P450 3A-Catalyzed O-Dealkylation of LapatinibInduces Mitochondrial Stress and Activates Nrf2

Marsha Rebecca Eno, † Bahaa El-Dien M. El-Gendy, ‡ and Michael D. Cameron,* †

† Department of Molecular Therapeutics, The Scripps Research Institute, Scripps Florida, 130 Scripps Way, Jupiter, Florida 33458, United States
‡ Chemistry Department, Faculty of Science, Benha University, Benha 13518, Egypt

ABSTRACT: Lapatinib (LAP), an oral tyrosine kinase inhibitor for the treatment of metastatic breast cancer, has been associated with idiosyncratic hepatotoxicity. Recent investigations have implicated the importance of P450 3A4/5 enzymes in the formation of an electrophilic quinone imine (LAPQI) metabolite generated through further oxidation of O-dealkylated lapatinib (OD-LAP). In the current study, hepatic stress was observed via mitochondrial impairment. OD-LAP caused a time- and concentration-dependent decrease in oxygen consumption in HepG2 cells, whereas LAP did not alter the oxygen consumption rate. Interestingly, however, HepG2 cells transfected with human P450 3A4 did exhibit mitochondrial dysfunction via P450 3A4-mediated metabolism of LAP to OD-LAP. OD-LAP-induced mitochondrial toxicity was enhanced upon depletion of intracellular GSH levels, demonstrating that cellular GSH levels are important in the protection of mitochondrial function against LAPQI. Given the nature of LAPQI and the importance of GSH levels in LAP-induced mitochondrial stress, the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) was evaluated, as this transcription factor induces the expression of NAD(P)H quinone oxidoreductase 1, glutathione S-transferase, UDP-glucuronosyltransferases, and glutathione synthetase, all of which might be expected to decrease the toxicity of LAP. Using a FRET-based target gene assay in HepG2 cells, OD-LAP was indeed found to activate Nrf2. Follow-up assays showed increased mRNA levels of Nrf2 target genes after a 4 h treatment with OD-LAP but not with LAP. LAP activation of Nrf2 was observed only when HepG2 cells were transduced with P450 3A4. The significance of Nrf2 protection was established in vivo in Nrf2-KO mice. Increased transaminase levels were found after a single LAP dose in both Nrf2-KO and control mice, indicating elevated hepatic necrosis, although transaminase levels reverted to baseline levels in the control mice upon repeat dosing. They continued to rise in Nrf2-KO mice, however, indicating the likelihood that Nrf2 plays a significant role in combatting the hepatotoxicity triggered by LAP.

INTRODUCTION

Drug-induced liver injury (DILI) is a significant health problem and can result in acute liver failure, the need for liver transplantation, or death. 2,3 Lapatinib (LAP), a dual tyrosine kinase inhibitor of ErbB-2 and EGFR, is approved for the treatment of advanced metastatic breast cancer; 2,4 however, treatment with LAP is associated with increased incidence of idiosyncratic hepatotoxicity. 2 In 2008, the FDA issued a black box warning for LAP following clinical reports of elevated liver enzymes and liver-related deaths during post market surveillance. 5,7 For this reason, LAP prescribing information includes information on liver chemistry monitoring and clinical management of hepatotoxicity. 8

Recently, it has been reported that a small population of LAP-treated patients had incidences of immune-mediated hepatotoxicity. Patients with HLA (human leukocyte antigen) polymorphisms HLA-DQA1*02:01 and HLA-DRB1*07:01 had an increased risk of serious hepatic toxicity (odds ratio, 14.0). 9 These findings suggest that LAP-induced hepatotoxicity has an immune component that may be partially due to the covalent modification of proteins by LAP. 9,10 It is important to note that LAP-induced liver injury has been observed in patients without these HLAs and that the majority of patients with the indicated HLAs do not develop serious liver injury.

