basis for elucidating

potential clinical drug interactions has been developed to guide the design of future clinical

studies with lapatinib.

Materials and Methods

Materials. GlaxoSmithKline Chemical Registry supplied [^{14}C]-lapatinib (55-57 mCi/mmol), GF120918 (Elacridar) and [^{3}H]-amprenavir (21 Ci/mmol). [^{3}H]-Cimetidine (20 Ci/mmol), [G-^{3}H]-digoxin (5 Ci/mmol), [^{3}H]-estradiol 17β-D-glucuronide (45.0 Ci/mmol) were purchased from GE Healthcare/Amersham Biosciences (Piscataway, NJ). [^{14}C]-p-Aminohippuric acid (53 mCi/mmol), [^{3}H]-prostaglandin F2α (155 Ci/mmol), [^{3}H]-estrone sulfate (57 Ci/mmol), and [^{3}H]-histamine (18 Ci/mmol) were supplied by Perkin Elmer Life Sciences (Boston, MA). [^{14}C]-Tetraethylammonium (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO) and [^{14}C]-uric acid (52 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO). Transwells (12-well, 11-mm diameter, 0.4 μm pores) were purchased from Corning Costar (Cambridge, MA).

Monolayer Efflux Studies. The polarized Madin-Darby canine kidney (MDCKII) cells heterologously expressing either human Pgp (MDCKII-MDR1 cell line) or BCRP (MDCKII-BCRP cell line) were used for the in vitro transport studies and were obtained from the Netherlands Cancer Institute (Amsterdam, Netherlands). Cell culture and transport studies were completed as previously described (Polli et al., 2001). Stock solutions of [^{14}C]-lapatinib, GF120918, and the positive control substrates [^{3}H]-amprenavir (Pgp substrate) and [^{3}H]-cimetidine (BCRP substrate) were prepared in DMSO. For substrate assessment, donor solutions with and without GF120918 (a Pgp and BCRP inhibitor) were prepared by diluting the stock solutions in transport medium (Fasted State Simulated Intestinal Fluid containing 25mM glucose; 134 mM NaCl, 29 mM NaH$_2$PO$_4$, 1.5 mM lecithin, and 5 mM sodium taurocholate, pH

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adjusted to 7.4 with NaOH, and osmolarity of approximately 290 mmol/kg). Receiver solutions

contained Dulbecco's Modified Eagle Medium (without Phenol Red), 25 mM HEPES and 1%

human serum albumin, with or without GF120918. The transport of [^{14}C]-lapatinib and positive

control substrates (amprenavir for Pgp and cimetidine for BCRP efflux) was measured in two

directions (apical to basolateral [A→B] and basolateral to apical [B→A]). Drugs were

quantified by liquid scintillation counting (LSC) using a TriCarb T2900 liquid scintillation

counter and Ultima Gold scintillation cocktail (Perkin Elmer, Boston, MA).

Pgp and BCRP Inhibition Assays. Cell culture and transport studies were completed as

described (Rautio et al., 2006). Lapatinib was tested in triplicate at a minimum of six

concentrations spanning 0.3 to 50 μM for Pgp and 0.01 to 3 μM for BCRP. Inhibition studies

were conducted 90 min using [^{3}H]-digoxin (27 nM) as the probe substrate for Pgp and [^{3}H]-

cimetidine (80 nM) for BCRP. [^{3}H]-digoxin and [^{3}H]-cimetidine were quantified by LSC.

GF120918 was used as a positive control inhibitor.

OATP, OAT, OCT and URAT Inhibition Assays. For OATP1B1 studies, Chinese Hamster

Ovary cell line heterologously expressing the human OATP1B1 transporter (CHO-OATP1B1)

was used and was obtained from the University of Zurich (Zurich, Switzerland). CHO-

OATP1B1 cells were seeded into 24-well assay plates (Becton Dickinson, Franklin Lakes, NJ) at

a density of 50000 cells/cm^2 in cell culture medium (Dulbecco's Modified Eagle Medium with

Glutamax (DMEM), 10% (v/v) fetal bovine serum, 0.5% (v/v) penicillin/streptomycin 10000

units/mL, 0.1% (v/v) L-proline 50 mg/mL and 0.7% (v/v) Geneticin 50 mg/mL). The

monolayers were used 2 days post seeding and induced for at least 24 hours prior to use with 5

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mm sodium butyrate. For inhibition studies, CHO-OATP1B1 monolayers were preincubated (37°C) for 15 to 30 minutes in 1 mL transport medium (Dulbecco's Phosphate Buffered Saline (DPBS) plus 1% DMSO) with or without lapatinib or rifamycin (positive control inhibitor). Following removal of preincubation solution, 400 μL of transport medium containing 20 nM [^{3}H]-estradiol 17β-D-glucuronide, with or without inhibitors, was added and the cells incubated at 37°C for 5 minutes. The inhibitor solution was removed, cells rinsed three times using 800 μL cold (4°C) DPBS, cells lysed with 400 μL of 1% (v/v) Triton X-100 and radioactivity quantified by LSC.

