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**An Unexpected Synergist Role of P-glycoprotein and Breast Cancer Resistance Protein on  
the CNS Penetration of the Tyrosine Kinase Inhibitor Lapatinib (GW572016).**

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DMD #24646

Running Title: Lapatinib CNS Disposition in *Mdr1/Bcrp1* Triple Knockout Mice

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Nonstandard abbreviations: 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; *Mdr1*, gene encoding the Pgp protein; Pgp, P-glycoprotein; *Bcrp1*, gene encoding Bcrp protein; Bcrp1, Breast cancer resistance protein;  $C_{ss}$ , concentration at steady state; i.v., intravenous;  $t_{1/2}$ , plasma half-life;  $C_{max}$ , maximum plasma concentration;  $C_{(0)}$ , extrapolated estimate of plasma concentration at end of bolus dose; CNS, central nervous system; ABC, ATP Binding Cassette; HER2, human epidermal receptor 2

DMD #24646

## Abstract

Lapatinib is a tyrosine kinase inhibitor approved for use in combination with capecitabine to treat advanced or metastatic breast cancers overexpressing human epidermal receptor 2 (HER2, ErbB2). This work investigated the role of P-glycoprotein (Pgp, the protein from the *Mdr1a/b* gene) and Breast cancer resistance protein (Bcrp, the protein from the *Bcrp1* gene) in modulating the CNS penetration of lapatinib at steady state conditions in FVBn mice (wild-type), *Mdr1a/b*(-/-), *Bcrp1*(-/-) and *Mdr1a/b*(-/-)/*Bcrp1*(-/-) knockout mice. Following an intravenous infusion of lapatinib for 24 hours to a targeted steady state plasma concentration of 700 ng/mL (0.3 mg/kg/h) or 7000 ng/mL (3 mg/kg/h), lapatinib brain-to-plasma ratios were approximately 3- to 4-fold higher in *Mdr1a/b*(-/-) knockout mice (ratio range 0.09 to 0.16) compared to wild-type mice (ratio range 0.03 to 0.04). There was no difference in the brain-to-plasma ratio in the *Bcrp1*(-/-) knockout mice (ratio range 0.03 to 0.04) compared to wild-type mice. In contrast, *Mdr1a/b*(-/-)/*Bcrp1*(-/-) triple knockout mice had a 40-fold higher brain-to-plasma ratio (ratio range 1.2 to 1.7), suggesting that Pgp and Bcrp work in concert to limit the brain-to-plasma ratio of lapatinib in mice. This finding has important potential consequences for the treatment of brain tumors in breast cancer patients treated with tyrosine kinase inhibitors as well as the basic understanding of ABC transporters expressed in the blood-brain barrier on the central nervous system disposition of drugs.

DMD #24646

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 epidermal growth factor receptor (EGFR; ErbB) 1 and human epidermal receptor (HER) 2 with IC<sub>50</sub> values of approximately 10 nM against the purified receptors in vitro, and potently inhibits growth of EGFR and/or HER2 overexpressing tumors both in vitro and in vivo. Lapatinib is approved for use in combination with other anti-cancer agents for the treatment of HER2-positive breast cancers (Geyer et al., 2006).

A particular challenge to the treatment of breast cancer, particularly HER2 overexpressing tumors, is central nervous system (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. Non-clinical data suggest that the brain concentrations of lapatinib and other TKIs are low due to efflux transporters in the blood-brain barrier (Breedveld et al., 2005; Heimberger et al., 2002; Kil et al., 2007; Polli et al., 2008). These non-clinical 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; Lin et al., 2008). 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 (de Vries et al., 2006).

The objective of this study was to establish the extent of attenuation of CNS penetration of

DMD #24646

lapatinib by P-glycoprotein (Pgp) and Breast Cancer Resistance Protein 1 (Bcrp1), which are ATP dependent efflux transporter proteins localized in the apical membrane of the blood-brain barrier. The mouse is an ideal model, as *Mdr1a/b(-/-)* and *Bcrp1(-/-)* knockout mice are well established to study Pgp and Bcrp1-dependent transport at the blood brain barrier.

