Human Metabolism of Lapatinib, a Dual Kinase Inhibitor: Implications for Hepatotoxicity

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ABSTRACT:

Lapatinib (Tykerb, Tyverb; GlaxoSmithKline) is an orally active dual tyrosine kinase inhibitor efficacious in combination therapy for patients with progressive human epidermal receptor 2-overexpressing metastatic breast cancer. However, clinically significant liver injury, which may be associated with lapatinib metabolic activation, has been reported. We describe the metabolism and excretion of [14C]lapatinib in six healthy human volunteers after a single oral dose of 250 mg and the potential relationships between metabolism and clinical hepatotoxicity. Overall, elimination showed high intersubject variability, with fecal elimination being the predominant pathway, representing a median of 92% of the dose with lapatinib as the largest component (approximate median 27% of the dose). In plasma, approximately 50% of the observed radioactivity was attributed to metabolites. Analysis of a 4-h pooled plasma extract identified seven metabolites related by an N- and α-carbon oxidation cascade. Fecal metabolites derived from three prominent pathways: N- and α-carbon oxidation, fluorobenzyl oxidative cleavage, and hydroxypridine formation. Several of the lapatinib metabolites can undoubtedly be linked to reactive species such as aldehydes or quinone imines. In addition to the contribution of these potentially reactive metabolites as suspects in clinical liver injury, the role of other disposition factors, including interaction with drug transporters, pharmacogenetics, or magnitude of the therapeutic dose, should not be discounted.

Introduction

Lapatinib (Tykerb, Tyverb; GlaxoSmithKline) is an orally active dual tyrosine kinase inhibitor of the epidermal growth factor receptor 1 (ErbB1) and human epidermal receptor 2 (HER2). The ErbB1 and HER2 transmembrane tyrosine kinase receptors are signaling proteins implicated in the pathogenesis of breast cancer that regulate cellular growth, proliferation, survival, and differentiation. Increased expression and activation of HER2, reported in approximately 20% of human breast cancers, is associated with a higher risk for recurrence of breast cancer and a poorer clinical outcome. Lapatinib acts intracellularly by reversibly binding to the cytoplasmic ATP-binding site of the kinase, preventing receptor phosphorylation and activation. Simultaneous inhibition of both ErbB1 and HER2 is an appealing therapeutic strategy compared with agents that inhibit ErbB1 or HER2 alone. At present, lapatinib is used in combination with capecitabine or letrozole in patients with progressive HER2-overexpressing metastatic breast cancer previously treated with an anthracycline, a taxane, and trastuzumab with efficacious doses at 1250 or 1500 mg q.d. (Boyd et al., 2005; Geyer et al., 2006; Johnston et al., 2006; Johnston and Leary, 2006; Moy and Goss, 2006).

Lapatinib has an acceptable safety profile for the treatment of breast cancer (Bence et al., 2005; Geyer et al., 2006). However, serious drug-induced liver injury (DILI) in patients with cancer receiving lapatinib has been reported (Moy et al., 2009). Hepatotoxicity has also been reported for other tyrosine kinase inhibitors including imatinib, and metabolic activation has been suggested as an underlying factor (Loriot et al., 2008; Duckett and Cameron, 2010).

In the case of the tyrosine kinase inhibitor E-2-methoxy-N-[3-[4-[3-methyl-4-(6-methyl-pyridin-3-yl)-phenylamino]-quinazolin-6-yl]-allyl]-acetamide (CP-724,714), direct mitochondrial toxicity and inhibition of bile salt efflux are reported to contribute to hepatotoxicity (Feng et al., 2009). In a recent study, a clinical pharmacogenetic investigation of patients taking lapatinib reported a significant association between the major histocompatibility complex HLA-DQA1*02:01 and ALT elevation (Spraggs et al., 2011). Associations between DILI and specific HLA polymorphisms have been observed for other drugs as well, such as amoxicillin-clavulanate, fluvoxacinil, ximelagatran, and ticlopidine (Russmann et al., 2010). Furthermore, DILI occurs at a higher frequency in compounds that have 50% or greater hepatic metabolism (Chalasani and Bjo¨rnsson, 2010), suggesting an important role for metabolites in hepatotoxicity. For lapatinib, the underlying mechanism of hepatotoxicity has not been fully elucidated; however, it is possible that a combination of factors, rather than a single component, may ultimately be responsible.

ABBREVIATIONS: ErbB, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; DILI, drug-induced liver injury; CP-724,714, E-2-methoxy-N-[3-[4-[3-methyl-4-(6-methyl-pyridin-3-yl)-phenylamino]-quinazolin-6-yl]-allyl]-acetamide; ALT, alanine amino-transferase; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; LSC, liquid scintillation counting; MS, mass spectrometry; BQL, below the limit of quantification.
Lapatinib absorption is incomplete and variable after oral administration and systemic exposure is increased when it is administered with food (Koch et al., 2009) with likely associated increases in hepatic exposures to lapatinib and its metabolites. Lapatinib undergoes extensive metabolism, primarily by CYP3A4 and CYP3A5, with minor contributions from CYP2C19 and CYP2C8 to a variety of oxidized metabolites. Recent in vitro experiments with lapatinib have shown that cytochrome P450-mediated O-dealkylation resulted in a phenol metabolite; further oxidation of the phenol could result in the formation of a quinone imine, capable of reacting with cellular proteins or glutathione (Teng et al., 2010). para-Hydroxylapatinib and other metabolites are also formed from the metabolism of other kinase inhibitors, dasatinib, gefitinib, and erlotinib (Duckett and Cameron, 2010). Lapatinib is known to be a substrate and inhibitor for the transporters breast cancer resistance protein (ABCG2) and P-glycoprotein (ABC1) in vitro and inhibits the hepatic uptake transporter organic anion-transporting polypeptide 1B1 at clinically relevant concentrations in vitro (Polli et al., 2008). It is therefore possible that lapatinib or its metabolites have the potential to inhibit other relevant hepatic transporters such as the bile salt efflux pump.