OAT, OCT and URAT inhibition screening studies were completed at Fuji Biomedix Co. Ltd. (Yamanashi, Japan). For OAT and OCT inhibition assays, transporter expressing S2 cells were seeded at a cell density of 10^5 cells/well in 24-well tissue culture plates individually expressing either OAT1, OAT2, OAT3, OAT4, OCT1, OCT2, OCT2-A, or OCT3; the parental S2 cell line is derived from the S2 portion of the renal proximal tubules and carries a temperature-sensitive simian virus 40 large T-antigen gene (Takeda et al., 2002). For URAT inhibition assays, transporter expressing HEK-293 cells were seeded in 24-well Biocoat plates (Becton Dickinson, Franklin Lakes, NJ) at a cell density of 10^5 cells/well. Transporter expressing cells were cultured for 2 days at 33°C (S2 cells) or 37°C (HEK293 cells) as described (Takeda et al., 2002). Lapatinib and positive control inhibitors (probenecid, quinidine and benzbromarone for OATs, OCTs, and URAT1, respectively) were dissolved in DMSO and then diluted to a final concentration of 30 μM into uptake medium (DPBS pH 7.5 for S2 cells or Hank's balanced salt solution (HBSS) pH 7.4 containing no Cl$^-$ for HEK293 cells) containing the radiolabeled substrates. Monolayers were incubated for 2 min (OAT1, OAT3 and OAT4), 0.5 min (OAT2),

15 min (OCT1), 5 min (OCT2 and OCT2-A), 1 min (OCT3) or 5 min (URAT1). After incubation, the solution was removed and uptake stopped by adding ice-cold DPBS (or HBSS). Cells were lysed with 0.1 M sodium hydroxide, lysate collected and radioactivity determined by LSC. Protein concentrations of cellular lysates were determined using a BCA Protein Assay Reagent (Pierce, Rockford, IL) as described by the manufacturer.

Animals Studies. All surgical and dosing procedures were done according to the approved Institutional Animal Care and Use Committee protocols.

Whole-body Autoradiography. The tissue distribution of radioactive drug-related material in the male Han Wistar rats (Charles Rivers Laboratories, Raleigh, NC) following a single oral dose of 10 mg/kg [^{14}C]-lapatinib (formulated in 0.5% (w/v) aqueous hydroxypropyl methyl cellulose containing 0.1% (v/v) Tween 80) was investigated using whole-body autoradiography at 0.5, 4, 8, 24 and 168 hours after dose administration. Tissue processing and image analysis were completed as described (Polli et al., 1999). Digital images, obtained by phosphorimaging, were quantified using Seescan Densitometry software (LabLogic Ltd, Sheffield, UK).

In Vivo Studies in GF120918-Treated Rats. A catheter was surgically placed into the femoral vein for collection of serial blood samples in Male Han Wistar rats and the animals allowed to recover after catheterization for a minimum of 24 hours. The oral dose suspensions for lapatinib and GF120918 were formulated fresh daily in a vehicle of 0.5% hydroxypropyl methyl cellulose and 0.1% Tween 80 at target concentrations of 2 mg/mL for lapatinib and 10 mg/mL for GF120918. Animals (245 to 300 grams) were pretreated with a single oral dose of vehicle or 50

mg/kg GF120918 followed 2 hours later by a single oral 10 mg/kg dose of lapatinib. Blood was collected in EDTA-containing tubes at 0.5, 1, 2, 4, 6, 12 and 24 hours and plasma isolated by centrifugation. Concentrations of lapatinib were determined in plasma using LC/MS.

In Vivo Studies in mdr1a/1b(-/-) Mice. Male wild-type FVB and Pgp-deficient mice weighing between 20 to 30 grams were purchased from Taconic Biotechnology, Germantown, NY. The dose solution of $[^{14}C]$-lapatinib was prepared on the day of use in a vehicle of 10% sulfobutyl ether-β cyclodextrin at a target concentration of 0.2 or 2 mg/mL. Mice received a single intravenous bolus (5 mL/kg) of $[^{14}C]$-lapatinib administered at a dose of 1 (100 μCi/kg) or 10 mg/kg (1000 μCi/kg). Blood and brain samples were collected at 0.5 and 2 hours. Blood samples were collected into tubes containing EDTA and plasma isolated by centrifugation. Radioactivity associated with plasma was measured by LSC.

Brains (without cerebellum) were weighed and homogenized by sonication in an equal volume to weight of water. Radioactivity associated with brain homogenate was measured by LSC. To confirm that the majority of radioactivity detected in plasma and brain homogenate was lapatinib, radiochemical profiling of a pooled group sample (n= 3 to 4 animals) was completed. Plasma (48 μL total) and brain homogenate (240 μL total) proteins were precipitated by the addition of four volumes of acetonitrile and precipitant removed by centrifugation. The resulting supernatants were evaporated to dryness and reconstituted in 1 part 70:30 methanol:acetonitrile and 3 parts 0.1% formic acid. Identification of the major peak was performed using LC/radio-LC/MS.

LC/MS Analysis.

General. The concentrations of lapatinib in standards and samples was determined by high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) using $[^{13}C_2, {}^2H_3, {}^{15}N]$-lapatinib. HPLC was performed using a Shimadzu LC-10A or Aglilent HP1100 system. Samples were analyzed by positive ion turbo ionspray LC/MS/MS with a PE/Sciex API 3000 or API 356. Raw data were analyzed with PE/Sciex Analyst 1.1 software. SMS 2000 (versions 1.2.7 to 1.4.0, GlaxoSmithKline) was used to calculate peak area ratios and to construct the calibration line from which concentrations of unknowns were interpolated.