## Materials and Methods

**In Vivo Studies in *Mdr1a/1b(-/-)*, *Bcrp1(-/-)* and *Mdr1a/1b(-/-)/Bcrp1(-/-)* Mice.** Procedures in this study were conducted in accordance with the US Animal Welfare Act, as amended in 1990 and the protocol was approved by the Institutional Animal Care and Use Committee. Jugular cannulated male FVBn (wild-type), *Mdr1a/b(-/-)*, *Bcrp1(-/-)* and *Mdr1a/b(-/-)/Bcrp1(-/-)* knockout mice were supplied by Taconic Farms (Germantown, NY). The mice weighed between 19 and 26 g at the start of the study. On arrival day, the jugular cannula was exteriorized and connected to a tether system that continuously infused saline (0.06 mL/h) to ensure cannula patency. Mice were fed LabDiet™ brand Certified Rodent Diet 5002 (PMI™ Nutrition International, Richmond, IN, USA) ad libitum, with free access to water. Temperature and humidity were maintained at  $70 \pm 10^\circ\text{F}$  and  $55 \pm 30\%$ , respectively and a 12-hour light/12-hour dark cycle was maintained.

The dose solution of lapatinib was prepared on the day of use in a vehicle of 10% sulfobutyl ether- $\beta$  cyclodextrin at a concentration of 0.15 or 1.5 mg/mL. Preliminary studies in wild-type mice indicated that the terminal plasma  $t_{1/2}$  of lapatinib following a single 10 mg/kg intravenous (i.v.) bolus dose ranged from about 4 to 6 hours, and  $C_{\max}$  and  $C_{(0)}$  ranged from about 1000 to 5000 ng/mL. A pilot study in wild-type mice confirmed that following constant rate i.v. infusion

DMD #24646

of lapatinib, steady state plasma concentrations of lapatinib were achieved within 16 hours (data not shown). Therefore, a standardized study design that include a 24 hour infusion at a rate of 60  $\mu\text{L/h}$  and a dose of 0.3 or 3.0 mg/kg/h to achieve a targeted steady state plasma concentration ( $C_{ss}$ ) of 700 or 7000 ng/mL was used for all strains of mice. The amount of dose formulation administered to each animal was determined by weighing the loaded dose syringe before and the emptied syringe after dose administration. The steady state concentrations were selected based on the human plasma  $C_{max}$  values (4.2  $\mu\text{M}$  or 2432 ng/mL) following a 1250 mg daily dose (Polli et al., 2008). This plasma concentration is well tolerated in mice. Two different plasma concentrations were included to determine if there is a concentration-dependent effect of lapatinib at the blood brain barrier (e.g., autoinhibition of transporters by lapatinib resulting in enhanced brain entry). After the 24 hour infusion, a single terminal blood sample (1 mL) was drawn from each animal by exsanguination via the vena cava under terminal anaesthesia and was transferred into tubes containing EDTA as the anticoagulant. The blood was centrifuged (5000 rpm, at 4°C for 10 min) to obtain plasma. Plasma samples were stored at -80°C until analysis. Brains were removed at the time of sacrifice, rinsed with ice-cold saline, frozen (e.g. solid carbon dioxide) and stored at -80°C until analysis.

### **LC/MS/MS Analysis.**

The concentration of lapatinib in mouse plasma and brain homogenate was determined by high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) using validated methods. Prior to analysis, mouse brains were weighed and homogenized with an equal volume of water. Plasma and brain homogenate samples were then extracted by protein precipitation using a solution of acetonitrile containing stable isotopically labeled lapatinib

DMD #24646

([<sup>2</sup>H<sub>3</sub><sup>15</sup>N<sup>13</sup>C<sub>2</sub>]-GW572016) as an internal standard. HPLC was performed by using a Shimadzu LC-10A VP HPLC. Chromatographic separation was achieved by using a mixture of aqueous 5 or 10mM ammonium acetate:acetonitrile on a Waters XBridge C<sub>18</sub> HPLC column. Samples were analyzed in positive mode by turbo ion spray MS/MS with an Applied Biosystems/MDS Sciex API 5000 (mouse brain homogenate) or an Applied Biosystems/MDS Sciex API 3000 (mouse plasma). The calibration range was 10 to 10000 ng/mL in mouse plasma and 1 to 1000 ng/mL in mouse brain homogenate. Data were integrated by using Applied Biosystems/MDS Sciex software Analyst v 1.4.1. SMS2000 (version 2.0, GlaxoSmithKline) was used to calculate peak area ratios (i.e., analyte/internal standard peak area ratios versus analyte concentration were constructed and a weighted  $1/x^2$  linear regression applied to the data) to construct the calibration lines from which the concentration of lapatinib in the study samples was determined. Descriptive statistics (mean, standard deviation) was performed on the data. Brain homogenate concentrations were converted to brain concentrations for calculations. Brain-to-plasma ratios between the groups were determined and compared.