Further insights into the mechanism of hepatotoxicity rely on understanding the human metabolism of lapatinib. Thus, we report our findings from a study of the metabolism and excretion of [14C]lapatinib in healthy human volunteers after administration of a single oral dose of 250 mg and the potential relationships between the metabolites of lapatinib and observed clinical hepatotoxicity.

Materials and Methods

Materials. [14C]Lapatinib, was prepared and analyzed by Isotope Chemistry, Chemical Development, GlaxoSmithKline, Stevenage, UK (Fig. 1). Analytical purity was determined to be 99.6% (w/w), and radiochemical purity (HPLC) was determined to be greater than 99%. The specific activity of the dose measured as free base was calculated to be 0.356 Ci/mg.

Clinical Mass Balance Study Design. The clinical portion of this study was conducted by Covance Inc. (Princeton, NJ), in accordance with Good Clinical Practice guidelines, the guiding principles of the Declaration of Helsinki, Covance standard operating procedures, the Nuclear Regulatory Commission regulations, and all applicable subject privacy requirements. All subjects provided written informed consent before participation. This was an open-label, single-dose, mass balance study in six healthy male or female subjects to determine the metabolic profile and routes of excretion of [14C]lapatinib in healthy human volunteers after administration of a single oral dose of 250 mg and the potential relationships between the metabolites of lapatinib and observed clinical hepatotoxicity.

Formulated Drug. A mixture of unlabeled lapatinib and [14C]lapatinib, equivalent to 250 mg of lapatinib free base (containing 89.1 μCi of 14C radioactivity), was dosed to each subject as an aqueous suspension (total volume of 80 ml of water). The specific activity of the dose measured as free base was calculated to be 0.356 μCi/mg.

Quantification of Lapatinib in Plasma. Quantification of Lapatinib in Plasma. Concentrations of lapatinib in plasma samples were determined with a validated analytical method, over the range 5 to 5000 ng/ml, by LC-MS/MS. Lapatinib was extracted from 25 μl of human plasma by protein precipitation using 80:20 (v/v) acetonitrile-10 mM ammonium acetate (pH 4.5) containing an isotopically labeled internal standard ([1H3,15N1,13C2]lapatinib). Extracts (2 μl) were injected onto a Hypersil-Keystone Fluophase PPP column (50 × 2.1 mm; Thermo Fisher Scientific, Waltham, MA), maintained at 40°C and eluted isocratically by using 10 mM ammonium acetate (pH 4.5)-acetonitrile-water (20:70:10) at a flow rate of 0.65 ml/min. Detection was performed by positive ion MS/MS using a TurboIon-Spray interface on an API 4000 mass spectrometer (Applied Biosystems, Waltham, MA) and positive ion tandem mass spectrometry (MS/MS) with collisionally activated dissociation (CAD). The extracted ion chromatograms (XIC) of the analyte and its internal standard were acquired in the positive ion mode with a m/z range 300 to 750.

Found concentrations of lapatinib were compared to the calibration standard (200, 400, 1000, and 2000 ng/ml) using linear regression with weights 1/x and with limits of quantitation and detection of 10 and 1 ng/ml, respectively. All plasma samples were kept at −80°C until analysis. The concentration-time profile of lapatinib obtained from a typical volunteer is shown in Fig. 2.

Urine was collected from each subject at 2, 4, 8, 12, 18, 24, 48, 72, 96, 120, 144, and 168 h postdose. Randomly selected urine samples were analyzed for the metabolites M1, M4, M5, M11, and M12. A total of 14 urine samples were collected for the metabolite and total radioactivity analysis. Each urine sample was weighed and placed in a prelabeled, 25-ml polypropylene storage tube for analysis of lapatinib (1 ml), total radioactivity (3 ml) and stored in an upright position at or below −20°C until analyzed.

Feces were collected predose and for 168 h postdose for pharmacokinetic analysis. Each fecal sample collection was weighed and placed in a prelabeled, sealable container in an upright position at or below −20°C until analyzed.

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Foster City, CA) with multiple reaction monitoring (m/z 581–m/z 365 for lapatinib and m/z 587–m/z 367 for the internal standard).

Radioanalytical Methods. All fecal sample combustions were performed in a model 307 Sample Oxidizer (PerkinElmer Life and Analytical Sciences, Waltham, MA). The resulting 14CO2 was trapped in Carbo-Sorb. Perma-Fluor scintillation cocktail and the radioactivity content were quantified by liquid scintillation counting (LSC). All samples, including plasma and urine, were directly counted with LSC by using Ultima Gold scintillation cocktail. The samples were counted in a model 1900TR or 2300TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences) for at least 5 min or 100,000 counts. The LSC data (counts per minute) were automatically corrected for counting efficiency by using an external standardization technique and an instrument-stored quench curve generated from a series of sealed quenched standards to obtain disintegrations per minute. The LSC data were corrected for background by subtracting the disintegrations per minute value measured from the analysis of a blank sample. Plasma and urine samples were thawed and homogenized and triplicate-weighted aliquots from each postdose sample (approximately 0.2 g each) were submitted for radioanalysis. The fecal samples were thawed and mixed with water (enough to create a slurry), and the total weight was recorded. The samples were homogenized with a probe-type homogenizer and triplicate-weighted aliquots (approximately 0.2 g each) were combusted and analyzed for radioactivity. If results for sample replicates (calculated as disintegrations per minute per gram or disintegrations per minute per milliliter of sample, as applicable) differed by more than 10% from the mean value, the sample was rehomogenized (where appropriate) and reanalyzed. This specification was met for all sample aliquots that had radioactivity greater than 500 dpm.

