Title: Detoxication vs. Bioactivation Pathways of Lapatinib In Vitro: UGT1A1 Catalyzes the Hepatic Glucuronidation of Debenzylated Lapatinib

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Abbreviations Used: AO, aldehyde oxidase; BCRP, breast cancer resistance protein; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor 1; HER2, human epidermal growth factor receptor 2; HLA, human leukocyte antigen; HLM, human liver microsomes; KHB, Krebs-Henseleit buffer; LC-MS/MS, liquid chromatography-tandem mass spectrometry; M1, debenzylated lapatinib; MRP2, multidrug resistance-associated protein 2; Nrf2, nuclear factor erythroid 2-related factor 2; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SRM, selected reaction monitoring; SULT, sulfotransferase; UDPGA, uridine 5'-diphosphate-glucuronic acid; UGT, UDP-glucuronosyltransferase; UPLC, ultra-performance liquid chromatography.
Abstract

O-Dealkylation of the tyrosine kinase inhibitor lapatinib by cytochrome P450 3A enzymes is implicated in the development of lapatinib-induced hepatotoxicity. Conjugative metabolism of the O-debenzylated phenolic metabolite of lapatinib (M1) via glucuronidation and sulfation is thought to be a major detoxication pathway for lapatinib in preclinical species (rat and dog), limiting formation of the quinoneimine reactive metabolite. Glucuronidation of M1 by human recombinant UDP-glucuronosyltransferases (UGTs) has been reported in vitro; however, the relative UGT enzyme contributions are unknown, and the inter-species differences in the conjugation vs. bioactivation pathways of M1 have not been fully elucidated. In the present study, reaction phenotyping experiments using human recombinant UGT enzymes and enzyme-selective chemical inhibitors demonstrated that UGT1A1 was the major hepatic UGT enzyme involved in lapatinib M1 glucuronidation. Formation of the M1-glucuronide by human liver microsomes from UGT1A1-genotyped donors was significantly correlated with UGT1A1 activity, as measured by 17β-estradiol 3-glucuronidation (R² = 0.90). Inter-species differences were found in the biotransformation of M1 in human, rat, and dog liver microsomal and S9 fractions via glucuronidation, sulfation, aldehyde oxidase-mediated oxidation, and bioactivation to the quinoneimine, trapped as GSH conjugate. Moreover, we demonstrated the sequential metabolism of lapatinib in primary human hepatocytes to the M1-glucuronide, M1-sulfate, and quinoneimine-GSH conjugate. M1 glucuronidation highly correlated with the rates of M1 formation, suggesting that O-dealkylation may be the rate-limiting step in lapatinib biotransformation. Inter-individual variability in the formation and clearance pathways of lapatinib M1 likely influences the hepatic exposure to reactive metabolites and may affect the risk for hepatotoxicity.
Significance Statement:

We used an integrated approach to examine the inter-individual and inter-species differences in detoxication vs. bioactivation pathways of lapatinib, which is associated with idiosyncratic hepatotoxicity. In addition to P450-mediated bioactivation, we report that multiple non-P450 pathways are involved in the biotransformation of the primary phenolic metabolite of lapatinib in vitro, including glucuronidation, sulfation, and aldehyde oxidase-mediated oxidation. UGT1A1 was identified as the major hepatic enzyme involved in lapatinib M1 glucuronidation, which may limit hepatic exposure to the potentially toxic quinoneimine.
Introduction

Lapatinib (Tykerb®) is an orally administered dual tyrosine kinase inhibitor of EGFR and HER2 approved for the treatment of advanced or metastatic HER2-positive breast cancer (Moy et al., 2007). Severe idiosyncratic liver injury has been associated with lapatinib in a subset of patients taking the drug, which limits its use in breast cancer therapy (Spraggs et al., 2011 and 2012). Several mechanisms, including drug metabolism, drug transport, and activation of immune responses, have been proposed to contribute to lapatinib-induced hepatotoxicity (Castellino et al., 2012); however, the actual mechanism(s) of the toxicity remain unclear.

Lapatinib is extensively metabolized in the liver by cytochrome P450 (CYP) 3A4 and CYP3A5 via three major routes: O-dealkylation, N-dealkylation, and N-hydroxylation (Castellino et al., 2012). Lapatinib and its O-debenzylated phenolic metabolite, M1, can also be oxidized by aldehyde oxidase (AO) on the quinazoline ring system to form “AO-M1” and M3, respectively (Dick 2018; Bissada et al., 2019). In human mass balance studies with [14C]lapatinib (250 mg single oral dose), the phenolic metabolites M1 and M3 were excreted in feces, accounting for a median of 3.9% and 3.3%, respectively, of the total dose; however, high inter-individual variability was reported (Castellino et al., 2012). The lapatinib N-hydroxylation and N-dealkylation pathways are thought to contribute to mechanism-based inactivation of CYP3A4 through formation of a nitroso intermediate leading to a metabolic intermediate complex with CYP3A4 (Teng et al., 2010; Takakusa et al., 2011).

The O-dealkylation pathway of lapatinib is implicated in the development of lapatinib-induced hepatotoxicity. Lapatinib M1 contains a para-hydroxyaniline moiety, similar to acetaminophen, which can undergo “phase II” conjugation via glucuronidation and sulfation or bioactivation to a reactive quinoneimine (Figure 1). The quinoneimine can readily react with glutathione (GSH) to form GSH conjugates and downstream cysteine conjugates (Teng et al.,
The electrophilic quinoneimine may covalently bind to proteins, leading to direct cell stress, haptenization, and/or activation of immune responses (Park et al., 2005; Castellino et al., 2012). We have previously shown that individual CYP3A activity influences the formation of lapatinib M1 and quinoneimine-GSH conjugates in human liver microsomes and primary human hepatocytes (Bissada et al., 2019). In the metabolically competent human hepatic HepaRG cell line (Aninat et al., 2006; Guillouzo et al., 2007), lapatinib M1 was more cytotoxic compared to the parent drug lapatinib; CYP3A4 induction in HepaRG cells enhanced lapatinib cytotoxicity and increased the formation of M1 and quinoneimine-cysteine conjugates (Hardy et al., 2014). Lapatinib M1 was also shown to induce mitochondrial stress and activate the antioxidant transcription factor Nrf2 in HepG2 cells (Eno et al., 2016). In patients treated with lapatinib, human leukocyte antigen (HLA) risk alleles were associated with the incidence of lapatinib-induced hepatocellular injury, suggesting involvement of immune-mediated toxicity (Spraggs et al., 2011, 2012).

Inter-individual and inter-species differences in the disposition of lapatinib may affect the risk of hepatotoxicity. Conjugative metabolism of lapatinib M1 via glucuronidation and sulfation is proposed as a detoxication pathway, reducing formation of the electrophilic quinoneimine (Castellino et al., 2012). In preclinical species (rat and dog), lapatinib O-dealkylation to the M1 phenol was the predominant metabolic clearance pathway in vivo, and glucuronide and sulfate conjugates of M1 were excreted into the bile (FDA, 2007; Castellino et al., 2012). However, these metabolic pathways for M1 have not yet been demonstrated in human subjects. Thus, whether glucuronide and sulfate conjugates of lapatinib M1 are formed in vivo in humans is not known. The lapatinib new drug application (FDA, 2007) indicates that human UDP-glucuronosyltransferases (UGTs): UGT 1A1, 1A3, 1A4, 1A8, 1A9, and 1A10, are capable of M1 glucuronidation in vitro; however, the relative enzyme contributions have not been described.
We hypothesize that the downstream metabolism of lapatinib M1 via conjugation vs. oxidation likely influences individual hepatic exposure to potentially toxic metabolites. The purpose of this investigation was to 1) identify the major UGT enzymes involved in lapatinib M1 glucuronidation, 2) compare the biotransformation pathways of lapatinib M1 between humans and preclinical species (rat and dog) in vitro, and 3) evaluate the sequential metabolism of lapatinib via conjugation vs. bioactivation. In this study, we used complementary reaction phenotyping approaches, including individual recombinant UGT enzymes, enzyme-selective chemical inhibitors, and commercially available UGT1A1-genotyped human liver microsomes, to characterize the glucuronidation pathway of lapatinib M1. We also examined the biotransformation of lapatinib M1 in liver microsomal and S9 fractions from human, rat, and dog. Further, we analyzed the sequential metabolism of lapatinib to phase II conjugates in primary human hepatocytes to evaluate the metabolic profile of lapatinib in an integrated system.
Materials and Methods

Chemicals and reagents. Lapatinib (free base, L-4899) and erlotinib (free base, E-4997) were purchased from LC Laboratories (Woburn, MA). Debenzylated lapatinib (M1) was chemically synthesized from lapatinib, as described previously (Teng et al., 2013). D<sub>4</sub>-debenzylated lapatinib (d<sub>4</sub>-M1) was synthesized by CoNCERT Pharmaceuticals, Inc., as described previously (Towles et al., 2016). PAPS (adenosine 3’-phosphate 5’-phosphosulfate lithium salt hydrate), magnesium chloride (MgCl<sub>2</sub>) hexahydrate, atazanavir, ketoconazole, and CYP3cide were purchased from Sigma Aldrich (St. Louis, MO). Reduced L-glutathione (GSH), dimethyl sulfoxide (DMSO), potassium phosphate monobasic, potassium phosphate dibasic, LC-MS grade Optima water, and LC-MS grade Optima acetonitrile were purchased from Fisher Scientific (Pittsburg, PA). Additional information regarding the preparation of chemical stock solutions and working solutions is provided in the Supplemental Materials and Methods section.