For non-radioactive in vivo samples, the chromatographic separation of lapatinib was performed using dual turbulent flow columns (Cohesive Turbo-C18 50 x 1 mm i.d.) and a Waters Symmetry C_{18} 50 x 2.1 mm i.d. analytical column. The Turbulent flow mobile phases consisted of two solvents: A) 10% acetonitrile and 90% 5 mM ammonium acetate, and B) 100% acetonitrile. The analytical mobile phase was 80% acetonitrile and 20% 5 mM ammonium acetate at a flow rate of 0.3 ml/min using a gradient profile.

For analysis of $[^{14}C]$-lapatinib and metabolites in mouse plasma and brain samples, the HPLC system was coupled with a Packard Flo One Radiomatic 500TR online radiodetector. Chromatography was performed using a Waters Symmetry C_{18} 4.6 x 150 mm, 5μm analytical column held at a temperature of 45°C. The mobile phases consisted of two solvents: A) water:formic acid, 99.9:0.1 (v/v), and B) methanol:acetonitrile:10mM ammonium acetate pH=4.5, 72:23:5 (v/v/v) at a flow rate of 1.0 ml/min using a gradient profile.

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Calculations.

For monolayer efflux studies, the transport rate of lapatinib and probe substrates was calculated using the following equation:

$$J = \frac{V(\mathrm{d}C/\mathrm{d}t)}{A}$$

where J is the transport rate (nmol/cm^2/h), V is the receptor volume (ml), C is the receiver drug concentration (nmol/ml), t is time in hours, and A is the membrane surface area (cm^2).

The passive permeability of lapatinib, probe substrates and Lucifer yellow in the presence of GF120918 was determined using the following equation as described previously (Rautio et al., 2006):

$$P_{7.4} = -\left(\frac{V_D V_R}{(V_D + V_R)At} \right) \ln\left\{ 1 - \frac{(V_D + V_R)C_R(t)}{(V_D C_D(t) + V_R C_R(t))} \right\} \times 10^7 \text{ nm/s}$$

where $P_{7.4}$ is the permeability coefficient at pH 7.4, V_D, V_R are donor and receiver well volumes, respectively (mL), A is the membrane surface area (cm^2), t is the incubation time (seconds), $C_R(t)$ is the measured concentration in the receiver well at time t (nmol/ml), $C_D(t)$ is the measured concentration in the donor well at time t (nmol/ml).

For Pgp and BCRP efflux inhibition and OATP uptake inhibition studies, the IC$_{50}$ values (the concentration of inhibitor required for 50% inhibition of the monlayer transport or cellular uptake rates) were calculated with GraFit (version 5.06, Erithacus Software Limited, London, UK) using:

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$$y = \frac{Range}{1 + \left(\dfrac{x}{IC_{50}}\right)^{s}} + \text{background}$$

where y = the rate of transport or uptake of an appropriate probe (expressed as a percentage of the uninhibited control), $Range$ = the rate of probe in the absence of test compound minus the background transport rate, s = is the slope factor, x = the inhibitor concentration (μM), background = the uninhibited rate of probe transport (expressed as a percentage of the total rate).

Pharmacokinetic Analysis. Non-compartmental pharmacokinetic parameters (AUC, T_{max}, C_{max}, and $t_{1/2}$) were obtained from the blood concentration-time profiles using WinNonlin, Enterprise Version 3.1 (Pharsight, Cary NC).

Results

<u>Pgp and BCRP Substrate Assays</u>. To determine whether [^{14}C]-lapatinib is a substrate for human

Pgp or BCRP, in vitro bidirectional transport across MDCKII monolayers expressing these

transporters was studied. In addition, the passive permeability was determined by inclusion of

GF120918, a Pgp and BCRP inhibitor. The efflux ratio for [^{14}C]-lapatinib across the MDCKII-

MDR1 monolayers was 15 and decreased to 0.33 in the presence of GF120918, consistent with

lapatinib being a substrate for Pgp (Table 1). The in vitro passive membrane permeability of

[^{14}C]-lapatinib was 11 nm/s ($P_{7.4}$ B→A + GF120918). For BCRP, the efflux ratio of [^{14}C]-

lapatinib across the MDCKII-BCRP monolayers was 2.6 and decreased to 1.0 in the presence of

GF120918, consistent with lapatinib being a substrate for BCRP. The passive membrane

permeability was 2.7 nm/s ($P_{7.4}$ B→A + GF120918).

<u>Pgp and BCRP Inhibition Assays</u>. The inhibition of Pgp (concentration range 0.3 to 50 µM) and

BCRP (concentration range 0.01 to 3 µM) by lapatinib was assessed by determining the B→A

transport of [^{3}H]-digoxin and [^{3}H]-cimetidine, respectively, across MDCKII-MDR1 or MDCKII-

BCRP monolayers. Lapatinib decreased the Pgp-mediated transport of [^{3}H]-digoxin up to 74%,

yielding an IC_{50} value of 3.9 µM (Table 2 and Figure 1A). Similarly, lapatinib decreased the

BCRP-mediated transport of [^{3}H]-cimetidine up to 82%, yielding an IC_{50} value of 0.025 µM

(Table 2 and Figure 1B).