**Pharmacokinetic and Statistical Analysis.** Plasma clearance (CL<sub>p</sub>) of lapatinib was calculated by using the equation  $CL = R(0)/C_{ss}$  where R(0) is the infusion rate (ng/h) and C<sub>ss</sub> is the plasma concentration at steady state (ng/mL). The CL<sub>p</sub> value obtained (mL/h) was then normalized to body weight and is reported as mL/min/kg. Mean, standard deviation, and Student's non-paired t-test calculations were performed by using Microsoft<sup>®</sup>Excel 2002 SP-3 (Microsoft Corporation, Redmond, WA). Values were declared statistically different when  $p \leq 0.05$ .

DMD #24646

## Results and Discussion

We have previously characterized the interaction of lapatinib as a substrate and inhibitor of human Pgp and BCRP transporters (Polli et al., 2008) as part of the effort to understand the impact of transporters on the efficacy, toxicity and distribution of this drug. Non-clinical studies on lapatinib and other TKIs suggest that brain concentrations are low due to efflux transporters in the blood-brain barrier (Polli et al. 2008, Breedveld et al., 2005; Heimberger et al., 2002; Kil et al., 2007), implying 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; Lin et al., 2008). This study was completed to assess the importance of the efflux transporters *Mdr1a/b* and *Bcrp1*, individually and together, on the lapatinib brain-to-plasma ratio under steady-state conditions using *Mdr1a/b(-/-)*, *Bcrp1(-/-)* and *Mdr1a/b(-/-)/Bcrp1(-/-)* knockout mice.

Lapatinib was delivered via i.v. infusion for 24 hours at a rate of 60  $\mu\text{L/h}$  and a dose of 0.3 or 3.0 mg/kg/h to reach a targeted steady state plasma concentration of 700 or 7000 ng/mL, respectively. The mean plasma  $C_{ss}$  of lapatinib achieved in the wild-type strain was  $730 \pm 410$  ng/mL and  $5097 \pm 2729$  ng/mL (Table 1). As these values were similar to the predicted values of 700 and 7000 ng/mL, these infusion conditions were used for the remaining studies with the knockout mice strains. Although this study was not designed to provide an in-depth analysis of impact of *Mdr1a/b* or *Bcrp1* deficiency on the plasma pharmacokinetics of lapatinib, analysis of the clearance values across the knockout strains may provide some insight into the possible role of transporters on the plasma clearance ( $CL_p$ ) of lapatinib. Overall, the mean  $CL_p$  values were moderate in comparison to hepatic plasma flow and ranged from 13 to 31 mL/min/kg. Following

DMD #24646

the low infusion rate (0.3 mg/kg/h), the CL<sub>p</sub> of lapatinib was statistically higher (1.5- to 2.5-fold) in all three knockout strains compared to wild-type mice, and CL<sub>p</sub> was statistically higher (~50%) in *Mdr1a/b(-/-)* mice compared to *Bcrp1(-/-)* mice. There was no difference in CL<sub>p</sub> in *Mdr1a/b(-/-)/Bcrp1(-/-)* triple knockout mice compared to either the *Mdr1a/b(-/-)* or *Bcrp1(-/-)* mice. In contrast, following the high infusion rate of 3 mg/kg/hr, CL<sub>p</sub> was not statistically different in any of the knockout strains compared to the wild type. However, CL<sub>p</sub> was statistically lower (about 25 to 45%) in *Mdr1a/b(-/-)* or *Bcrp1(-/-)* mice compared to the *Mdr1a/b(-/-)/Bcrp1(-/-)* triple knockout mice. As lapatinib is cleared mainly by metabolism in mice (data not shown) and the addition of GF120918 does not change the systemic exposure of an oral dose of lapatinib to rats (Polli et al., 2008), it is not immediately clear why CL<sub>p</sub> was impacted by loss of Pgp and/or Bcrp1 activity. It is possible that the profile of the metabolic enzymes differs across these strains of mice; this has been previously described for these strains (Schuetz et al, 2000). Further characterization of the metabolic and transporter profiles in the knockout strains relative to the wild-type animal is warranted.