Corning® Gentest™ UGT Reaction Mix solution A (25 mM UDPGA) and solution B (5X UGT assay buffer with alamethicin), product numbers 451300 and 451320, respectively, were purchased from Corning Life Sciences (Tewksbury, MA). NADPH regenerating system solution A (26 mM NADP+, 66 mM glucose-6-phosphate, and 66 mM MgCl<sub>2</sub> in water) and solution B (40 U/ml glucose 6-phosphate dehydrogenase in 5 mM sodium citrate), product numbers 451220 and 451200, respectively, were also purchased from Corning Life Sciences. An enzyme mix of β-glucuronidase and arylsulfatase obtained from Helix pomatia in saline was purchased from Sigma Aldrich (catalog number 10127060001). All other materials, chemicals, and reagents utilized in the experiments were obtained from commercial sources and were of analytical grade or higher.
Liver microsomes, liver S9 fractions, and recombinant enzymes. Pooled human liver microsomes from 150 donors (mixed gender; 20 mg protein/ml; catalog number 452117; lot numbers 38294 and 38295) were purchased from Corning Life Sciences (Tewksbury, MA). Human liver microsomes from six individual donors genotyped for UGT1A1 were purchased from Sekisui XenoTech, LLC. (Kansas City, KS). The sample size was based on the number of commercially available UGT1A1-genotyped human liver microsomes. Donor information is shown in Supplemental Table S1. Donors included two males (M) and four females (F); the median age was 52.5 years (range 32-62 years). UGT1A1 genotype and microsomal enzyme activity values were provided by XenoTech. UGT1A1*1/*1 donor lots were 710412 (M) and 810002 (F); UGT1A1*1/*28 donors were 710414 (M), 710415 (F) and 710425 (F); the UGT1A1*28/*28 donor was 710411 (F). Pooled human liver S9 fraction from 150 donors (mixed gender; 20 mg/ml; catalog number 452116; lot number 38292) was purchased from Corning Life Sciences. Pooled dog liver microsomes (pool of 3 male Beagle dogs; 20 mg protein/ml; catalog number 452601; lot number 9350001), pooled rat liver microsomes (pool of approximately 79 Sprague-Dawley rats; 20 mg protein/ml; catalog number 452501; lot number 9259001), pooled dog liver S9 fraction (20 mg protein/ml; catalog number 452693; lot number 8253001), and pooled rat liver S9 fraction (20 mg protein/ml; catalog number 452591; lot number 9217001) were purchased from Corning Life Sciences. The following human recombinant UGT enzymes (Supersomes™, 5 mg protein/ml) were purchased from Corning Life Sciences: UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17 (catalog numbers: 456411, 456413, 456414, 456416, 456407, 456418, 456419, 456410, 456424, 456427, 453323, 456435, 456437, respectively).

Glucuronidation of lapatinib M1 in pooled human liver microsomes. Lapatinib M1 (2 μM) was incubated with pooled human liver microsomes (0.1 mg protein/ml) in purified deionized water supplemented with 2 mM UDPGA and alamethicin (UGT solutions A and B).
The samples were pre-incubated for 5 minutes at 37°C in a temperature-controlled shaking water bath. After the 5-minute pre-incubation, UDPGA (final concentration 2 mM) was added to initiate the reactions, and the reaction mixtures were returned to the 37°C shaking water bath. Control incubations were without UDPGA. The final incubation volume was 200 µl, and the final organic solvent concentration was 1% (0.1:0.9 % DMSO/acetonitrile, v/v). After time points at 0, 30, and 60 minutes, 400 µl of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard) was added to quench the reactions and precipitate the protein. The solutions were mixed with a vortex device and then centrifuged at 20,000 x g at 4°C for 20 minutes. A 200-µl aliquot of the clear supernatant was transferred to a separate LC-MS vial, and the samples were stored at -20°C prior to analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS).

Additional experiments were conducted to further examine the time-course of M1 glucuronidation in pooled human liver microsomes. M1 (2 µM) was incubated with pooled human liver microsomes (0.1 mg protein/ml) over various time points (0, 5, 10, 20, 30, 45, 60 minutes). Reactions were supplemented with 2 mM UDPGA and alamethicin, and the final reaction volume was 1.0 ml. After each time point, a 100-µl aliquot of the reaction mixture was removed and combined with an equal volume (100 µl) of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard). Samples were prepared as described above, and formation of M1-glucuronide conjugates was analyzed by LC-MS/MS. Product formation was linear up to 30 minutes.

**Glucuronidation of lapatinib M1 by recombinant UGT enzymes.** Metabolic incubations with human recombinant UGT enzymes (Supersomes™) were similar to those described above with pooled human liver microsomes. Briefly, M1 (0.2 and 2 µM) was incubated
with individual recombinant UGT enzymes (UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17) and pooled human liver microsomes (0.2 mg protein/ml) in purified deionized water supplemented with 2 mM UDPGA and alamethicin. The samples were pre-incubated for 5 minutes at 37°C in a shaking water bath. After the 5-minute pre-incubation, UDPGA (2 mM) was added to initiate the reactions, and the samples were returned to the 37°C shaking water bath. Control incubations were without UDPGA. The final incubation volume was 200 μl, and the final organic solvent concentration was 1% (0.1:0.9 % DMSO/acetonitrile, v/v).

After a 30-minute incubation period, 400 μl of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard) was added to quench the reactions and precipitate the protein. The solutions were mixed with a vortex device and then centrifuged at 3700 x g at 4°C for 20 minutes. A 200-μl aliquot of the clear supernatant was transferred to separate LC-MS vials, and the samples were stored at -20°C prior to LC-MS/MS analysis.

**Effect of UGT1A1 inhibition on lapatinib M1 glucuronidation.** Experiments were conducted to confirm the role of UGT1A1 in the glucuronidation of M1 using UGT1A1 inhibitors atazanavir and erlotinib (Zientek and Youdim, 2015), similar to the methods described by Zientek et al. (2016). M1 (2 μM) was incubated with 150-donor pooled human liver microsomes (0.1 mg protein/ml) in the presence and absence of UGT1A1 inhibitors atazanavir (1 and 10 μM) and erlotinib (3 and 10 μM). Vehicle control incubations were without inhibitors in the presence of solvent (1:9 DMSO/methanol, v/v). The final organic solvent concentration in the incubations was 0.2% DMSO, 0.9% acetonitrile, 0.9% methanol. The final reaction volume was 200 μl.

Reactions were initiated with the addition of UDPGA (2 mM) and were conducted for 30 minutes in a shaking water bath at 37°C. Following the incubation period, an equal volume (200 μl) of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard) was added to quench the reactions and precipitate the protein. Samples were processed as described above prior to LC-
MS/MS analysis. Formation of M1-glucuronide conjugates was compared to vehicle control incubations without inhibitors.