<u>OATP1B1, OAT, OCT and URAT Inhibition Assays.</u> The inhibition of the OATP1B1 uptake

transporter by lapatinib (concentration range 0.01 to 30 µM) was investigated by determining the

uptake of [^{3}H]-estradiol 17β-D-glucuronide ([^{3}H]-EG) in the CHO-OATP1B1 cell line.

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Lapatinib reduced the uptake of [^{3}H]-EG by OATP1B1 by 70%, yielding an IC$_{50}$ value of 4.0 μM (Table 2 and Figure 1C).

The inhibitory effect of lapatinib on a panel of human renal transporters was investigated in S2 cells stably expressing organic anion transporter 1 (OAT1), OAT2, OAT3, or OAT4, the organic cation transporter 1 (OCT1), OCT2 (isoform a), OCT2-A (isoform b) or OCT3, and HEK293 cells expressing urate transporter 1 (URAT1). For each transporter, transfected cells were incubated with the radiolabeled substrate in the absence or presence of 30 μM of lapatinib (Table 3). Lapatinib reduced the uptake of [^{3}H]-estrone sulfate via OAT3 to 40.2% of the control activity, while 30 μM probenecid (the positive control inhibitor) reduced the uptake of [^{3}H]-estrone sulfate to 12.9% of the control activity. In contrast, the uptake of probe substrates for OAT1, OAT2, OAT4, OCT1, OCT2, OCT2-A, OCT3 and URAT1 were all greater than 62% in the presence of 30 μM of lapatinib, demonstrating little to no inhibition (Table 3).

Whole-body Autoradiography in Rats. The tissue distribution of [^{14}C]-lapatinib was determined in male rats by using whole-body autoradiography at 0.5, 4, 8, 24 and 168 hours after oral administration. The absorption of radioactivity following a single oral dose of 10 mg/kg [^{14}C]-lapatinib yielded low tissue radioactivity concentrations relative to the dose (Figure 2); high levels of radioactivity were associated with the gastrointestinal tract. However, the radioactivity that was absorbed was widely distributed into tissues with the exception of brain and was cleared from most tissues by 24 hours post dose, mainly by biliary elimination (Table 4). Tissues with the highest radioactivity included the liver, kidney, and harderian gland. Only low levels of radioactivity were detected in the central nervous system (CNS) at any time (brain-to-plasma

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ratios <0.13). In contrast to brain parenchyma, cerebrospinal fluid contained measurable levels of radioactivity. Using muscle as a reference tissue (Polli et al., 1999), the brain-to-muscle ratio was low (0.15 to 0.2) demonstrating that the low CNS concentrations were not due to lapatinib's inability to partition into tissues; therefore, another mechanism must account for the limited brain concentrations. Finally, the harderian gland, which expresses high levels of BCRP, had tissue-to-plasma ratios of 0.31 to 3.71 demonstrating that [^{14}C]-lapatinib was able to distribute into this tissue.

Effect of Efflux Transporters on the Systemic Exposure of Lapatinib. To initially evaluate the influence of efflux transporters (e.g., Pgp and BCRP) on the systemic exposure of lapatinib, a GF120918-treated rat model was selected. This model offers several advantages over mouse models such as the ability to inhibit multiple efflux transporters at one time, a serial blood collection design for pharmacokinetic calculation, requirement of very few animals (n=6), reduced cost and less time to complete the study. Rats were pretreated with vehicle (0.5% hydroxypropyl methyl cellulose and 0.1% Tween 80) or a single oral dose of 50 mg/kg GF120918 followed 2 hours later by a single oral 10 mg/kg dose of lapatinib. The systemic pharmacokinetics (AUC, C_{max} and T_{max}) of lapatinib in the GF120918 pretreated animals were similar to the vehicle treated animals (Table 5), suggesting that efflux transporter have a limited role in the intestinal absorption of lapatinib.

Effect of Efflux Transporters on the CNS Exposure of Lapatinib. A study was conducted to investigate the influence of Pgp on the CNS penetration of [^{14}C]-lapatinib in male wild-type (FVB) and Pgp-deficient (mdr1a/1b-/-) mice. Blood and brain samples were collected at 0.5 and

2 hours after either a 1 or 10 mg/kg intravenous dose of [^{14}C]-lapatinib. LC radio-chromatographic profiling and LC/MS of a pooled sample demonstrated that 73 to 84% (0.5 hours) and 77 to 99% (2 hours) of radioactivity in plasma was [^{14}C]-lapatinib, and that 92 to 99% (0.5 hours) and 89 to 99% (2 hours) of radioactivity in brain was [^{14}C]-lapatinib. After intravenous dosing, there were no notable differences in the plasma concentrations of [^{14}C]-lapatinib between Pgp-deficient and wild-type FVB mice at either time point or dose level (Table 6); this observation is consistent with the study completed with GF120918 showing no influence of Pgp on systemic exposure. The mean brain-to-plasma ratios of radioactivity at 0.5 and 2 hours in wild-type FVB mice given a 1 mg/kg dose were 0.04 and 0.09, demonstrating low CNS exposure of lapatinib; these results are in agreement with the WBA data (Figure 2). In contrast, the mean brain-to-plasma ratios at 0.5 and 2 hours in wild-type FVB mice given a 10 mg/kg dose were 0.22 and 0.25, demonstrating that the entry of [^{14}C]-lapatinib into the brain after an intravenous bolus was sensitive to dose. In this instance, the 10-fold change in dose yielded a 2.7- to 5.0-fold change in brain-to-plasma ratios, suggesting inhibition of an efflux pathway(s) with increasing intravenous doses of lapatinib.