Metabolite Radiochemical Profiling. The radiochemical profiles of 14C]lapatinib-derived radioactivity were determined for selected fecal homogenate and plasma extracts by using HPLC combined with radiochemical flow detection or fractionation and off-line counting. HPLC-radiometric data were generated on-line by using a 500TR series flow scintillation analyzer and Ultima-Flo M scintillation fluid (PerkinElmer Life and Analytical Sciences) with a scintillant flow rate of 1 ml/min. For off-line analysis, representative fecal homogenate and plasma extracts were fractionated into 96-well plates by using HPLC. A portion of each fraction was transferred to a separate 96-well plate and combined with scintillation cocktail for liquid scintillation counting. Reconstructed radiochemical profiles were compiled from the resulting data. Plates containing the original fecal homogenate, plasma extract fractions, and other selected samples were analyzed by using nanoelectrospray mass spectrometry. Off-line radiodetection was performed with either a Wallac 1450 MicroBeta liquid scintillation analyzer or a Tri-Carb 2500TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences). Liquid scintillant was added at 150 μl/well (Ultima-Flo M). Data tables (radioactivity concentration and percentage of dose) were generated by DEBRA (version 5.2a; LabLogic, South Yorkshire, UK), an automated and validated data capture and management system for studies using radiolabeled test material.

Four plasma pools were prepared for extraction. Three pools were used to generate LC-radiochemical profiles, and the fourth pool was used for LC-MS analysis. The first pool contained equal volumes of 4-h postdose plasma from all six subjects. The second pool contained equal volumes of 3-, 4-, and 6-h postdose plasma from a single female subject. The third pool contained equal volumes of 2.5-, 3-, and 8-h postdose plasma from three male subjects. The fourth pool contained equal volumes of 1.5-, 2-, 3-, 4-, and 6-h postdose plasma from a different female subject. In all cases, 1 volume of human plasma was extracted with 2 volumes of methanol and 2 volumes of acetonitrile. The extract was vigorously mixed to a vortex followed by centrifugation for approximately 5 min. The supernatant was removed, and the volume was reduced to approximately 0.1 ml under either a stream of nitrogen at ambient temperature or reduced to dryness overnight using a Savant SpeedVac (Thermo Fisher Scientific) on the low drying setting. The dried extract was reconstituted with 100 μl of the starting mobile phase for the method. Approximately 90 μl of sample extract was injected onto the HPLC column.

Because of the limited amount of radiocarbon associated with many of the plasma samples, analyses were conducted by using HPLC with fraction collection followed by liquid scintillation counting. Fractions collected from the analysis of human plasma produced an average background of approximately 9 dpm with use of the Wallac 1450 MicroBeta liquid scintillation analyzer. A 3:1 signal/noise ratio (27 dpm or approximately 23 ng equivalents) was chosen as the limit of quantitation for off-line liquid scintillation counting.

**TABLE 2**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Urine</th>
<th>Feces</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects (n = 6)</td>
<td>1.2</td>
<td>91.8</td>
<td>93.0</td>
</tr>
<tr>
<td>Male (n = 3)</td>
<td>1.0</td>
<td>93.6</td>
<td>94.6</td>
</tr>
<tr>
<td>Female (n = 3)</td>
<td>1.3</td>
<td>89.9</td>
<td>91.5</td>
</tr>
</tbody>
</table>

**TABLE 3**

Percentage of cumulative radioactivity recovered from six healthy male and female subjects after a single 250-mg oral suspension dose of [14C]lapatinib

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Feces</th>
<th>Urine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–24 h</td>
<td>10.5</td>
<td>1.16</td>
<td>1.01</td>
</tr>
<tr>
<td>0–48 h</td>
<td>45.3</td>
<td>1.16</td>
<td>1.16</td>
</tr>
<tr>
<td>0–72 h</td>
<td>74.9</td>
<td>1.16</td>
<td>1.16</td>
</tr>
<tr>
<td>0–96 h</td>
<td>78.4</td>
<td>1.16</td>
<td>1.16</td>
</tr>
<tr>
<td>0–120 h</td>
<td>90.8</td>
<td>1.16</td>
<td>1.16</td>
</tr>
<tr>
<td>0–144 h</td>
<td>91.4</td>
<td>1.16</td>
<td>1.16</td>
</tr>
<tr>
<td>0–168 h</td>
<td>91.8</td>
<td>1.16</td>
<td>93.1</td>
</tr>
</tbody>
</table>

**FIG. 3.** Representative 4-h pooled plasma extract HPLC-radiochemical chromatogram from healthy subjects after a single oral administration of [14C]lapatinib (250 mg).

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**Pharmacokinetic parameters**

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Lapatinib</th>
<th>Total Radioactivity</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (mg/l)</td>
<td>0.33 (0.15–1.07)</td>
<td>0.72 (0.40–1.69)</td>
<td></td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>4.05 (2.53–5.97)</td>
<td>4.03 (3.03–5.97)</td>
<td></td>
</tr>
<tr>
<td>AUC, (h · mg/l)</td>
<td>3.58 (2.31–13.0)</td>
<td>6.79 (4.51–23.0)</td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>14.8 (11.5–20.5)</td>
<td>5.59 (4.50–14.6)</td>
<td>0.54 (0.47–0.57)</td>
</tr>
<tr>
<td>Urinary recovery</td>
<td>1.15 (0.49–1.61)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% dose)</td>
<td>27.0 (2.68–66.9)</td>
<td>91.7 (60.3–98.3)</td>
<td>0.33 (0.04–0.72)</td>
</tr>
<tr>
<td>Fecal recovery</td>
<td>93.0 (61.6–99.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% dose)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Value probably underestimated because of detection limits for radioactivity.