Following a constant rate intravenous administration of lapatinib for 24 hours, lapatinib brain and plasma levels were measured by LC/MS/MS. Although there was a statistical difference in the brain-to-plasma ratios in the *Bcrp1(-/-)* mice compared to wild-type mice after the low dose infusion (Table 1), the difference was small. The brain-to-plasma ratios were 0.03 to 0.04 for both mice strains; these ratios are similar to blood contamination of brain tissue. In the *Mdr1a/b(-/-)* mice, there was a modest 3- to 4-fold increase in the brain-to-plasma ratios (0.09 and 0.16 for the 0.3 and 3 mg/kg/h infusion rates, respectively) compared to wild-type mice. Further, there was some evidence of a concentration dependent effect in the *Mdr1a/b(-/-)* mice

DMD #24646

with animals receiving the higher infusion concentration of lapatinib having a 1.80-fold larger brain-to-plasma ratio (0.16 vs. 0.09) compared with the lower infusion concentration. Even with this increased brain entry of lapatinib in the *Mdr1a/b(-/-)* mice, the brain-to-plasma ratio remained well below that of unity, suggesting other mechanisms (e.g., permeability, plasma protein binding, other efflux transporters) influence lapatinib entry into the brain.

A recent study by de Vries et al. (2007) using *Mdr1a/b(-/-)/Bcrp1(-/-)* knockout mice demonstrated that the two transporters work together to limit the plasma exposure and brain penetration of topotecan, a topoisomerase I inhibitor used in the treatment of ovarian, lung and cervical cancers. In that study, the brain-to-plasma ratio of topotecan increased 3.7-fold in the triple knockout mice compared to 2.0-fold increase in the *Mdr1a/b(-/-)* or 0.65-fold increase in the *Bcrp1(-/-)* knockout mice. The authors also noted differences in the plasma AUC values (0.75- to 2.4-fold) and plasma clearance of topotecan in knockout mice compared to wild-type mice, an observation consistent with the differences in clearance values observed in our study with lapatinib. That report, along with the limited individual effects of *Mdr1a/b* and *Bcrp1* efflux on the lapatinib brain-to-plasma ratio, raised interest as to the possible combined effects of a triple *Mdr1a/b* and *Bcrp1* gene knockout on the brain-to-plasma ratio of lapatinib. In contrast to the modest changes observed in *Mdr1a/b(-/-)* and *Bcrp1(-/-)* mice, the lapatinib steady state infusion brain-to-plasma ratio in *Mdr1a/b(-/-)/Bcrp1(-/-)* mice was 40-fold higher compared to the wild-type mice. The brain-to-plasma ratios were greater than unity (1.2 and 1.7 for the 0.3 and 3 mg/kg/h infusion rates, respectively) suggesting that the CNS penetration of lapatinib is not restricted by protein binding or passive permeability; this agrees with the wide distribution of radiocarbon to all tissues except brain in the rat whole body autoradiography study following an

DMD #24646

oral dose of [<sup>14</sup>C]lapatinib (Polli et al., 2008). Of note, there was no concentration-dependent effect in brain to plasma ratios observed with the triple knockout animals, unlike that seen in the *Mdr1a/b(-/-)* and *Bcrp1(-/-)* animals (this study; Polli et al., 2008). These results suggest that *Mdr1a/b* and *Bcrp1* have a combined effect to control the brain entry of lapatinib in mice.

The finding that *Mdr1a/b* and *Bcrp1* have a combined effect on the brain entry of drugs has important potential consequences for the treatment of brain tumors in breast cancer patients treated with tyrosine kinase inhibitors as well as the basic understanding of ABC transporters in the CNS disposition of drugs. For lapatinib, this information provides insight into possible future strategies to enhance the brain and tumor delivery of this drug. These approaches may include co-administration with a dual Pgp and BCRP inhibitor such as elacridar (Breedveld et al., 2006) or improved brain penetration of future TKIs by designing out efflux by Pgp and BCRP during lead optimization. In the case of lapatinib, the high brain-to-plasma ratio achieved in the triple knockout mice suggests that the high plasma protein binding (>99%) and modest passive permeability (Polli et al., 2008) are not limiting CNS entry in vivo. The 40-fold increase in the brain-to-plasma ratio in the triple knockout mice further highlights the need to understand the possible synergistic role of efflux transporters in the blood-brain barrier for drug delivery, as well as the role these proteins have at maintaining this barrier under normal and disease situations. For example, it appears that the blood-brain barrier is fully intact and functional in the triple knockout mice based on the observation that these animals are healthy, reproduce well and do not have any obvious adverse behaviors (e.g., seizures, altered gait). However, a more detailed investigation of the effect the triple knockout has on the expression of other proteins / transporters and characterization of the blood-brain barrier tight junctions is warranted.