In Pgp-deficient mice given a 1 mg/kg dose, the mean brain-to-plasma ratios of [^{14}C]-lapatinib at 0.5 and 2 hours were 0.24 and 0.56. At a dose of 10 mg/kg, the mean brain-to-plasma ratios at 0.5 and 2 hrs were 0.54 and 0.94. The brain-to-plasma ratios in Pgp-deficient mice were 2.6- to 6.4-fold greater than the brain-to-blood ratios in wild-type mice. Further, the brain-to-plasma ratios of [^{14}C]-lapatinib in Pgp-deficient mice remained less than 1.0, suggesting that other efflux pathways or mechanisms influencing lapatinib CNS distribution may be present.

Discussion

Tyrosine kinase inhibitors are a new class of anti-cancer drugs (Rowinsky, 2004). A number of small molecule TKIs have been approved, including lapatinib (Tykerb, GW572016), imatinib (Gleevec; STI571), sunitinib (Sutent; SU11248), erlotinib (Tarceva; OSI-774), gefitinib (Iressa; ZD1839), dasatinib (Sprycel, BMS-354825) and sorafenib (Nexavar, Bay 43-9006). Lapatinib is a novel member of the 4-anilinoquinazoline class of TKIs approved for the treatment of breast cancer (Johnston and Leary, 2006). The unique feature of lapatinib is that it is an inhibitor of both EGFR1 and HER2 with in vitro IC_{50} values near 10 nM. In addition, as a large (molecular weight = 581) and lipophilic (clogP = 5.1) molecule, lapatinib is typical of drugs that interact with ABC efflux and SLC transporters (Chang et al., 2006). It was therefore of interest to investigate the role of these transporters in lapatinib's disposition and drug interactions.

Lapatinib was a substrate for Pgp and BCRP, as well as an inhibitor of Pgp, BCRP and OATP (IC_{50} values of 3.9, 0.025 and 4.0 μM, respectively). These IC_{50} values are within the range of peak plasma concentrations following a 1250 mg daily doses (4.2 μM or 2432 ng/mL) suggesting a potential for transporter-mediated drug interactions. However, lapatinib also exhibits extensive plasma protein binding (>99%), which could diminish this potential. In contrast to the inhibition of ABC transporters, lapatinib displayed little to no inhibition of OAT, OCT and URAT transporters. These observations are consistent with lapatinib's physicochemical properties and the pharmacophores for these transporters (Chang et al., 2006). Further, lapatinib undergoes negligible renal elimination (<5% of the dose) in rats, dogs and humans (GSK Data on file). Therefore, the potential for pharmacokinetic interactions between

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lapatinib and drugs that are substrates or inhibitors of OAT, OCT and URAT transporters is predicted to be low.

Currently, there are few reports describing the interactions of other TKIs with drug transporters. Imatinib and gefitinib are substrates and inhibitors of Pgp and BCRP (Ozvegy-Laczka et al, 2004). Gefitinib inhibited Pgp-mediated transport of calcein-AM with an IC_{50} of 3.05 μM and BCRP-mediated transport of Hoechst 3342 with an IC_{50} of 0.29 μM (Ozvegy-Laczka et al, 2004; Leggas et al, 2006). Imatinib inhibited Pgp-mediated transport of calcein-AM with a Ki value of 18.3 μM and BCRP-mediated transport of Hoechst 3342 with a Ki value of 0.9 μM (Ozvegy-Laczka et al, 2004; Hamada et al., 2003). Intracellular concentrations of imatinib have also been shown to be influenced by OCT1 (White et al., 2006). Little information on the interaction of other TKIs such as sunitinib, erlotinib, dasatinib, or sorafenib with ABC or SLC transporters has yet been published.

This characterization of the interaction of lapatinib with transporters, together with other preclinical data, is a first step towards understanding 1) the impact of transporters on the efficacy, toxicity and pharmacokinetics of lapatinib and 2) the involvement of transporters in drug interactions between lapatinib and other anti-cancer agents. This is especially relevant in oncology where tumor penetration and drug combinations are essential for successful therapy. Previous studies in mouse xenografts indicated 5-fold higher concentrations of lapatinib in tumor compared to plasma (Clark et al, 2002). Although the presence and function of transporters was not determined in that experiment, it is possible, based on data presented in this report, that lapatinib could enhance its own distribution into tumors by inhibiting efflux.

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Drug transporters have also been implicated in TKI-associated toxicities. The ABCG2 C421A genotype is associated with diarrhea induced by gefitinib, independent of any change in pharmacokinetics (Cusatis et al, 2006). Phase I clinical trials have shown that lapatinib is well tolerated, with diarrhea and rash being the most frequent toxicities (Johnston and Leary, 2006; Bence et al., 2005). To date, no relationship has been clearly demonstrated between ABCB1 or ABCG2 genotype and the pharmacokinetics or toxicity of lapatinib (Zaks et al., 2006).