The hepatotoxicity of LAP can be attributed to the multifactorial nature of idiosyncratic toxicity and can be due to a combination of immune-mediated and chemical-induced toxicities that involve direct, acute, and reactive-metabolite-mediated toxicities involving drug bioactivation and formation of drug–protein adducts as well as oxidative stress that involves mitochondrial dysfunction. Acute chemical-induced toxicity may provide danger-associated molecular patterns that are required to produce an immune response. 2

LAP was demonstrated to undergo extensive metabolism by P450 3A4/5 enzymes, resulting in O-dealkylation, N-dealkylation, and N-hydroxylation metabolites (Figure 1). 11 While the mechanism by which LAP causes hepatotoxicity is unknown, reactive LAP metabolites have been identified such as its O-dealkylated metabolite (OD-LAP). OD-LAP possesses...
a \( p \)-hydroxyanilide group that upon oxidation generates an electrophilic reactive metabolite, lapatinib-quinone imine (LAPQI), which can be trapped with glutathione (GSH). Recently, LAP metabolism and excretion studies have been performed on human volunteers that have highlighted the potential relationship of LAP’s reactive metabolites to its clinical hepatotoxicity. For instance, Chan et al. disclosed that LAP may inactivate P450 3A5 by the adduction of LAPQI to the apoprotein. Thus, LAP-induced hepatotoxicity could be linked to the cytochrome P450-catalyzed formation of LAPQI, which, like other electrophiles that cause hepatotoxicity, such as acetaminophen’s \( N \)-acetyl-\( p \)-benzoquinone imine, has the potential to covalently modify hepatic proteins and deplete GSH, further exacerbating oxidative stress.

A number of hepatotoxic drugs that were withdrawn from the market were found to impair mitochondrial function. In mammalian cells, the mitochondria are responsible for generating the majority of cellular ATP by the process of oxidative phosphorylation. This is needed for normal cell function, including the ability to respond to endogenous or exogenous stress. Drugs that impair mitochondrial function decrease cell viability, and depending on the severity of the drug insult, this can lead to organ or tissue damage. The liver is exposed to a high level of toxic species, including toxic metabolites, and is thus a major target for tissue-specific toxicity. In response to electrophilic and oxidative stresses, activation of the erythroid 2-related factor 2 (Nrf2) pathway induces the expression of several cytoprotective genes through a \( cis \)-acting antioxidant response element (ARE). Nrf2 is

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**Figure 1.** Reactive metabolites of lapatinib.
regulated through proteosomal degradation. In the presence of electrophiles, Nrf2 ubiquitination is decreased and it hetero-
dimerizes with a small musculo-aponeurotic factor protein (Maf); this complex binds to an ARE to promote the transcription of numerous cytoprotective genes, including, but not limited to, NAD(P)H quinone oxidoreductase 1 (NQO-1), heme oxygenase (HO-1), glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), glutathione synthetase (GS), and glutamate-cysteine ligase (GCL). These important detoxifying and biotransformative enzymes in the elimination of potentially harmful chemicals. Of particular relevance with respect to LAP-induced hepatotoxicity is NQO-1, which can reduce the electrophilic metabolite LAPQI to its corresponding hydroquinone, which is not an electrophile. Additionally, GS and GCL are involved in GSH synthesis, and as we demonstrate in this article, cellular GSH levels are important factors in determining the extent of the mitochondrial toxicity caused by LAP metabolites.

The current study demonstrates for the first time that the OD-LAP metabolite of the tyrosine kinase inhibitor LAP causes mitochondrial dysfunction in a time- and concentration-dependent manner. Mitochondrial dysfunction was observed under physiologically relevant concentrations and may be expected to lead to cellular toxicity. Importantly, the expected OD-LAP-mediated toxicity is likely minimized through the activation of Nrf2, which was demonstrated to protect against LAP-induced hepatocellular damage when evaluated in Nrf2-KO and control mice.

EXPERIMENTAL PROCEDURES

Materials. Reagents and solvents were purchased at the highest available grade and purity. Lapatinib ditosylate was purchased from Toronto Research Chemicals (Toronto, Canada) and Biotang Inc., TSZ Chem (Lexington, MA). OD-LAP was chemically synthesized from LAP as described previously. Stock solutions of LAP and OD-LAP were prepared in DMSO. Midazolam, galactose, formic acid, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN) was purchased from Fisher Scientific (Fair Lawn, NJ), HEPES, from Amresco (Solon, OH), and pen-strep, blasticidin, and puromycin, from Life Technologies (Carlsbad, CA).

Cell Culture Conditions. High-Glucose Growth Media. The human hepatoma cell line HepG2 was purchased from ATCC (Manassas, VA) and maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies 1056-010) containing 25 mM glucose and GlutaMAX. The cell culture medium was supplemented with 10% (v/v) heat-activated fetal bovine serum, 0.1 mM nonessential amino acids (NEAA), 25 mM HEPES, and 100 μg/mL penicillin–streptomycin. The cells were cultured in 5% CO2, at 37°C. The CellSensor ARE-bla HepG2 cell line (Life Technologies) was maintained in the above growth media with the addition of 5 μg/mL blasticidin.