**Metabolism of lapatinib M1 in UGT1A1-genotyped human liver microsomes.**

Metabolic incubations with human liver microsomes from pooled and individual UGT1A1-genotyped donors were similar to those described above for liver microsomes with slight modifications. Briefly, M1 (2 \( \mu \)M) was incubated with pooled and individual genotyped human liver microsomes (0.2 mg protein/ml) in 100 mM potassium phosphate (pH 7.4) supplemented with 5 mM GSH, NADPH regenerating system, and 2 mM UDPGA. The samples were pre-incubated for 5 minutes at 37°C in a shaking water bath. Reactions were initiated with the addition of UDPGA and NADPH, and samples were incubated for 30 minutes at 37°C in a shaking water bath. Control incubations were without NADPH and UDPGA. The final incubation volume was 200 \( \mu \)l, and the final organic solvent concentration was 1% (0.1:0.9 % DMSO/acetonitrile, v/v). After a 30-minute incubation period, 200 \( \mu \)l of ice-cold acetonitrile containing 100 ng/ml \( \text{d}_4 \)-M1 (internal standard) was added to quench the reactions and precipitate the protein. The solutions were mixed with a vortex device and then centrifuged at 3700 x g at 4°C for 20 minutes. Approximately 350 \( \mu \)l of the clear supernatant was transferred to separate vials, and the solvent was evaporated to dryness using a vacuum centrifugation system to concentrate the samples. The sample residue was re-dissolved in 100 \( \mu \)l of 80:20 water/acetonitrile (v/v), mixed with a vortex device, centrifuged at 20,000 x g at room temperature, and the clear supernatant was transferred to LC-MS vials for LC-MS/MS analysis.

**Metabolism of lapatinib M1 in pooled liver microsomes from human, rat, and dog.**

M1 (2 \( \mu \)M) was incubated with pooled human, rat, and dog liver microsomes (0.1 mg protein/ml) in 100 mM potassium phosphate (pH 7.4) supplemented with 5 mM GSH, NADPH regenerating
system, and 2 mM UDPGA. The samples were pre-incubated for 5 minutes at 37°C in a shaking water bath. Reactions were initiated by the addition of NADPH and UDPGA, and incubations were conducted at 37°C for 30 minutes. Control incubations were conducted in the absence of cofactors NADPH and UDPGA. After a 30-minute incubation period, reactions were stopped by the addition of an equal volume (200 μl) of ice-cold acetonitrile containing internal standard (100 ng/ml d₄-M1), and the samples were processed similar to the methods described above for experiments with \textit{UGT1A1}-genotyped human liver microsomes.

**Metabolism of lapatinib M1 in pooled human liver S9 fraction.** Initial studies were conducted to examine the time-course of M1 sulfation in pooled human liver S9 fraction. M1 (2 μM) was incubated with 150-donor pooled human liver S9 fraction (2 mg protein/ml) in 100 mM potassium phosphate, pH 7.4. Magnesium chloride (final concentration 2.5 mM) was added to stimulate SULT activity (Bansal and Lau, 2016). Reactions were initiated by addition of PAPS (final concentration 0.1 mM), and the final reaction volume was 1.0 ml. Control incubations were without PAPS. Incubations were conducted over time (0, 5, 10, 15, 20, 30, 45, 60 minutes) in a shaking water bath at 37°C. At each time point, a 100-μl aliquot of the reaction mixture was removed and combined with an equal volume (100 μl) of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard). Samples were processed as described above for experiments with recombinant UGT enzymes. Formation of M1-sulfate conjugates was analyzed by LC-MS/MS. Product formation was linear up to 10 minutes of incubation.

**Metabolism of lapatinib M1 in pooled liver S9 fractions from human, rat, and dog.** M1 (1 μM) was incubated with pooled human, rat, and dog liver S9 fractions (2.0 mg protein/ml) in 100 mM potassium phosphate, pH 7.4, supplemented with MgCl₂ (2.5 mM), PAPS (0.1 mM), and UDPGA (2 mM). Control incubations were without PAPS and UDPGA. The final reaction
volume was 500 μl. Reactions were initiated with addition of substrate, and the incubations were conducted in a shaking water bath at 37°C. At time points: 0, 5, 10, 15, and 20 minutes, an 80-μl aliquot was removed from the reaction mixtures and combined with an equal volume (80-μl) of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard). Samples were mixed with a vortex device, placed on ice, and centrifuged at 3700 x g at 4°C for 20 minutes to pellet the protein. The clear supernatant (approximately 100 μl) was transferred to a clean LC-MS vial for analysis of substrate depletion and metabolite formation. Samples were stored at -20°C prior to LC-MS/MS analysis. Relative levels of M1 and M1 metabolites (M1-glucuronide, M1-sulfate, and M3) were measured by LC-MS/MS analysis (see LC-MS/MS analysis below). The percentage (%) of remaining M1 substrate was calculated based on the M1 peak area ratio at time 0 minutes.

**Deconjugation of M1-glucuronide and M1-sulfate by β-glucuronidase/sulfatase**

The susceptibility of the M1-glucuronide and M1-sulfate conjugate to be hydrolyzed by β-glucuronidase and sulfatase, respectively, was assessed using a method similar to that described by Huang et al. (2017). To form the M1-glucuronide, M1 (2 μM) was incubated with pooled human liver microsomes (0.2 mg/ml) in the presence of UDPGA (2 mM) and alamethicin for 30 minutes at 37°C. For the M1-sulfate, M1 (2 μM) was incubated with pooled human liver S9 fraction (2.0 mg/ml) in the presence of PAPS (0.1 mM) and MgCl₂ (2.5 mM) for 30 minutes at 37°C. Following the incubation period, reactions were quenched by addition of an equal volume (400 μl) of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard). The solutions were mixed with a vortex device and then centrifuged at 20,000 x g at 4°C for 20 minutes. Approximately 700-750 μl of the clear supernatant was transferred to a separate clean vial, and the solvent was evaporated to dryness using a vacuum centrifugation system. The sample residue was re-dissolved in 240 μl of 10 mM ammonium acetate buffer (pH 5.0) and mixed with
a vortex device for 30 seconds. The samples were aliquoted into three vials of 80 µl each; 20 µl of β-glucuronidase/arylsulfatase solution was added to the first aliquot for a 1:5 dilution (v/v, final volume 100 µl) according to the supplier’s instructions; 20 µl of 10 mM ammonium acetate buffer was added to the second (control without β-glucuronidase/arylsulfatase) and third (untreated) aliquots for a final volume of 100 µl in each vial. Samples (aliquots 1 and 2) were incubated with β-glucuronidase/sulfatase or buffer control for 4 hours at 37°C. Following the incubation, 300 µl of ice-cold acetonitrile containing 100 ng/ml d₄-M1 was added. The same volume (300 µl) of internal standard was added to untreated samples (aliquot 3) without the 4-hour incubation for comparison. The samples were centrifuged at 20,000 x g at room temperature, and the clear supernatant (200 µl) was transferred to LC-MS vials and stored at -20°C prior to LC-MS/MS analysis. Relative levels of the M1-glucuronide and M1-sulfate were compared with and without incubation with β-glucuronidase/sulfatase.

**Metabolism of lapatinib in primary human hepatocytes.** Pooled cryopreserved human hepatocytes (LiverPool®) from 5 donors (mixed gender) were purchased from BioIVT, as described previously (Bissada et al., 2019). Briefly, lapatinib (1 and 10 µM) was incubated with pooled human hepatocytes (0.5 x 10⁶ cells/ml) in suspension in Krebs-Henseleit buffer (InVitroGRO KHB, BioIVT) in a 24-well collagen-coated plate for 2 hours at 37°C on a temperature-controlled plate-shaker (Bissada et al., 2019). Control incubations were without cells. Incubations were conducted in duplicate per condition. Following the incubation period, an equal volume (0.5 ml) of ice-cold acetonitrile containing 100 ng/ml d₄-M1 (internal standard) was added to each well, and the cell suspension was transferred to clean microcentrifuge tubes, mixed with a vortex device, and centrifuged for 20 minutes at 20,000 x g at 4°C. The supernatant was transferred to separate microcentrifuge tubes, and the solvent was evaporated to dryness under nitrogen gas (N₂) using a TurboVap evaporation system (Biotage, Charlotte,
NC). The sample residue was re-dissolved in 200 µl of 80:20 LC-MS grade water/LC-MS grade acetonitrile, and the samples were mixed with a vortex device and centrifuged for 5 minutes at 20,000 x g at room temperature (Bissada et al., 2019). Lapatinib metabolites from the clear supernatant were analyzed by LC-MS/MS analysis, as described below.