Intestinal efflux transporters can attenuate the bioavailability of substrates with limited passive permeability, such as lapatinib. In Phase I trials, patients receiving concomitant medications capable of inhibiting Pgp (sertraline, paroxetine) or BCRP (omeprazole, folic acid) displayed no alteration in systemic exposure to lapatinib (data on file at GSK). This suggests that lapatinib systemic exposure is not significantly impeded by intestinal efflux transporters, despite its low/moderate permeability, and is consistent with the finding that GF120918 had no effect on lapatinib disposition in rats. However, recently it has been suggested that the food effect on lapatinib bioavailability in cancer patients may involve inhibition of efflux transporters (Reddy et al, 2007). An alternate explanation for the food effect is enhanced solubility of lapatinib with food leading to increased absorption.

A particular challenge to the treatment of breast cancer, particularly HER2 over-expressing tumors, is CNS metastases. Up to 35% of patients with HER2-positive advanced breast cancer relapse due to intracranial disease despite control of the peripheral tumors (Weil et al., 2005). CNS tumors are difficult to treat due to limited brain and/or tumor exposure of most anti-cancer

agents. The preclinical data presented here suggest that brain concentrations of lapatinib are low in normal, healthy animals due to efflux transporters in the blood-brain barrier. Like lapatinib, gefitinib and imatinib also display low CNS penetration due to efflux transporters (Breedveld et al., 2005; Heimberger et al., 2002; Kil et al., 2007). These preclinical data imply that TKIs may have limited utility for the treatment of brain tumors in humans. However, both lapatinib and gefitinib have been shown in clinical studies to reduce CNS tumor growth (Geyer et al., 2006; Roggero et al., 2005; Peck, 2006). It is possible that disruption of the BBB by tumors circumvents the usual protective function of transporters or that inhibition of efflux by these agents enhances their own accumulation upon repeat dosing.   Clinical and preclinical investigations of the accumulation and efficacy of lapatinib in CNS tumors are ongoing to address this critical issue.

A second key aspect of the information presented here is to better anticipate the potential for drug interactions between lapatinib and other anti-cancer agents, resulting in altered pharmacokinetics, efficacy or toxicity. The concept of inhibiting efflux transporters to enhance oral delivery is well-established (Breedveld et al., 2006). Co-administration with lapatinib could conceivably enhance the systemic exposure of other anti-cancer agents by inhibiting gastrointestinal efflux or biliary/renal excretion.   The clinical utility of such interactions is currently under investigation with agents such topotecan, a BCRP substrate (Molina et al., 2007).

When used in combination, the therapeutic index of cytotoxic agents can be altered by TKI-mediated inhibition of efflux transporters, which can provide a resistance mechanism in tumor cells and a protective mechanism in sensitive normal cells. This is illustrated by studies with

docetaxel and paclitaxel, two efflux transporter substrates that are important first line treatments for breast cancer. In a Phase I study (EGF10021) that examined the combination of lapatinib given daily plus docetaxel given once every 3 weeks, neutropenia produced by docetaxel was observed at doses lower than those generally seen when docetaxel is given alone. Despite the potential for lapatinib to inhibit Pgp-mediated transport and CYP3A4-dependent metabolism of docetaxel, no increase in systemic exposure of this taxane was observed. This is consistent with reports that, following intravenous administration, docetaxel pharmacokinetics are not greatly influenced by more potent Pgp inhibitors such as MS209 and zosuquidar (Fracasso et al., 2004; Dieras et al., 2005). However, potent CYP3A4 inhibitors, such as ketoconazole, reduced docetaxel clearance requiring a dose adjustment (Engles et al., 2004). Therefore, the enhanced neutropenia of the lapatinib-docetaxel combination is currently hypothesized to be attributed to inhibition of efflux transporters in blood cell progenitors and not the result of a pharmacokinetic drug interaction.

In another Phase I study (EGF10009) (Jones et al., 2004) that examined the combination of lapatinib given daily and paclitaxel given every 3 weeks, the duration of peripheral neuropathy produced by paclitaxel appeared to be affected by the concentration of lapatinib. Previous reports have associated this toxicity of paclitaxel with reduced efflux of drug from neurons by Pgp (Sissung et al., 2006). These observations suggest that lapatinib may have inhibited Pgp-mediated efflux of paclitaxel from this sensitive normal cell type. However, combined administration of these agents also produced clinical activity in patients who had failed prior treatment with taxanes. The potential for synergy with this combination awaits further examination in larger clinical trials.

In conclusion, lapatinib interacts with a number of ABC and SLC transporters shown to influence treatment outcome by altering tumor resistance and the disposition/toxicity profile for a number of anti-cancer drugs. These studies, in conjunction with other research, provide a mechanistic basis for the role of efflux and uptake transporters in lapatinib disposition and drug interactions. Studies are ongoing to further clarify the clinical importance of transporters on the efficacy, disposition, toxicity and drug interactions of lapatinib.