DMD #24646

Finally, Pgp is well documented to influence the brain entry of many drugs (Breedveld et al., 2006). In contrast, the role of BCRP/*Bcrp1* is still being clarified, with few examples demonstrating that this transporter significantly attenuates the CNS disposition of drugs (de Vries et al., 2007; Breedveld et al., 2006; Polli et al., 2008). The results from this study with lapatinib, along with that for topotecan (de Vries et al., 2007), highlight the need to understand further the role of BCRP/*Bcrp1* and other transporters in the blood brain barrier, and how these transporters interact with Pgp, which appears to be the dominant efflux transporter in the barrier. The triple knockout model will be a useful tool to elucidate these mechanisms.

In conclusion, *Mdr1a/b* and *Bcrp1* work in combination to modulate the CNS penetration of lapatinib in mice, resulting in a 40-fold increase in brain-to-plasma ratio compared to wild-type mice. Further research into the interplay between these and other transporters is warranted, as this finding provides unique insight into future cancer treatments with TKIs as well as an increased understanding of ABC transporters on the CNS disposition of drugs.

DMD #24646

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DMD #24646

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DMD #24646

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DMD #24646

TABLE 1. Summary of the Plasma and Brain Concentrations and Plasma Clearance of Lapatinib in *Mdr1a/1b* (-/-), *Bcrp1* (-/-), *Mdr1a/b* (-/-)/*Bcrp1* (-/-) Knockout Mice and FVBn (wild-type) Mice Following a Constant Rate Intravenous Infusion of Lapatinib at 0.3 and 3 mg/kg/h.

| Lapatinib Infusion Rate: 0.3 mg/kg/h     |                |                                |                              |                                |                              |
|--|----------------|--------------------------------|------------------------------|--------------------------------|------------------------------|
| Genotype                                 | Mice Per Group | Plasma C <sub>ss</sub> (ng/mL) | Brain Concentration (ng/mL)  | Brain-to-Plasma Ratio          | Plasma Clearance (mL/min/kg) |
| FVBn                                     | 5              | 730 ± 410                      | 18 ± 9.1                     | 0.03 ± 0.01                    | 13 ± 6.1                     |
| <i>Mdr1a/b</i> (-/-)                     | 4              | 239 ± 43 <sup>a, b</sup>       | 21 ± 3.3 <sup>c</sup>        | 0.09 ± 0.02 <sup>a, b, c</sup> | 31 ± 4.2 <sup>a, b</sup>     |
| <i>Bcrp1</i> (-/-)                       | 5              | 394 ± 74 <sup>a</sup>          | 17 ± 3.1 <sup>d</sup>        | 0.04 ± 0.01 <sup>a, d</sup>    | 20 ± 4.6 <sup>a</sup>        |
| <i>Mdr1a/b</i> (-/-)/ <i>Bcrp1</i> (-/-) | 5              | 296 ± 150 <sup>a</sup>         | 319 ± 67 <sup>a</sup>        | 1.2 ± 0.42 <sup>a</sup>        | 28 ± 13 <sup>a</sup>         |
| Lapatinib Infusion rate: 3 mg/kg/h       |                |                                |                              |                                |                              |
| FVBn                                     | 4              | 5097 ± 2729                    | 194 ± 65                     | 0.04 ± 0.02                    | 20 ± 13                      |
| <i>Mdr1a/b</i> (-/-)                     | 3              | 4463 ± 811 <sup>c</sup>        | 732 ± 421 <sup>a, b, c</sup> | 0.16 ± 0.06 <sup>a, b, c</sup> | 15 ± 2.3 <sup>c</sup>        |
| <i>Bcrp1</i> (-/-)                       | 5              | 6447 ± 2731 <sup>d</sup>       | 253 ± 23 <sup>a, d</sup>     | 0.04 ± 0.01 <sup>d</sup>       | 13 ± 4.4 <sup>d</sup>        |
| <i>Mdr1a/b</i> (-/-)/ <i>Bcrp1</i> (-/-) | 5              | 2840 ± 867                     | 4938 ± 1823 <sup>a</sup>     | 1.7 ± 0.50 <sup>a</sup>        | 23 ± 6.2                     |

Data are presented as the mean ± standard deviation

Statistical difference when p value ≤ 0.05.

<sup>a</sup> FVBn compared to *Mdr1a/b*(-/-), *Bcrp1*(-/-), or *Mdr1a/b*(-/-)/*Bcrp1*(-/-).

<sup>b</sup> *Mdr1a/b*(-/-) compared to *Bcrp1*(-/-).

<sup>c</sup> *Mdr1a/b*(-/-)/*Bcrp1*(-/-) compared to *Mdr1a/b*(-/-).

<sup>d</sup> *Mdr1a/b*(-/-)/*Bcrp1*(-/-) compared to *Bcrp1*(-/-).