Cryopreserved human hepatocytes (CryostaX Geneknown™) from CYP3A5-genotyped donors were purchased from XenoTech (Bissada et al., 2019). Cryopreserved human hepatocytes were from a pool of 3 donors for each CYP3A5 genotype: CYP3A5*3/*3, CYP3A5*1/*3, and CYP3A5*1/*1 (lot numbers 1510230, 1510229, and 1510228, respectively). Lapatinib (1 and 10 µM) was incubated with CYP3A5-genotyped human hepatocytes according to the methods described above for 5-donor pooled hepatocytes. Incubations were conducted in replicates of 4-5 per hepatocyte lot. In a separate series of experiments, lapatinib (10 µM) was incubated in suspension with single-donor cryopreserved human hepatocytes from 15 individual CYP3A5-genotyped donors, as described by Bissada et al. (2019). Single-donor cryopreserved human hepatocytes were purchased from BioIVT. Individual donor information and experimental methods were reported previously (Bissada et al., 2019).

**LC-MS/MS Analysis.** Lapatinib and lapatinib metabolites were analyzed by LC-MS/MS similar to the methods described previously (Bissada et al., 2019; Towles et al., 2016; Hardy et al., 2014). Two LC-MS/MS systems were used for metabolite analysis from independent experiments (see below); comparisons of metabolite formation within the same set of experiments were conducted using the same LC-MS/MS system for consistency.

LC-MS/MS system 1 was used for the detection of lapatinib M1 and M1 metabolites from experiments with recombinant enzymes, liver microsomes, and liver S9 fractions. LC-MS/MS
system 1 consisted of a Thermo TSQ Quantum triple quadrupole mass spectrometer, and a Waters Acquity ultra-performance liquid chromatography (UHPLC) system equipped with a temperature-controlled autosampler and column oven. Mobile phase A consisted of LC-MS-grade Optima water with 0.1% formic acid (v/v). Mobile phase B consisted of LC-MS grade Optima acetonitrile with 0.1% formic acid (v/v). A 10-μl aliquot of sample was injected into the equilibrated system with a flow rate of 0.3 ml/min, and metabolite separation was achieved with a Phenomenex Kinetex C18 octadecylsilane column (2.6 μm, 50 x 2.1 mm, 100 Å) (Phenomenex, Torrance, CA). The column oven temperature was 40°C. Details describing the LC gradient program and MS/MS instrument parameters for LC-MS/MS system 1 are provided in the Supplemental Materials and Methods section. Based on the chromatographic conditions stated in the Supplemental Methods for LC-MS/MS system 1, M1 and M1 metabolites eluted from 1.8-2.0 minutes. LC-MS/MS system 2 was used for analysis of lapatinib metabolites from experiments with cryopreserved human hepatocytes. LC-MS/MS system 2 consisted of a Shimadzu Prominence XR UHPLC coupled to a Shimadzu MS/MS 8030 triple quadrupole mass spectrometer (Bissada et al., 2019). The LC-MS/MS method for metabolite analysis and quantitation of M1 from the experiments with primary human hepatocytes was described by Bissada et al. (2019).

Selected reaction monitoring (SRM) was employed to selectively detect lapatinib M1 and its metabolites. The following precursor-to-product ion transitions were analyzed in positive ion mode, and the precursor ions represent the respective [M+H]^+ species: lapatinib M1 (m/z 473 > 350), M1-glucuronide conjugate (m/z 649 > 473), M1-sulfate conjugate (m/z 553 > 473), M3 (m/z 489 > 366), M1-derived quinoneimine-GSH conjugate (m/z 778 > 655), and internal standard, d4-M1 (m/z 477 > 352), similar to the methods described previously (Bissada et al., 2019; Towles et al., 2016; Hardy et al., 2014). In the absence of authentic standards for the M1-
glucuronide conjugate, M1-sulfate conjugate, M3, and quinoneimine-GSH conjugate, the LC-SRM peak area or peak area ratios (the ratio of analyte peak area/internal standard peak area) were used to measure relative metabolite levels. The LC-MS/MS data were analyzed using Xcalibur 3.0 software (Thermo Scientific) and LabSolutions 5.93 software (Shimadzu) for LC-MS/MS systems 1 and 2, respectively.

**Structural characterization of the quinoneimine-GSH conjugate**

To confirm the structural identity of the quinoneimine-GSH conjugate, we conducted additional studies with M1 and d₄-M1 as substrates. M1 and d₄-M1 (2 μM), were incubated with pooled human liver microsomes (0.2 mg/ml) in the presence of GSH (5 mM) and NADPH regenerating system for 30 minutes at 37°C. The resulting quinoneimine-GSH conjugates derived from M1 and d₄-M1 were analyzed by LC-MS/MS in positive ion mode using collision dissociation (collision energy 29 V) for the predicted precursor ions: M1-GSH conjugate (m/z 778) and d₄-M1-GSH conjugate (m/z 782). Based on the major product ions observed from MS/MS analysis, the following precursor-to-product ion transitions were used for SRM analysis of M1 and d₄-M1 derived quinoneimine-GSH conjugates: M1-derived GSH conjugate (m/z 778 > 655) and d₄-M1-derived GSH conjugate (m/z 782 > 657). SRM analysis of the M1-derived GSH conjugate has been described previously (Bissada et al., 2019; Towles et al., 2016).

**Data analysis.** The LC-MS/MS data (integrated peak areas for each analyte of interest) were exported to Microsoft Excel for initial analysis, and the data were further analyzed and graphed using GraphPad Prism 8 software (GraphPad, San Diego, CA). As noted above, metabolite peak area ratios were determined by the ratio of analyte peak area to internal standard, d₄-M1, peak area. Statistical analyses were performed using GraphPad Prism 8 to calculate descriptive statistics: mean and standard deviation (SD). GraphPad Prism 8 software was also used for linear regression and Pearson r correlation analyses and for Grubbs’ outlier
analysis (Alpha = 0.05). Substrate depletion data were natural log (Ln) transformed and analyzed by non-linear regression using a one-phase decay model to estimate the \textit{in vitro} depletion rate constants ($k$) for M1 using GraphPad Prism 8. For experiments with \textit{UGT1A1}-genotyped human liver microsomes, sample size was based on the number of commercially available samples. Statistical significance was considered at $p < 0.05$.

\section*{Results}

\textit{Glucuronidation of lapatinib M1 in pooled human liver microsomes}

Incubation of lapatinib M1 (2 $\mu$M) with pooled human liver microsomes supplemented with UDPGA resulted in an increase in M1-glucuronide product formation over time (\textbf{Figure 2}). In a more detailed analysis examining the time-course of M1 glucuronidation, product formation was linear up to 30 minutes (data not shown); thus, 30 minutes was selected as the incubation time for subsequent experiments.

LC-MS/MS analysis of the M1-glucuronide is shown in \textbf{Supplemental Figure S1}. The M1-glucuronide has a predicted [M+H]$^+$ at $m/z$ 649 in positive ion mode. Collision-induced dissociation of the precursor ion $m/z$ 649 yielded the product ion spectrum shown. Loss of the glucuronic acid moiety resulted in the aglycone fragment at $m/z$ 473 [M+H – 176]$^+$, which corresponds to M1. Neutral loss of 176 Da is characteristic of glucuronide conjugates. Secondary fragmentation yielded the product ion $m/z$ 350, which corresponds to cleavage at the secondary amine of lapatinib M1 (Castellino et al., 2012).

\textit{Formation of lapatinib M1-glucuronide by recombinant UGT enzymes}
Reaction phenotyping experiments were conducted to identify the major UGT enzymes involved in M1 glucuronidation. UGT1A1 and UGT1A8 were the major enzymes to form the M1-glucuronide, with minor contributions from UGT1A3, UGT1A9, and UGT1A10 (Figure 3). These data are consistent with a previous report regarding M1 glucuronidation (FDA, 2007). While UGT1A8 is expressed extra-hepatically, UGT1A1 is highly expressed in the liver (Guillemette et al., 2014). Thus, UGT1A1 is likely the primary hepatic enzyme involved in glucuronidation of lapatinib M1.

Effect of UGT1A1 inhibition on lapatinib M1 glucuronidation

In incubations with pooled human liver microsomes, UGT1A1 inhibitor atazanavir (1 and 10 µM) reduced formation of the M1-glucuronide to (mean ± SD) 59.1 ± 7.1 % and 20.1 ± 11.6 %, respectively, compared to control levels without inhibitor. Erlotinib (3 and 10 µM) decreased M1-glucuronide formation to 40.7 ± 2.0 % and 18.2 ± 6.2%, respectively, compared to control levels (Figure 4).