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Legends for Figure:

Figure 1. Inhibition of Pgp, BCRP and OATP1B1 by lapatinib. The inhibition of Pgp (A) and BCRP (B) by lapatinib was assessed by determining the B→A transport of [^{3}H]-digoxin and [^{3}H]-cimetidine, respectively, across MDCKII-MDR1 or MDCKII-BCRP monolayers. (C) The inhibition of the OATP1B1 by lapatinib was investigated by determining the uptake of [^{3}H]-estradiol 17β-D-glucuronide ([^{3}H]-EG) in the CHO-OATP1B1 cell line.

Figure 2. Whole-body autoradiogram of a male rat 4 hours after a single oral administration of [^{14}C]-lapatinib at a dose of 10 mg /kg in 0.5% (w/v) aqueous hydroxypropyl methyl cellulose containing 0.1% (v/v) Tween 80. Tissue processing and image analysis were completed as described (Polli et al., 1999) and digital images were obtained by phosphorimaging. Abbreviations: bf- brown fat; sc- spinal cord; lv-liver; th- thymus; sg- salivary gland; lma-mandibular lymph nodes; ed- epdidymis; bug- bulbo-urethral gland; re- rectum; bdr- bladder.

Table 1: Results of Transport Studies for 3 μM [^{14}C]-Lapatinib in MDCKII-MDR1 or MDCKII-BCRP Cell Monolayers

MDCKII Cell Line [a]	GF120918 [b]	Rate A→B (pmoles/min/cm^2)	Rate B→A (pmoles/min/cm^2)	Apical Efflux Ratio	A→B Mass Balance (%)	B→A Mass Balance (%)	$P_{7.4}$ (nm/s)
MDR1	-	0.06 ± 0.01	0.98 ± 0.06	15	85 ± 5.9	88 ± 0.2	
MDR1	+	0.61 ± 0.03	0.20 ± 0.01	0.33	82 ± 5.3	100 ± 1.0	11 ± 0.2
BCRP	-	0.027 ± 0.004	0.071 ± 0.002	2.6	92 ± 4.7	95 ± 2.7	
BCRP	+	0.046 ± 0.008	0.047 ± 0.001	1.0	90 ± 5.4	97 ± 3.8	2.7 ± 0.2

[a] Data is the mean ± standard deviation from three monolayers over 90 min for MDR1 and 180 min for BCRP expressing MDCKII monolayers.

All donor compartments contained Lucifer yellow (100 μM) to determine monolayer integrity. Amprenavir served as a positive control for Pgp efflux and cimetidine for BCRP efflux. The measured radiochemical purity of [^{14}C]-lapatinib was >97% and no metabolic or chemical degradation was detected during the studies.

[b] GF120918 was used in both donor and receiver compartments at 2 μM for Pgp and 5 μM for BCRP.

Table 2. Inhibition of Human ABC Transporters by Lapatinib.

Transporter	IC$_{50}$ (μM)	Substrate
Pgp	3.9 (0.6) [a]	[^{3}H]-digoxin
BCRP	0.025 (0.003)	[^{3}H]-cimetidine
OATP1B1	4.0 (2.1)	[^{3}H]-estradiol 17β-D-glucuronide

[a] Data is the mean (± standard deviation) from triplicate well.

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Table 3. Inhibition of Human Solute Carriers by 30 μM Lapatinib.

Transporter	% Control Activity [a]	Substrate
OAT 1	78.7 (2.2)	$[^{14}C]$-p-aminohippuric acid
OAT 2	86.4 (2.1)	$[^{3}H]$-prostaglandin F2α
OAT 3	40.2 (0.3)	$[^{3}H]$-estrone sulfate
OAT 4	119 (3.5)	$[^{3}H]$-estrone sulfate
OCT 1	62.1 (1.1)	$[^{14}C]$-tetraethylammonium
OCT 2	88.4 (4.6)	$[^{14}C]$-tetraethylammonium
OCT 2A	70.3 (3.5)	$[^{14}C]$-tetraethylammonium
OCT 3	113 (4.2)	$[^{3}H]$-histamine
URAT1	79.8 (2.1)	$[^{14}C]$-uric acid

[a] Data is the mean (± standard deviation) from triplicate well from a single concentration screening experiment.

Table 4. Mean Tissue Concentrations and Tissue-to-Blood Ratios of Radioactivity in Male Rats After A Single Oral Administration of 10 mg/kg [^{14}C]-Lapatinib.

Tissue	Concentration (µg equivalents/gm tissue)				Tissue-to-Blood Ratio			
	0.5 hr	4 hr	8 hr	24 hr	0.5 hr	4 hr	8 hr	24 hr
Blood	0.26	0.92	0.31	BLQ	1.00	1.00	1.00	NC
Brain	BQL	0.09	0.04	BQL	NC	0.10	0.13	NC
Cerebrospinal Fluid	0.03	0.36	0.29	BQL	0.12	0.39	0.94	NC
Harderian Gland	0.08	1.22	1.15	0.62	0.31	1.33	3.71	NC
Heart	0.21	1.13	0.54	BQL	0.77	1.23	1.74	NC
Kidney	0.71	3.40	1.77	0.16	2.73	3.70	5.71	NC
Liver	1.51	5.55	2.47	0.22	5.81	6.03	7.97	NC
Muscle	0.03	0.46	0.26	BQL	0.12	0.50	0.84	NC

Abbreviations: BLQ = below limit of quantification; NC = not calculated

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Table 5. Pharmacokinetic Parameters of Lapatinib after a Single Oral Dose of 10 mg/kg to Male Rats 2 hours after

Pretreatment With a Single Oral Dose of Vehicle or 50 mg/kg GF120918.