---

**TABLE 1**

Excretion balance results by sex

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Urine</th>
<th>Feces</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>%dose</td>
<td>%dose</td>
<td>%dose</td>
<td>%dose</td>
</tr>
<tr>
<td>All subjects (n = 6)</td>
<td>1.2</td>
<td>91.8</td>
<td>93.0</td>
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<td>94.6</td>
</tr>
<tr>
<td>Female (n = 3)</td>
<td>1.3</td>
<td>89.9</td>
<td>91.5</td>
</tr>
</tbody>
</table>
### Table 4

**Analytical reference data for lapatinib and metabolites**

<table>
<thead>
<tr>
<th>Proposed Structure</th>
<th>[M + H]⁺ (m/z)</th>
<th>Characteristic Fragment Ions (m/z)</th>
<th>¹H-NMR (600 MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapatinib (GW572016)</td>
<td>581</td>
<td>458, 393, 379, 365, 350</td>
<td>¹H NMR (D₂O: ACN) δ ppm 3.05 (s, 3H), 3.54 (m, 2H), 3.58 (m, 2H), 4.40 (s, 2H), 5.20 (s, 2H), 6.80 (d, J = 3.43 Hz, 1H), 7.08 (d, J = 3.43 Hz, 1H), 7.08–7.11 (m, 1H), 7.21 (d, J = 8.88 Hz, 1H), 7.20–7.24 (m, 1H), 7.28 (d, J = 7.47 Hz, 1H), 7.40 (dq, 1H), 7.48 (dd, J = 8.95, 2.57 Hz, 1H), 7.74 (d, J = 2.65 Hz, 1H), 7.84 (d, J = 8.56 Hz, 1H), 8.35 (dd, J = 8.72, 1.71 Hz, 1H), 8.60 (s, 1H), 8.76 (d, J = 1.40 Hz, 1H)</td>
</tr>
<tr>
<td>M1</td>
<td>473</td>
<td>393, 379, 367, 351, 350</td>
<td>¹H NMR (DMSO-d₆) δ ppm 3.03 (s, 3H), 3.01–3.07 (m, 2H), 3.27–3.35 (m, 2H), 3.86–3.92 (br s, 2H), 6.51 (br d, J = 2.80 Hz, 1H), 7.00 (d, J = 8.72 Hz, 1H), 7.06 (d, J = 3.11 Hz, 1H), 7.14 (br s, 1H), 7.57 (dd, J = 8.72, 2.49 Hz, 1H), 7.77 (d, J = 8.72 Hz, 1H), 7.86 (d, J = 2.49 Hz, 1H), 8.13 (dd, J = 8.72, 1.56 Hz, 1H), 8.50 (s, 1H), 8.78 (d, J = 1.87 Hz, 1H), 9.85 (br s, 1H), 10.06 (br s, 1H)</td>
</tr>
<tr>
<td>M2</td>
<td>473</td>
<td>364, 363, 336, 335, 329, 301</td>
<td>¹H NMR (DMSO-d₆) δ ppm 5.26 (s, 2H), 7.15–7.20 (m, 1H), 7.24–7.27 (m, 1H), 7.28 (d, J = 9.34 Hz, 1H), 7.30–7.32 (m, 1H), 7.33 (m, J = 6.85 Hz, 1H), 7.35 (dd, J = 8.20, 2.80 Hz, 1H), 7.76 (dd, J = 9.01, 2.50 Hz, 1H), 7.82 (d, J = 8.72 Hz, 1H), 8.02 (d, J = 2.49 Hz, 1H), 8.04 (d, J = 8.72 Hz, 1H), 8.30 (d, J = 3.11 Hz, 1H), 8.50 (dd, J = 8.88, 1.71 Hz, 1H), 8.56 (s, 1H)</td>
</tr>
<tr>
<td>M3</td>
<td>489</td>
<td>366, 323</td>
<td>¹H NMR (600 MHz, DMSO-d₆) δ ppm 2.96 (t, J = 6.80 Hz, 2H), 3.01 (s, 3H), 3.25 (t, J = 6.80 Hz, 2H), 3.78 (s, 2H), 6.41 (d, J = 3.11 Hz, 1H), 6.83 (d, J = 3.11 Hz, 1H), 7.00 (d, J = 8.72 Hz, 1H), 7.20 (d, J = 8.41 Hz, 1H), 7.50 (dd, J = 8.72, 2.18 Hz, 1H), 7.83 (d, J = 2.18 Hz, 1H), 7.90 (dd, J = 8.72, 1.24 Hz, 1H), 8.53 (s, 1H), 9.73 (s, 1H)</td>
</tr>
<tr>
<td>M4</td>
<td>475</td>
<td>366, 365</td>
<td>Not acquired (authentic standard available)</td>
</tr>
<tr>
<td>Proposed Structure</td>
<td>$[M+H]^{+}$ (m/z)</td>
<td>Characteristic Fragment Ions (m/z)</td>
<td>$^1$H-NMR (600 MHz)</td>
</tr>
<tr>
<td>--------------------</td>
<td>------------------</td>
<td>----------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>M5</td>
<td>579</td>
<td>470, 391, 363, 327, 263</td>
<td>$^1$H NMR (600 MHz, DMSO-d$_6$) δ ppm 2.97 (s, 3H), 3.88 (t, 2H), 4.98 (t, $J = 7.03$ Hz, 2H), 5.27 (s, 2H), 7.15–7.20 (m, 1H), 7.30 (m, 1H), 7.31 (d, $J = 9.24$ Hz, 1H), 7.32–7.34 (m, 1H), 7.44–7.48 (m, 1H), 7.90 (dd, $J = 8.83$, 2.41 Hz, 1H), 8.04 (d, $J = 8.84$ Hz, 1H), 8.07 (d, $J = 8.23$ Hz, 1H), 8.07 (d, $J = 2.61$ Hz, 1H), 8.16 (dd, $J = 8.63$, 1.00 Hz, 1H), 8.26 (dd, $J = 8.83$, 2.41 Hz, 1H), 8.86 (br s, 1H), 8.95 (d, $J = 2.61$ Hz, 1H), 9.37 (br s, 1H)</td>
</tr>
<tr>
<td>M6 ($\Delta$$N_7$-C$_6$-nitron)</td>
<td>595</td>
<td>489, 471, 380, 363, 350</td>
<td>$^1$H NMR (DMSO-d$_6$) δ ppm 3.05 (s, 3H), 3.75 (t, $J = 6.23$ Hz, 2H), 4.43 (t, $J = 6.54$ Hz, 2H), 5.26 (s, 2H), 7.18 (td, $J = 8.70$, 2.60 Hz, 1H), 7.29 (d, $J = 9.03$ Hz, 1H), 7.30 (m, 1H), 7.32 (d, $J = 3.74$ Hz, 1H), 7.33 (d, $J = 8.72$ Hz, 1H), 7.45–7.49 (m, 1H), 7.76 (dd, $J = 8.82$, 2.61 Hz, 1H), 7.81 (d, $J = 3.43$ Hz, 1H), 7.83 (d, $J = 8.72$ Hz, 1H), 8.03 (d, $J = 2.49$ Hz, 1H), 8.22 (dd, $J = 8.41$, 1.56 Hz, 1H), 8.28 (~s, 1H), 8.58 (s, 1H), 8.86 (d, $J = 1.56$ Hz, 1H), 9.97 (s, 1H), stereochemistry of nitron set by NOE</td>
</tr>
<tr>
<td>M7 ($\Delta$$N_7$-C$_8$-nitron)</td>
<td>595</td>
<td>489, 471, 380, 363, 350</td>
<td>Unstable during isolation</td>
</tr>
<tr>
<td>M8</td>
<td>597</td>
<td>491, 459, 382, 364, 350, 335, 306</td>
<td>$^1$H NMR LC-NMR (D$_2$O: ACN) δ ppm 3.00 (s, 3H), 3.17 (br t, $J = 7$ Hz, 2H), 3.39 (br t, $J = 6.77$ Hz, 2H), 3.99 (s, 2H), 5.23 (s, 2H), 6.53 (d, $J = 3.43$ Hz, 1H), 7.02 (d, $J = 3.43$ Hz, 1H), 7.06–7.11 (m, 1H), 7.20 (d, $J = 9.03$ Hz, 1H), 7.20–7.25 (m, 1H), 7.28 (d, $J = 7.32$ Hz, 1H), 7.37–7.43 (m, 1H), 7.50 (dd, $J = 8.64$, 2.73 Hz, 1H), 7.76 (d, $J = 2.49$ Hz, 1H), 7.82 (d, $J = 8.72$ Hz, 1H), 8.32 (dd, $J = 8.56$, 1.71 Hz, 1H), 8.58 (s, 1H), 8.70–8.72 (m, 1H)</td>
</tr>
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HPLC method 1 (used for analysis of plasma). The chromatographic system comprised an Agilent 1100 binary pump (Agilent Technologies, Santa Clara, CA) and autosampler, manual fraction collection, column oven (45°C), UV detector (277 nm), and Waters Symmetry C18 column (4.6 mm × 150 mm, 5 μm) (Waters, Milford, MA). The mobile phase consisted of water-formic acid (0.1%, v/v) (solvent A) and methanol-acetonitrile (10 mM ammonium acetate, pH 4.5, 72:23:5, v/v/v) (solvent B). A flow rate of 1 ml/min was maintained for the entire run. The following 90-min linear gradient was used: start at 15% B for 10 min, increase to 35% B over 20 min, increase to 50% B over 10 min, increase to 60% B over 10 min, increase to 75% over 10 min, and increase to 90% B over 5 min. The column was reequilibrated after each injection. HPLC column recoveries were determined for selected samples by collecting the total HPLC column eluate, determining the total amount of radioactivity recovered, and comparing that with the amount of radioactivity injected onto the column.