Effect of UGT1A1 variation on lapatinib M1 glucuronidation

Based on the finding that UGT1A1 was the major hepatic UGT enzyme involved in lapatinib M1 glucuronidation, we explored M1 glucuronidation in human liver microsomes from UGT1A1-genotyped donors. The UGT1A1*1 wild-type allele, which contains six TA repeats in the UGT1A1 gene promoter, results in fully functional enzyme activity; whereas, the common UGT1A1*28 variant allele, which contains seven TA repeats, results in “low promoter activity” and reduced enzyme expression (Guillemette et al., 2014).

To test whether UGT1A1 genetic variation affects M1 glucuronidation, M1-glucuronide formation was compared in a small set of human liver microsomes from individual donors.
genotyped for *UGT1A1*. The sample size was based on the number of commercially available *UGT1A1*-genotyped human liver microsomes, and only six donors were available. Donor information is shown in Supplemental Table S1. The donor genotypes were *UGT1A1*/*1*/*1* (n = 2); *UGT1A1*/*1*/*28* (n = 3); and *UGT1A1*/*28*/*28* (n = 1). UGT1A1 activity for each donor was characterized by XenoTech based on the marker reaction 17β-estradiol 3-glucuronidation, and CYP3A activity was measured based on testosterone 6β-hydroxylation and midazolam 1'-hydroxylation (XenoTech). One donor lot 710414 (*UGT1A1*/*1*/*28*) had markedly higher UGT1A1 activity (2290 pmol/mg protein/min 17β-estradiol 3-glucuronidation) compared to other donors. This donor lot 710414 also had the highest CYP3A activity (10000 pmol/mg protein/min testosterone 6β-hydroxylation and 991 pmol/mg protein/min midazolam 1'-hydroxylation) among the individual liver microsomal samples (Supplemental Table S1).

**Figure 5** shows a comparison of lapatinib M1 glucuronidation and quinoneimine-GSH conjugate formation between human liver microsomal fractions from individual *UGT1A1*-genotyped donors as well as pooled human liver microsomes. The relative levels of M1-glucuronide varied 4.8-fold (range, mean ± SD peak area ratio: 0.75 ± 0.19 to 3.6 ± 0.77) between human liver microsomes from pooled and individual genotyped donors (Figure 5A). Donor lot 710414 was identified as an outlier among *UGT1A1*/*1*/*28* donors based on lapatinib M1 glucuronidation (Grubbs’ outlier analysis; data not shown). Donor lot 710414 (*UGT1A1*/*1*/*28*), which had the highest UGT1A1 activity, formed the highest relative levels of M1-glucuronide; while donor lot 710411 (*UGT1A1*/*28*/*28*), which had the lowest UGT1A1 activity, had the lowest levels of M1-glucuronide. Notably, formation of the M1-glucuronide was highly correlated with UGT1A1 activity, as measured by 17β-estradiol 3-glucuronidation (R² = 0.90, p = 0.0010, n = 7) (Figure 6A).
When comparing the relative levels of the quinoneimine-GSH conjugate among 
UGT1A1-genotyped donors, levels of the quinoneimine-GSH conjugate varied 4.2-fold (range, 
mean ± SD peak area ratio: 0.007 ± 0.002 to 0.028 ± 0.005) between human liver microsomes 
from pooled and individual genotyped donors (Figure 5B). Donor lot 710414 (UGT1A1*1/*28) 
had the highest levels of quinoneimine-GSH conjugate. As noted above, this donor also had the 
highest CYP3A activity. Formation of the quinoneimine-GSH conjugate was positively correlated 
with CYP3A activity, as measured by testosterone 6β-hydroxylation (R² = 0.78, p = 0.0087, n = 
7) (Figure 6B). Lower levels of quinoneimine-GSH conjugate were also observed in microsomal 
incubations without NADPH supplementation (Figure 5B). To further investigate this 
observation, we conducted a subsequent analysis by incubating pooled human liver 
microsomes with lapatinib M1 in the presence and absence of NADPH and with buffer only. The 
results demonstrated that the generation of quinoneimine-GSH conjugate in pooled human liver 
microsomes was primarily NADPH-dependent (Supplemental Figure S2). Formation of the 
quinoneimine-GSH conjugate in the absence of NADPH supplementation was minimal, and no 
quinoneimine-GSH conjugate was detected in incubations of lapatinib M1 with buffer alone 
without human liver microsomes (Supplemental Figure S2).

Structural characterization of the quinoneimine-GSH conjugate

Additional studies comparing LC-MS/MS analysis of quinoneimine-GSH conjugates 
derived from M1 and d₄-M1 provided further support for the structural identity of the 
quinoneimine-GSH conjugate. The predicted precursor ion [M+H]⁺ of the M1-derived GSH 
conjugate is m/z 778. A 4-Da mass increase in the precursor ion (m/z 782) is expected for the 
deuterium-labeled quinoneimine-GSH conjugate derived from d₄-M1. The MS/MS product ion 
spectra from analysis of the M1-derived GSH conjugate (m/z 778) and d₄-M1-derived GSH 
conjugate (m/z 782) are shown in Supplemental Figure S3. Consistent with previous reports 
(Teng et al., 2010; Hardy et al., 2014), neutral loss of 123 Da from the lapatinib portion of the
M1-derived quinoneimine-GSH conjugate yielded the major product ion \( m/z \) 655 (Figure S3A). Cleavage of lapatinib about the secondary amine is a characteristic fragmentation pattern for the molecule resulting in neutral loss of 123 Da (Teng et al., 2010; Castellino et al., 2012). Neutral loss of 125 Da from the deuterium-labeled portion of the \( d_4 \)-M1 quinoneimine-GSH conjugate yielded the major product ion \( m/z \) 657 (Figure S3B). We have previously detected the M1- and \( d_4 \)-M1-derived GSH conjugates through neutral loss scanning of 129 Da (data not shown). Neutral loss of 129 Da is characteristic for GSH conjugates based on cleavage of the pyroglutamic acid moiety (Evans et al., 2004); however, neutral loss of 123 Da from the lapatinib portion of the molecule generated the most prominent product ion from MS/MS analysis of the M1-derived GSH conjugate (Teng et al., 2010; Hardy et al., 2014). SRM analysis of the M1-derived GSH conjugate \( (m/z \ 778 > 655) \) and the \( d_4 \)-M1-derived GSH conjugate \( (m/z \ 782 > 657) \) formed from human liver microsomal incubations is shown in Supplemental Figure S4.

Species comparison of microsomal metabolism of lapatinib M1

Next, we examined species differences in the metabolic profile of lapatinib M1 in liver tissue fractions. We compared the relative extent of M1 glucuronidation vs. quinoneimine-GSH conjugate formation in liver microsomal fractions from human, rat, and dog. Figure 7 shows the results from this analysis. In microsomal incubations containing UDPGA and NADPH, the relative levels of the M1-glucuronide formed by human, rat, and dog liver microsomes were (mean ± SD peak area ratio) 2.7 ± 0.3, 1.4 ± 0.7, and 5.2 ± 0.9, respectively. Overall, M1-glucuronide formation was highest in liver microsomal incubations from dog compared to rat and human in the following order (from highest to lowest relative levels): dog > human > rat (Figure 7A).

The relative levels of quinoneimine-GSH conjugate formed were similar between human, rat, and dog liver microsomes in incubations containing both NADPH and UDPGA (Figure 7B).
However, the extent of quinoneimine-GSH conjugate formation differed between species when comparing microsomal incubations supplemented with NADPH alone without UDPGA. The GSH conjugate was 3.2-fold higher in human liver microsomes (mean ± SD: 0.055 ± 0.010 peak area ratio) compared to rat liver microsomes (0.017 ± 0.006 peak area ratio) and 1.4-fold higher in human liver microsomes compared to dog liver microsomes (0.040 ± 0.009 peak area ratio). Overall, in incubations containing NADPH alone (-UDPGA), GSH conjugate formation was highest in liver microsomal incubations from human compared to rat and dog in the following order (from highest to lowest relative levels): human > dog > rat (Figure 7B).