Predose Treatment	AUC$_{(0-all)}$ (ng.h/mL)	Cmax (ng/mL)	Tmax (h)	t½ (h)
Vehicle	2094 (644) [a]	725 (227)	0.88 [0.5-1.0]	1.08 (0.06)
GF120918 (50 mg/kg) [b]	2152 (1405)	533 (493)	0.75 [0.5-1.0]	2.78 (1.14)

[a] Data are the mean (± standard deviation) from four male animals, except for Tmax which represents the range.

[b] GF120918 treatment consisted of a single oral 50 mg/kg dose given 2 hours prior to lapatinib dosing.

Table 6. Brain-to-Plasma Ratios of [^{14}C]-Lapatinib after a Single Intravenous Dose of 1 or 10 mg/kg to Male Wild-Type and Pgp-Deficient Mice
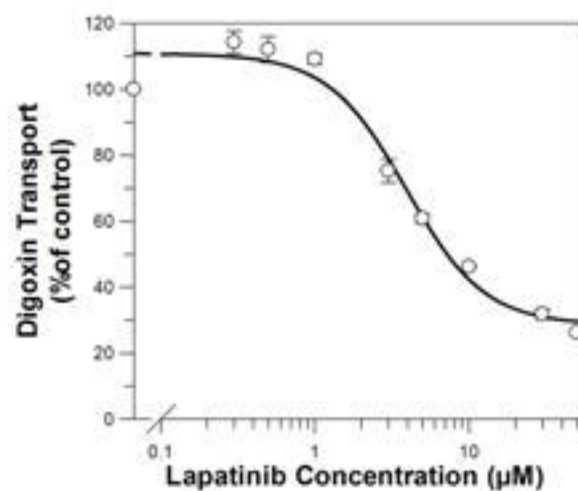
Dose (mg/kg)	Time (hr)	Wild-Type FVB Mice			Mdr1a/1b(-/-) Mice			KO/WT Brain-to-Plasma Ratio
		Plasma Concentrations (ng/mL) [a]	Brain Concentrations (ng/gm)	Average Brain-to-Plasma Ratio	Plasma Concentrations (ng/mL)	Brain Concentrations (ng/gm)	Average Brain-to-Plasma Ratio [1]	
1	0.5	312 (35)	13.9 (3.7)	0.04 (0.01)	429 (16)	102 (11)	0.24 (0.03)	5.53
	2	68.9 (13.4)	6.26 (2.62)	0.09 (0.02)	76.3 (14.6)	41.9 (7.9)	0.56 (0.15)	6.36
10	0.5	4559 (745)	1012 (116)	0.22 (0.06)	4916 (314)	2640 (239)	0.54 (0.06)	2.63
	2	1869 [b] (1502, 2235)	463 (332,594)	0.25 (0.22,0.27)	1871 (414)	1762 (595)	0.94 (0.20)	2.86

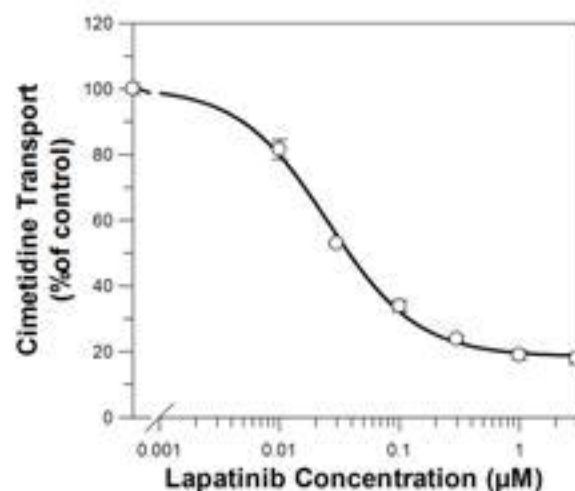[a] Data are the mean (± standard deviation) from three male animals.

[b] Data are the mean and range from two male animals due to a miss dose of one animal.
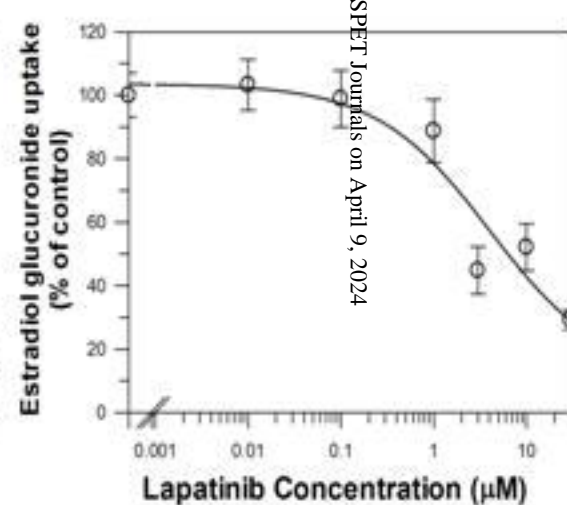
Figure 1

A.

B.

C.

Figure 2.