HPLC method 2 (used for analysis of plasma to resolve N-oxidation products). The chromatographic system and column were the same as for method 1. The mobile phase consisted of water-formic acid (0.1% v/v) (solvent A) and acetonitrile containing 0.1% formic acid (v/v) (solvent B). A flow rate of 1 ml/min was maintained for the entire run. The following 90-min linear gradient was used: start at 27% B and hold for 5 min, increase to 28% B over 5 min, increase to 38% B over 20 min, and increase to 90% B over 2 min. The column was reequilibrated after each injection.

For fecal radiochemical profiles, time points were selected for each subject such that at least 90% of the total radioactivity over the sample collection...
period was represented. Approximately 1 to 5 g of each selected fecal homogenate were extracted (liquid-liquid) with ethanol-acetonitrile-sample (3:3:1, v/v/v). The extraction vessels were mixed to a vortex and shaken for approximately 1 h, and the supernatant was removed and reduced to dryness with a Savant SpeedVac. The dried extract was reconstituted with dimethyl sulfoxide-methanol-acetonitrile (2:1:1, v/v/v) before HPLC analysis. Selected fecal homogenates, representing >90% of the radiocarbon recovered in feces for each subject, were analyzed by HPLC-radio profiling method 1. No urine samples were analyzed by HPLC because of the low percentage of the dose (median 1.2%) recovered in the urine.