In addition, for incubations with human and dog liver microsomes, the relative levels of GSH conjugate formation differed when comparing reaction conditions with and without NADPH and UDPGA. Specifically, the quinoneimine-GSH conjugate was 2.8-fold higher in human (0.055 ± 0.010 vs. 0.019 ± 0.003 peak area ratio) and dog (0.040 ± 0.009 vs. 0.014 ± 0.003 peak area ratio) liver microsomal incubations supplemented with NADPH alone, compared to the levels formed in the presence of both NADPH and UDPGA. The differences in the levels of GSH conjugate formed in the presence of NADPH with and without UDPGA suggest that glucuronidation is a competing pathway for M1 metabolism vs. bioactivation of M1 to the quinoneimine. The levels of GSH conjugate formed in rat liver microsomes did not significantly differ between incubations supplemented with NADPH in the presence (0.017 ± 0.006 peak area ratio) or absence of UDPGA (0.014 ± 0.007 peak area ratio).

Liver S9 fractions contain microsomal enzymes (e.g. P450s and UGTs) and cytosolic enzymes (e.g. SULTs and AO); thus, we used liver S9 fractions from human, rat, and dog to evaluate the metabolic profile of M1 in a more integrated system. For this analysis, we monitored the depletion of M1 and the formation of M1-glucuronide and M1-sulfate over time in liver S9 fractions supplemented with UDPGA (UGT cofactor) and PAPS (SULT co-substrate).
We also analyzed the formation of M3, a metabolite generated from AO-mediated metabolism of lapatinib M1 (Castellino et al., 2012; Dick 2018). The results from this analysis are shown in Figure 8. Representative LC-SRM chromatograms of M1, M3, M1-sulfate, and M1-glucuronide are shown in Supplemental Figure S5. The overall rate of M1 depletion was highest in the human liver S9 fraction, compared to rat and dog (Figure 8A). The estimated one-phase decay rate constants, $k$, for M1 depletion in incubations with human, rat, and dog liver S9 fractions supplemented with PAPS and UDPGA were 0.23 minute$^{-1}$, 0.09 minute$^{-1}$, and 0.13 minute$^{-1}$, respectively. Dog liver S9 formed higher relative levels of M1-glucuronide, compared to human and rat (Figure 8B), while human liver S9 formed higher relative levels of M1-sulfate, compared to rat and dog (Figure 8C). M3 formation was markedly higher in human liver S9 fraction compared to rat (Figure 8D). Dogs lack liver AO activity (Terao et al., 2006); thus, as expected, M3 was not formed in the dog liver S9 fraction (Figure 8D).

Deconjugation of M1-glucuronide and M1-sulfate by $\beta$-glucuronidase/sulfatase

The M1-glucuronide and M1-sulfate were susceptible to hydrolysis by $\beta$-glucuronidase and arylsulfatase. As shown in Supplemental Figure S6, the M1-glucuronide was reduced in samples incubated with $\beta$-glucuronidase/arylsulfatase, compared to control incubations without hydrolysis. Similar findings were observed from hydrolysis of the M1-sulfate (Supplemental Figure S7). These results provide strong evidence for the structural assignment of the M1-glucuronide and M1-sulfate.

Sequential metabolism of lapatinib in primary human hepatocytes

Human hepatocytes provide an integrated system for drug metabolism studies because they contain drug metabolizing enzymes, transporters, and enzyme cofactors at physiologically relevant concentrations. The maximum total plasma concentration ($C_{max}$) of lapatinib at...
therapeutic doses is approximately 5 μM; the hepatic concentrations of lapatinib are predicted to be 5-10-fold higher compared to concentrations in plasma (Hudachek and Gustafon, 2013). Therefore, we examined the sequential metabolism of lapatinib (1 and 10 μM) in cryopreserved pooled human hepatocytes leading to the formation of M1-glucuronide and M1-sulfate, and we examined the bioactivation pathway of lapatinib leading to formation of the reactive quinoneimine, detected as a GSH conjugate. The results from this analysis demonstrated that M1, M1-glucuronide, and M1-sulfate as well as the quinoneimine-GSH conjugate were formed in hepatocytes in a concentration-dependent manner (Figure 9).

We have previously shown that lapatinib M1 formation from lapatinib was significantly correlated with CYP3A activity (Bissada et al., 2019). M1 formation was higher in pooled human hepatocytes from CYP3A5 expressers (CYP3A5*1/*3 and CYP3A5*1/*1 donors), which had higher CYP3A activity, compared to CYP3A5*3/*3 donors, which have low or no CYP3A5 expression (Kuehl et al., 2001) and had lower CYP3A activity (Bissada et al., 2019). As a follow up to this analysis, we examined the sequential metabolism of lapatinib leading to M1 glucuronidation and sulfation in pooled human hepatocytes from CYP3A5-genotyped donors. The results from this analysis are shown in Figure 10. The higher relative formation of M1-glucuronide in CYP3A5 expressers mirrors the 2.4-fold higher levels of M1 formed from lapatinib in CYP3A5*1/*3 and CYP3A5*1/*1 donors, compared to CYP3A5*3/*3 donors, as reported previously (Bissada et al., 2019). On the other hand, the mean relative levels of the M1-sulfate conjugate varied between CYP3A5*1/*3 and CYP3A5*1/*1 donors compared to CYP3A5*3/*3 donors.

Correlation of M1-glucuronide with lapatinib M1 formation from lapatinib in human hepatocytes
Next, we evaluated the relationship between lapatinib M1 formation and M1-glucuronidation in 15 individual-donor primary human hepatocytes incubated with lapatinib (10 μM). The relative levels of the M1-glucuronide varied 71-fold between donors, and M1-glucuronide formation was highly correlated with the rates of M1 formation ($R^2 = 0.88$, $p < 0.0001$, $n = 15$) (Figure 11).

Discussion

Lapatinib O-dealkylation to the phenolic metabolite M1 is one of the major routes of lapatinib metabolic clearance in vivo. Other routes of lapatinib metabolism include N- and α-carbon oxidation to form a series of circulating metabolites (Castellino et al., 2012). Hepatobiliary disposition of lapatinib M1 via bioactivation, phase II conjugation, and/or interaction with drug efflux transporters is implicated in the hepatotoxicity of lapatinib (Polli et al., 2008; Castellino et al., 2012). Conjugation of M1 to glucuronic acid or sulfate is thought to be a detoxication pathway to reduce subsequent bioactivation of M1 to the reactive quinoneimine metabolite, which may covalently modify cysteine residues of proteins leading to cellular injury (Park et al., 2005). In this study, we conducted a detailed investigation of lapatinib M1 conjugative and oxidative metabolism using relevant hepatic in vitro systems to evaluate the inter-species and inter-individual differences in these downstream clearance pathways.

To our knowledge, this is the first study to demonstrate that UGT1A1 is the major hepatic UGT enzyme involved in the glucuronidation of lapatinib M1. Several lines of evidence from complementary reaction phenotyping experiments support this conclusion: 1) screens with a panel of human recombinant UGT enzymes demonstrated that UGT1A1 (hepatic) and UGT1A8 (extrahepatic) generated the highest relative levels of M1-glucuronide; 2) UGT1A1 inhibitors atazanavir and erlotinib reduced M1-glucuronide formation in pooled human liver microsomes; and 3) formation of the M1-glucuronide in human liver microsomes from $UGT1A1$-
genotyped donors was highly correlated with UGT1A1 activity. The observation that donor 710414 (UGT1A1*1/*28) had the highest UGT1A1 activity compared to other donors suggests that factors besides UGT1A1 genotype likely influence UGT1A1 activity (Peterkin et al., 2007). Lot 710414 was an outlier with respect to M1-glucuronide formation compared to other UGT1A1*1/*28 donors. This donor had high enzyme activities for both UGT1A1 and CYP3A, suggesting that other factors, such as enzyme induction and/or regulatory gene variants, may have contributed to the high metabolic activity (Gardner-Stephen et al., 2004; Sugatani et al., 2005); however, the medical history of this donor is unknown. UGT1A3*2 polymorphism is also linked to UGT1A1*28 and causes increased UGT1A3 activity (Cho et al., 2012). A limitation of this study was the small sample size of commercially available UGT1A1-genotyped human liver microsomes. Future investigations with a larger number of samples are needed to further explore the genotype-phenotype relationships between UGT1A1 and lapatinib M1 glucuronidation.