Identification of Metabolites. Structural characterization by MS was performed using selected sample isolates by nanoelectrospray infusion on a quadrupole time of flight mass spectrometer (Waters) operating in positive ion mode. Collision energies were ramped between 20 and 40 eV. The optimum energy for each metabolite was selected. Leucine enkephalin (2 μg/ml) spiked into the sample at 10% (v/v). Lapatinib metabolites were characterized by the accurate mass measurement of the full scan protonated molecular ion and collision-induced dissociation fragmentation. All human lapatinib metabolites had previously been fully characterized by MS and NMR from preclinical studies or synthesis. Synthetic standards were used for comparison of chromatographic retention times as well as characteristic MS fragmentation patterns.

Analysis and identification of drug-related material in human plasma were also performed by using LC-MS with HPLC method 1. This system consisted of an Agilent 1100 binary pump, autosampler, column oven (45°C), and UV detector (λ 277 nm) interfaced with a Sciex API 150 (Applied Biosystems) single quadrupole mass spectrometer with an electrospray ion source (positive ion) operated in full scan mode (m/z 100–900). Chromatographic retention times and protonated molecular ions (m/z) were used with synthetic standards to characterize lapatinib metabolites.

Results

Pharmacokinetics of Lapatinib and Total Radioactivity. Lapatinib was well tolerated with no clinically significant changes in laboratory values, vital signs, or serious adverse events observed. The median plasma concentration-time profiles and pharmacokinetic parameters of total unchanged lapatinib and radioactivity in healthy volunteers (n = 6) after a single 250-mg oral dose of [14C]lapatinib are shown in Fig. 2 and Table 1, respectively. The median time to reach peak plasma concentration (T\text{max}) was approximately 4 h for both lapatinib and total radioactivity. The highest concentrations (C\text{max}) in plasma of lapatinib and total radioactivity achieved were 0.33 mg/l (range 0.15–1.07) and 0.72 mg/l (range 0.40–1.69), respectively. Radioactivity levels at 24 h were below the limits of quantification, whereas lapatinib levels were determined to 72 h postdose.

Mass Balance and Excretion in Urine and Feces. In healthy male and female subjects (n = 6) after a single 250-mg oral suspension dose of [14C]lapatinib, the median total recovery of radioactivity was 93.1% (range 61.7–99.7%). Two subjects had overall recoveries of 61.7 and 71.5% of the administrated radioactivity, whereas the other four subjects had overall recoveries greater than 90%. The low recovery for these two subjects does not appear to be due to inadequate duration of sampling or the analytical methodology and remains unexplained (Roffey et al., 2007). Because of the variability in recovery of total radioactivity, data are best represented by the median. There were no notable differences in mass balance, based on the subject’s gender (Table 2). Forty-eight hours after dose administration, approximately 50% of the total recovered radioactivity was excreted. Fecal excretion was the predominant route of elimination, accounting for a median of 91.8% (range 60.3–98.4%) of the recovered dose. Excretion of radioactivity in feces occurred gradually over 168 h (Table 3). Urinary excretion was minimal (median 1.16%; range 0.49–1.61%).

Metabolite Profiling and Characterization. Recovery of radioactivity after the extraction of human plasma was determined to be >90%. However, only a single quantifiable LC radioactive peak, identified as lapatinib by MS analysis, was observed in a multisubject pooled 4-h plasma extract (Fig. 3). The amount of radioactivity associated with the single peak accounted for approximately half of the total radioactivity extracted from the plasma on the basis of the LC-MS/MS quantitative analysis of lapatinib. It was estimated that the limit of radiochemical quantification, in this chromatogram was approximately 5% of the total radioactivity of the pooled plasma sample (23 ng equivalents) or approximately 10% of the peak area associated with lapatinib. Various plasma pools consisting of time points from 1.5 to 8 h also demonstrated that lapatinib was the only quantifiable radioactive peak present in the chromatogram.

To account for the estimated 50% of the radioactivity in the plasma extract that was not quantifiable in the radiochemical chromatogram, LC-MS with a secondary chromatographic method was used to detect and identify drug-related material. Seven metabolites in addition to lapatinib were identified, including a hydroxylamine (M8), two oximes (stereoisomers, M9 and M10), two nitrones (rogoisomers, M6 and M7), an aldehyde (M11), and a carboxylic acid (M12) (Table 4). These metabolites originate from an oxidation cascade of the aliphatic secondary amine and include contributions from N- and α-carbon oxidation (Scheme 1). A composite of extracted ion chromatograms obtained from the LC-MS analysis of pooled human plasma (female subject, pool 1.5–6 h) after a single oral dose of [14C]lapatinib is shown in Fig. 4. These metabolites and potentially other metabolites present at low concentrations appear to represent the majority of the total radioactivity not accounted for by lapatinib. No metabolites containing the chlorophenol moiety (M1 and M3) were detected in plasma. In addition, it should be noted that not all of the minor drug-related components observed by LC/MS were characterized.

The fecal collection periods selected for radiochemical profiling represented 94.7 ± 2.3% of the dose recovered in the feces. Radioactivity extraction efficiencies from the fecal homogenates were 86.2 ± 9.5%. The HPLC-radiochemical chromatograms of the fecal homogenates showed a high degree of variability among subjects and were characterized by the presence of a few notable metabolites and many minor metabolites. Of the total 18 radiochemical peaks that were quantifiable in at least one radiochemical profile, only two peaks had a median of greater than 10% of the dose. Representative HPLC-radiochemical chromatograms of fecal extracts are shown in Fig. 5. These two chromatograms contained all of the notable metabolites but demonstrate the variability in the relative proportions of metabolites present.

Lapatinib was present in all fecal homogenates with a median value of 27.0% of the dose; however, the range was between 2.7 and 66.9%. The subject with the lowest amount of lapatinib (2.7%) also had poor overall recovery of radioactivity (71.5%). Two significant metabolites, M1 and M3, were characterized as oxidative cleavage products resulting in the formation of phenol metabolites. The median value for the phenol, M1, was 3.9% of the dose and ranged from below the limit of quantification (BQL) to 19.2%. M3, a phenol that is also hydroxylated on the quinazoline moiety, constituted a median 3.3% of the dose (range from BQL to 17.8%). The one subject who had values BQL for both M1 and M3 also had poor overall recovery of cumulative radioactivity (61.7%). The carboxylic acid metabolite, M12, was present in the fecal homogenate profiles of all subjects. The median amount of this metabolite was 14.0% (0.4–32.5%) and was the only metabolite with a median value greater than 10% of the dose. The most structurally novel metabolite quantified in the fecal homogenates was the intramolecular cyclized hydroxypyridine, M2, related
to the pyridinium salt, M5, which was observed as a small partially resolved shoulder of lapatinib in some chromatograms. The median amount of M2 was 6.3% of the dose, ranging from BQL to 14.5%. Other metabolites represented in Scheme 1 were detected by LC-MS in the fecal homogenate but were below the limit of quantification. Evidence of the N-dealkylated metabolite, M4, was also observed by LC-MS. In this regard, MS fragmentation of lapatinib provides useful insights into its biotransformation pathways (Fig. 6).

**Discussion**

Lapatinib is an orally active dual tyrosine kinase inhibitor of ErbB1 and HER2 currently approved for use in the treatment of breast cancer. Lapatinib has an acceptable safety profile for its therapeutic indication; however, hepatotoxicity (ALT or aspartate transaminase > 3 times the upper limit of normal and total bilirubin > 2 times the upper limit of normal) has been observed in clinical trials (<1% of patients) and postmarketing experience (GlaxoSmithKline, Tykerb product information, 2007, http://us.gsk.com/products/assets/us_tykerb.pdf). Recent advances in molecular toxicology have established a basis for understanding the mechanisms of hepatotoxins at the chemical and cellular levels with drug metabolism studies providing a logical framework to link in vitro and whole animal studies to man (Park et al., 2006). Thus, understanding the metabolism and distribution of lapatinib in humans provides a platform to investigate the origins of hepatotoxicity and hypothesis building. The metabolism and excretion of [14C]lapatinib were studied in healthy human volunteers, after administration of a single oral dose of 250 mg. This study was conducted at a subtherapeutic dose; there-
fore, the relative quantities of metabolites formed may not be reflective of the clinical setting in which saturation of biotransformation or transport pathways may occur. Nevertheless, this study provides a key understanding of lapatinib metabolism in humans and further highlights the potential complex relationships between the metabolites and clinical hepatotoxicity.

Although some quantitative details of the mass balance and radiochemical profiling were limited by the high degree of variability, an understanding of lapatinib metabolism in humans has emerged. Lapatinib is extensively metabolized, as exemplified by diverse biotransformations to form metabolites, many of which are linked through common pathways. All the metabolites were distributed across all subjects in feces or plasma, with none of the metabolites, by themselves, consistently accounting for a significant quantity of the administered dose. Three main biotransformation pathways for lapatinib were identified in humans. The first involves the formation of the phenol moieties by oxidative cleavage of the fluorobenzyl group. These metabolites were observed in feces but were not detected in plasma. In the preclinical species (rat and dog), this pathway predominates, with biliary elimination of M1 as both glucuronide and sulfate conjugates account for a large portion of the dose (S. Castellino, G. Bowers, J. Sigafoos, and D. Borts, unpublished observations). Likewise, in humans, biliary excretion of the corresponding conjugates could be anticipated; however, the proportion of the dose cleared by this oxidative cleavage pathway is greatly reduced relative to that in the preclinical species.

Multiple pathways initiated by N- and \( \alpha \)-carbon oxidations may be involved in accounting for the metabolites derived from the secondary
amine. Oxidation of the secondary aliphatic nitrogen leads to a hydroxylamine, M8 (Scheme 1). Further oxidation of the hydroxylamine followed by elimination gives rise to two geometric nitroine isomers (M6 and M7) and represents a branching point in the cascade. Hydrolysis of the $\Delta$-N$_2$C$_8$ nitroine metabolite, M7, would produce a primary hydroxylamine and methylsulfonyl acetaldehyde. Further oxidation of the hydroxylamine would lead to a nitroso intermediate that tautomerizes to produce the two observed stereomeric oxime isomers (M9 and M10). In addition, hydrolysis of the $\Delta$-N$_2$C$_6$ nitroine, M6, would result in the corresponding furfuraldehyde metabolite, M11, a potentially reactive electrophilic intermediate implicated in a similar cascade starting with a piperazine moiety (Rodriguez et al., 1999). A final oxidation step to the corresponding carboxylic acid appears to be the terminal step in the cascade. The carboxylic acid metabolite, M12, was quantitatively the most significant, suggesting that formation of the $\Delta$-N$_2$C$_6$ nitroine and a-carbon oxidation at C6 to generate the furfuraldehyde, M11, is the favored pathway. Spectroscopic evidence for the hydrolysis of nitrones M6 and M7 to the furfuraldehyde, M11, and a primary hydroxylamine, respectively, was obtained from chromatographically isolated nitrones (data not shown).

In addition, a-carbon oxidation pathways could also contribute to the formation of metabolites M9, M10, M11, and M12. The N-dealkylated species, M4, resulting from a-carbon oxidation (C8) to the carbinol amine followed by hydrolysis, was not observed as a major metabolite. However, the observed low levels of M4 could be attributed to its intermediacy in two further biotransformation pathways: 1) oxidation leading to a primary hydroxylamine followed by further oxidation to the nitroso intermediate, which tautomerizes to the oximes, M9 and M10, and 2) cyclization resulting in M2. Oxidation of the other a-carbon (C6) to generate the carbinol amine followed by elimination can also contribute to the formation of the furfuraldehyde metabolite, M11 (Scheme 1).