UGT1A1 is abundantly expressed in the liver and is known to be highly polymorphic. UGT1A1 catalyzes the glucuronidation of bilirubin and other endogenous substrates (i.e. β-estradiol, bile acids) as well as xenobiotics (i.e. irinotecan active metabolite SN-38) (Guillemette et al., 2014). Variations in the number of TA-repeats in the UGT1A1 gene promoter contribute to individual differences in UGT1A1 expression (Guillemette et al., 2014). The UGT1A1*1 wild-type allele, which contains six TA-repeats, results in fully functional enzyme activity; whereas, the common UGT1A1*28 variant allele, which contains seven TA-repeats, results in “low promoter activity” and decreased UGT1A1 expression. The homozygous UGT1A1*28/*28 genotype is associated with Gilbert’s syndrome, a form of mild unconjugated hyperbilirubinemia (Bosma et al., 1995). Genetic variation in UGT1A1 has been shown to impact the pharmacokinetics and adverse effect profile of drugs, such as the chemotherapeutic irinotecan and the antiretroviral atazanavir (Iyer et al., 2002; Gammal et al., 2016). In addition, lapatinib, as well as several other
kinase inhibitors (e.g., regorafenib and sorafenib), have been characterized as potent inhibitors of UGT1A1 (Zhang et al., 2015; Miners et al., 2017; Qosa et al., 2018; Lv et al., 2019; Korprasertthaworn et al., 2019).

Species differences have been reported in the O-dealkylation and subsequent conjugative metabolism of lapatinib M1 in humans vs. preclinical species (rat and dog) (Castellino et al., 2012). In the present study, incubation of lapatinib M1 with dog liver microsomes generated the highest levels of the M1-glucuronide compared to human and rat liver microsomes. Whereas in incubations with NADPH alone, human liver microsomes generated the highest levels of quinoneimine-GSH conjugate compared to rat and dog liver microsomes. The differences in the levels of quinoneimine-GSH conjugate formed in the presence of NADPH with and without UDPGA suggest that glucuronidation is a competing pathway for microsomal M1 metabolism vs. bioactivation to the quinoneimine reactive metabolite.

Species differences between human, rat, and dog liver S9 fractions were observed in the substrate depletion and metabolite formation from lapatinib M1 via glucuronidation, sulfation, and AO-mediated oxidation. The estimated depletion rate constants indicated that the overall rate of M1 depletion was highest for the human liver S9 fraction, as compared to rat and dog. The relative levels of M1-glucuronide formation were higher in dog liver S9 compared to human and rat, and M1-sulfate formation was higher in human liver S9, compared to rat and dog. M3 formation was higher in human liver S9 fraction compared to rat. M3 was not formed in liver S9 fraction from dog, which lack liver AO activity (Terao et al., 2006). The results from incubation of lapatinib M1 with human liver S9 fraction suggest that the combined activities of AO, UGTs, and SULTs may be involved in the hepatic metabolism of lapatinib M1.
In human mass balance studies with [14C]lapatinib, unconjugated M1 and M3 were excreted in feces, representing a median of 3.9% (ranging from below the limit of quantitation to 19.2%) and 3.3% (ranging from below the limit of quantitation to 17.8%), respectively, of the total dose (Castellino et al., 2012). AO has been previously shown to convert lapatinib M1 to M3 in vitro (Dick, 2018; Bissada et al., 2019). We have also demonstrated that AO-mediated metabolism of lapatinib to “AO-M1” was inversely related to formation of the M1 phenol in primary human hepatocytes (Bissada et al., 2019). In preclinical studies reported from rats administered lapatinib, M1-sulfate conjugates represented the major (72%) drug-related material excreted in bile; whereas, M1-glucuronide conjugates represented 16% of the drug-related material excreted in bile (FDA, 2007).

Herein, the studies conducted in primary human hepatocytes provide an overview of the sequential metabolism of lapatinib in vitro, specifically through M1 formation and subsequent conjugative pathways. Lapatinib O-dealkylation to form M1 is catalyzed by both CYP3A4 and CYP3A5 (Teng et al., 2010; Chan et al., 2012; Towles et al., 2016). We have previously shown that increased rates of M1 formation were associated with increased individual CYP3A activity (Bissada et al., 2019). In the present study, formation of the M1-glucuronide was higher in human hepatocytes from CYP3A5 expressers (CYP3A5*1/*3 and CYP3A5*1/*1 donors), compared to CYP3A5*3/*3 donors (“non-expressers”), which mirrored the higher levels of M1 formation from lapatinib in CYP3A5 expressers compared to non-expressers, reported previously (Bissada et al., 2019). Hepatocytes from CYP3A5*1/*3 and CYP3A5*1/*1 donors, had higher CYP3A activity (Bissada et al., 2019), and the increased formation of lapatinib M1 in CYP3A5*1/*3 and CYP3A5*1/*1 donors likely contributed to the higher levels of M1-glucuronide conjugate formation, compared to CYP3A5*3/*3 donors. Higher levels of M1-sulfate conjugate were also found in CYP3A5*1/*3 donors, compared to CYP3A5*3/*3 donors; however, this relationship was not observed with CYP3A5*1/*1 donors. The reason for this discrepancy is not
known. Additional factors may contribute to individual differences in the generation of the M1-sulfate conjugate, and the specific SULT enzyme(s) involved are unknown.

Lapatinib M1 was extensively metabolized by glucuronidation in single-donor cryopreserved human hepatocytes incubated with lapatinib. The relative levels of M1-glucuronide generated were significantly correlated with the rates of M1 formation by individual donors. These findings indicate a direct relationship between M1 formation and subsequent conjugation to the M1-glucuronide, which suggests that M1 formation may be the rate-limiting step in this metabolic pathway. Once formed, M1 is likely efficiently cleared, at least in part, by glucuronidation. Whether lapatinib M1 undergoes glucuronidation and sulfation in vivo in humans remains unknown. Glucuronide conjugates formed in the liver are often transported into bile by the efflux transporter multidrug resistance-associated protein 2 (MRP2), and once emptied into the intestine, glucuronide conjugates can be deconjugated by bacterial β-glucuronidases (Guillemette et al., 2014). The resulting aglycone species can be reabsorbed in the intestine and undergo enterohepatic cycling or be eliminated into the feces (Guillemette et al., 2014). Sulfate conjugates can be hydrolyzed by sulfatases (Coughtrie et al., 1998). Further studies will be necessary to understand the roles of these conjugative metabolism and transport pathways in the human disposition of lapatinib in vivo.

In summary, this study has investigated the detoxication vs. bioactivation pathways of lapatinib, which is associated with idiosyncratic hepatotoxicity. Lapatinib O-dealkylation by CYP3A enzymes leads to the formation of the phenolic metabolite M1, which can undergo bioactivation to form the chemically reactive, potentially toxic quinoneimine. We report that, in addition to P450-mediated bioactivation, multiple non-P450 pathways are involved in the biotransformation of lapatinib M1 in vitro, including glucuronidation, sulfation, and AO-mediated oxidation, which may represent detoxication pathways. The findings from reaction phenotyping
studies support the conclusion that UGT1A1 is the major hepatic enzyme that catalyzes the glucuronidation of lapatinib M1 to produce the M1-glucuronide conjugate, and UGT1A1 activity influences the extent of M1 glucuronidation. Moreover, inter-individual and inter-species differences in the biotransformation pathways of lapatinib likely influence the hepatic exposure to potentially toxic metabolites.

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Authorship contributions

Participated in research design: Nardone-White and Jackson
Conducted experiments: Nardone-White, Bissada, Abouda, and Jackson
Performed data analysis: Nardone-White and Jackson
Wrote or contributed to the writing of the manuscript: Nardone-White and Jackson
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Figure Legends

Figure 1. Metabolic pathway of lapatinib via O-dealkylation and subsequent metabolism.

Figure 2. Time-course of lapatinib M1-glucuronide formation. Lapatinib M1 (2 μM), was incubated with pooled human liver microsomes (0.1 mg protein/ml) for 0, 30, and 60 min in the presence and absence of UGT cofactor UDPGA. Formation of the M1-glucuronide conjugate was analyzed by LC-MS/MS using selected reaction monitoring (m/z 649 > 473). Relative metabolite levels were measured by the peak area ratio of M1-glucuronide to internal standard (d₄-M1, m/z 477 > 352). Results are presented as the mean ± SD from one experiment conducted in triplicate.

Figure 3. Lapatinib M1 glucuronidation by human recombinant UGT enzymes. Lapatinib M1, at (A) 0.2 μM and (B) 2 μM, was incubated with individual recombinant UGT enzymes (Supersomes™) or pooled human liver microsomes (0.2 mg protein/ml) for 30 min in the presence of UDPGA. Relative levels of M1-glucuronide conjugate were measured by LC-MS/MS analysis using selected reaction monitoring (m/z 649 > 473). Peak area ratios of M1-glucuronide to internal standard (d₄-M1, m/z 477 > 352), are shown. Results are presented as the mean ± SD from (A) two or (B) one experiment(s) conducted in triplicate each.