The third pathway of significance results in hydroxypyridine (M2) formation, consistent with an initial bioactivation of the dihydrofuran ring followed by intramolecular cyclization involving the secondary amine. Loss of the ethyl sulfone moiety from the pyridinium salt M5 results in the stable hydroxy pyridine (Scheme 2). Likewise, M4 could undergo oxidative cyclization to form M2 directly. An alternative mechanism for the formation of the pyridinium salt involves photooxidation (Kuo and Shih, 1991) through a common dicarbonyl intermediate (Dalvie et al., 2002) (Scheme 3). Experiments with preclinical feline homogenates spiked with lapatinib demonstrated that formation of the pyridinium salt, M5, was proportional to the sample manipulation time under normal laboratory conditions. Thus, M2 and its precursor, M5, may have resulted from metabolism, the isolation process, or a combination of both.

Overall, the data from this study demonstrate that elimination of lapatinib is predominantly metabolic, with the majority of the dose excreted as metabolites in feces. Variability in the extent of metabolism reflects that observed in systemic exposure to lapatinib. Clinically, the metabolism of lapatinib is predominantly mediated by CYP3A because both ketoconazole (CYP3A inhibitor) and carbamazepine (CYP3A inducer) significantly alter lapatinib exposures (Smith et al., 2009).

CYP3A is the predominant cytochrome P450 in both the intestinal epithelium and liver (Thummel et al., 1997) where it contributes to the

SCHEME 2. Proposed enzymatic mechanism leading to the formation of hydroxypyridine metabolite (M2).
large first-pass metabolism of lapatinib. In addition, there is considerable interindividual variability in expression and activity of CYP3A (Guengerich, 1999). Because of its involvement in the metabolism of lapatinib, differences in CYP3A-mediated metabolism may have important consequences that influence the development of clinical hepatotoxicity, which could arise because of different genetic or environmental factors. Several lapatinib metabolites could potentially form reactive electrophilic intermediates that could contribute to hepatotoxicity. A recent in vitro investigation demonstrated that lapatinib (50 μM) undergoes CYP3A4- and CYP2C8-mediated O-dealkylation to generate M1 as well as the formation of glutathione and cysteinylglycine conjugates (Teng et al., 2010), the latter conjugates being formed from the reaction of glutathione with the putative quinone imine intermediate.

Furthermore, lapatinib was suggested to be a mechanism-based inactivator of CYP3A4, through formation of the quinone imine that covalently modifies the CYP3A4 apoprotein and/or heme moiety. The authors proposed this route as a potential mechanism to explain clinical hepatotoxicity findings (Teng et al., 2010). Another in vitro investigation characterized the metabolic intermediate complex formation of CYP3A4 by lapatinib to be quasi-reversible and mediated through metabolites from N-oxidation rather than the quinone imine (Takakusa et al., 2011). These studies provide good insight into highlighting possible mechanisms; however, these elements may only be a part of the process, and a better understanding of the underlying complexities is essential to establish a link from in vitro experiments to hepatotoxicity (Obach et al., 2008). In vivo studies in preclinical species and in vitro human investigations suggest that the O-dealkylation product, the phenol (M1), is extensively glucuronidated and sulfated. In the toxicity species (rat and dog), hepatotoxicity was not observed at clinically relevant exposures of lapatinib. In addition, the predominant metabolic route of elimination favors formation of M1, suggesting an efficient protective mechanism to potentially reactive intermediates. Nevertheless, a recent pharmacogenetic analysis of patients with metastatic breast cancer experiencing liver injury while taking lapatinib reported a significant association with the major histocompatibility complex HLA-DQA1*02:01 and ALT elevation (Spraggs et al., 2011). This finding suggests an activation of the adaptive immune system, in a small subset of patients, with HLA-DQA1*0201 to cause liver injury. Other drugs have shown similar
HLA associations that may be explained as being due to covalent binding to proteins by the drug itself or metabolites to form a hapten, which is recognized by specific HLA proteins, resulting in inflammatory tissue damage (Chesson et al., 2008).

If the lapatinib metabolites are potential suspects in clinical liver injury, interdependency on other disposition factors such as interaction with drug transporters should not be discounted (Scheme 4). In plasma, only metabolites emanating from oxidation of the secondary aliphatic nitrogen or α-carbons appear and only in small quantity. All other metabolites are found in the feces, suggesting that an efficient biliary elimination route is present, with liver exposure being significantly different from what is represented in plasma. The roles that transporters have in the disposition and drug interactions of lapatinib have been well characterized (Polli et al., 2008), and it is plausible that transporters are involved either directly or indirectly in the underlying mechanism of hepatotoxicity. The interruption of bile salt efflux has been reported as a cause for cholestatic liver damage (Pauli-Magnus and Meier, 2006), and inhibition of the bile salt export pump transportер by lapatinib and its metabolite M1 has recently been investigated (C. MacLauchlin, G. Bowers, R. Brown, S. Castellino, G. Generaux, R. Groselcose, R. Miller, D. Newall, L. Webster, and J. Polli, unpublished observations). Feng et al. (2009) suggested a combination of direct cytotoxicity to mitochondria and inhibition of bile salt efflux to explain the clinical hepatotoxicity observed by a structurally similar tyrosine kinase inhibitor. In relation to both mechanisms of toxicity, the authors cite the role uptake and efflux transporters play in controlling intracellular hepatic concentrations. This also may be an important factor to consider for lapatinib and its metabolites, especially considering the clinical doses (1250 and 1500 mg q.d.) compared with the single 250-mg dose administered in this study and major dependence on hepatic metabolism for clearance and excretion.

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Authorship Contributions

Participated in research design: Castellino, Koch, and Bowers.

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Wrote or contributed to the writing of the manuscript: Castellino, Koch, Bowers, and MacLauchlin.

References


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