Figure 4. Effect of UGT1A1 inhibition on lapatinib M1-glucuronide formation. Lapatinib M1 (2 μM) was incubated with pooled human liver microsomes (0.1 mg protein/ml) supplemented with UDPGA for 30 min in the presence and absence of UGT1A1 inhibitors atazanavir (1 and 10 μM) and erlotinib (3 and 10 μM). Relative levels of M1-glucuronide conjugate (m/z 649 > 473) were measured by LC-MS/MS analysis. The percentage (%) of M1-glucuronide conjugate formation
was compared to vehicle control incubations without inhibitors (mean ± SD peak area ratio: 5.25 ± 0.80). Results are presented as the mean ± SD from one experiment conducted in triplicate.

**Figure 5.** Formation of lapatinib M1-glucuronide and quinoneimine-GSH conjugate in UGT1A1-genotyped human liver microsomes. Lapatinib M1 (2 μM) was incubated with pooled and individual genotyped human liver microsomes (0.2 mg protein/ml) for 30 min in the presence and absence of NADPH and UDPGA supplemented with GSH (5 mM). Relative levels of (A) M1-glucuronide conjugate \( m/z 649 > 473 \), and (B) quinoneimine-GSH conjugate \( m/z 778 > 655 \) were measured by LC-MS/MS analysis. Peak area ratios of analyte to internal standard \( (d_4\text{-M1}, m/z 477 > 352) \), are shown. Values are presented as the mean ± SD from two independent experiments conducted in triplicate each for six individual donors and pooled HLM. Donor lot numbers and UGT1A1 genotypes are shown: UGT1A1*1/*1 (n = 2); UGT1A1*1/*28 (n = 3); and UGT1A1*28/*28 (n = 1). Sample size was based on the number of commercially available UGT1A1-genotyped human liver microsomes.

**Figure 6.** Correlation of lapatinib M1-glucuronide and quinoneimine-GSH conjugate formation with UGT1A1 and CYP3A activity, respectively, in human liver microsomes. (A) Correlation of lapatinib M1-glucuronide formation with UGT1A1 activity, measured by 17β-estradiol 3-glucuronidation. (B) Correlation of quinoneimine-GSH conjugate formation with CYP3A activity, measured by testosterone 6β-hydroxylation. Values are presented as the mean from two independent experiments conducted in triplicate for each of six individual donors and pooled HLM (n = 7 total). Donor genotypes were UGT1A1*1/*1 (n = 2); UGT1A1*1/*28 (n = 3); and UGT1A1*28/*28 (n = 1). Donor lot 710414 is highlighted as an outlier among UGT1A1*1/*28 donors with respect to high M1 glucuronidation (Grubbs’ outlier analysis). Sample size was based on the number of commercially available UGT1A1-genotyped human liver microsomes.
UGT1A1 and CYP3A enzyme activity values are from XenoTech. GraphPad Prism 8 software was used for linear regression, Pearson r correlation, and Grubbs’ outlier analyses.

**Figure 7.** Formation of lapatinib M1-glucuronide and quinoneimine-GSH conjugate in liver microsomes from human, rat, and dog. Lapatinib M1 (2 μM) was incubated with pooled liver microsomes from human, rat, and dog (0.1 mg protein/ml) for 30 min with and without cofactors NADPH and UDPGA in the presence of GSH (5 mM). Relative levels of (A) M1-glucuronide conjugate (m/z 649 > 473), and (B) quinoneimine-GSH conjugate (m/z 778 > 655) were measured by LC-MS/MS analysis. Peak area ratios of analyte to internal standard (d₄-M1, m/z 477 > 352) are shown. Results are presented as the mean ± SD from two experiments conducted in triplicate each.

**Figure 8.** Time-course for lapatinib M1 depletion and metabolite formation in human, rat, and dog liver S9 fractions. Lapatinib M1 (1 μM) was incubated with pooled human, rat, and dog liver S9 fractions (2.0 mg protein/ml) for 0, 5, 10, 15, and 20 min. Incubations were supplemented with MgCl₂ (2.5 mM), PAPS (0.1 mM), and UDPGA (2 mM). Control incubations were without PAPS and UDPGA. Relative levels of M1 and M1 metabolites were measured by LC-MS/MS analysis using selected reaction monitoring for: A) M1 (m/z 473 > 350), B) M1-glucuronide conjugate (m/z 649 > 473), C) M1-sulfate conjugate (m/z 553 > 473), and D) M3 (m/z 489 > 366). The percentage (%) of remaining M1 substrate was calculated based on the M1 peak area ratio at time 0 min (A). Peak area ratios of metabolite to internal standard (d₄-M1, m/z 477 > 352), are shown (B-D). Results are presented as the mean ± SD from one experiment conducted in triplicate.

**Figure 9.** Phase II metabolism of lapatinib in cryopreserved human hepatocytes. Lapatinib (1 μM and 10 μM) was incubated with pooled human hepatocytes in suspension for 2 hours.
Cryopreserved hepatocytes were from a pool of 5 donors (mixed gender). Control incubations were with no cells. Relative metabolite levels were measured by LC-MS/MS analysis using selected reaction monitoring: A) lapatinib M1 (m/z 473 > 350), B) M1-glucuronide (m/z 649 > 473), C) M1-sulfate (m/z 553 > 473), and D) quinoneimine-GSH conjugate (m/z 778 > 655). Metabolite peak areas are shown. Bars are the mean from one experiment conducted in duplicate.

**Figure 10.** Phase II metabolism of lapatinib M1 in CYP3A5-genotyped cryopreserved human hepatocytes. Lapatinib (1 µM and 10 µM) was incubated with pooled human hepatocytes in suspension for 2 hours. Cryopreserved human hepatocytes were from a pool of 3 donors for each CYP3A5 genotype: CYP3A5*3/*3, CYP3A5*1/*3, and CYP3A5*1/*1. Relative metabolite levels were measured by LC-MS/MS analysis for M1-glucuronide (m/z 649 > 473) and M1-sulfate (m/z 553 > 473). Formation of M1-glucuronide (A-B) and M1-sulfate (C-D) from hepatocyte incubations with 1 µM lapatinib (A,C) and 10 µM lapatinib (B,D) is shown as the peak area for each metabolite. Results are presented as the mean ± SD from one experiment conducted in 4-5 replicates per hepatocyte lot. The peak area value for one of four replicates from incubations with 1 µM lapatinib in CYP3A5*3/*3 hepatocytes was identified as an outlier based on Grubbs’ outlier analysis (GraphPad Prism 8 software); thus, this outlier was excluded.

**Figure 11.** Correlation of lapatinib M1-glucuronide to M1 formation in cryopreserved human hepatocytes. Lapatinib (10 µM) was incubated in suspension with cryopreserved human hepatocytes from 15 individual donors (n = 15). Metabolite formation was measured by LC-MS/MS analysis. M1 formation was quantified using an authentic chemical standard (Bissada et al., 2019). Peak area ratios of M1-glucuronide to internal standard (d4-M1, m/z 477 > 352), are shown. Results are presented as the mean values for each donor from experiments conducted
in triplicate. Formation of M1-glucuronide was analyzed for correlation with lapatinib M1 formation by linear regression analysis and Pearson $r$ correlation using GraphPad Prism 8 software.
Figure 1.
Figure 2.

![Graph showing M1-glucuronide (peak area ratio) over time (min) with data points for +UDPGA and -UDPGA](image-url)
Figure 3.

A.

B.
Figure 4.

![Graph showing %Control M1-glucuronide for different treatments: Vehicle (control), Atazanavir (1 µM), Atazanavir (10 µM), Erlotinib (3 µM), Erlotinib (10 µM).]
Figure 5.

A.

B.
Figure 6.

A.

![Graph A](image)

B.

![Graph B](image)
Figure 7.

A.

B.
Figure 8.

A.

B.

C.

D.
Figure 9.

A. Lapatinib M1 (peak area)

B. M1-glucuronide (peak area)

C. M1-sulfate (peak area)

D. GSH conjugate (peak area)
Figure 10.

A.

B.

C.

D.
Figure 11.

![Graph showing M1-glucuronide (peak area ratio) vs. M1 (pmol/min/million cells). The correlation coefficient (r) is 0.94, R² is 0.88, and P < 0.0001.](image)