Assay Media. High-glucose growth media was used without antibiotics.

Galactose Media. DMEM deprived of glucose (Sigma cat. no. D5030) supplemented with 10 mM galactose, 1 mM sodium pyruvate, and 25 mM HEPES and with the pH adjusted to 7.4 was used.

Unbuffered Media. DMEM base powder (Sigma cat. no. D5030) dissolved in sterile water supplemented with 1.0 mM sodium pyruvate, 25 mM glucose, and 2.5 mM Glutamax and with the pH adjusted to 7.4 using NaOH was used.

Production of Lentivirus and Transduction of HepG2 and CellSensor ARE-bla HepG2 Cells. Lentiviruses, lentiviral plasmids, and reagents were obtained from GeneCopoeia. The P450 3A4 (Ex-Z0804-Lv121), P450 3A5 (I031-Lv121), and control vector with EGFP (green fluorescent protein) (EX-EGFP-Lv121) plasmids were transformed into One Shot ShB3 chemically competent E. coli cells (Life Technologies) and purified using a plasmid maxi-prep kit (Qiagen, Valencia, CA). Two days before transfection, 293TN packaging cells (System Biosciences) were plated in a 10 cm dish with DMEM and 10% heat-inactivated FBS. Forty-eight hours after seeding, 293TN packaging cells were transfected with lentiviral vectors encoding P450 3A4, P450 3A5, or EGFP control vector, using the Lenti-Pac HIV expression packaging kit (HPK-LvTR-20, GeneCo-

poea) according to the manufacturer’s instructions. Two days post-transfection, lentivirus-containing culture medium was concentrated using a Vivaspin 20 column (100 000 MWCO PES), sterile filtered by passing through a 0.2 μm filter, and added to the target cells (HepG2 cells or CellSensor ARE-bla Hep G2 cells). Transduced cells were selected with 1.0 μg/mL puromycin.

Functional Characterization of Transduced HepG2 and CellSensor ARE-bla HepG2 Cells. To measure P450 3A4 and P450 3A5 activity, assays were performed by incubating HepG2 or CellSensor ARE-bla HepG2 cells (120 000 cells/well) in 24-well plates with midazolam as the probe substrate. Midazolam was added at 5, 10, and 20 μM. The final solvent concentration in the incubation medium did not exceed 0.2% DMSO (v/v) and ACN with 0.1% formic acid (solvent-B) and ACN with 0.1% formic acid (solvent-B) run at a flow rate of 0.4 mL/min. Linear gradients were used as follows: 0–0.3 min = 5% B; 2.5–3.0 min = 95% B; and 3.1–6.1 = 5% B. 1′Hydroxymidazolam and dextrotransformation was detected using the m/z 342.2 −→ 203.1 and m/z 258.2 −→ 157.2 mass transitions, respectively. The optimized declustering potential and collision energy were 80 and 58 eV for 1′hydroxymidazolam and 95 and 38 eV for dextrotransformation, respectively.

Measurement of NADH/NADPH Levels. NADH/NADPH levels in HepG2 and HepG2-3A4 cells were measured using the Vybrant MTT cell proliferation kit (Life Technologies). Cells (1 × 10^4 cells/mL) in 100 μL of phenol-red-free high-glucose growth medium were plated in a 96-well plate, and the next day, they were incubated with various concentrations of LAP (10 and 50 μM), OD-LAP (10, 30, and 100 μM), or vehicle control (0.1% DMSO). After 24 h incubation in a 5% CO2 humidified incubator at 37°C, the cells were incubated with MTT following the manufacturer’s instructions. Two days post-transfection, lentivirus-containing culture medium was concentrated using a Vivaspin 20 column (100 000 MWCO PES), sterile filtered by passing through a 0.2 μm filter, and added to the target cells (HepG2 cells or CellSensor ARE-bla Hep G2 cells). Transduced cells were selected with 1.0 μg/mL puromycin.

ATP Level Measurement. Intracellular ATP levels were measured using the mitochondrial Tox-Glo assay (Promega), in accordance with the manufacturer’s instructions. In brief, HepG2 cells were plated at a density of 300 000 cells/well in a 96-well plate in the presence of various concentrations of LAP (10 μM to ~625 nM) or OD-LAP (50 μM to ~750 nM). Cells were grown in standard high-glucose medium but were washed and serially dosed in 100 μL of serum-free, galactose-containing medium. ATP detection substrate (100 μL) was added, and luminescence was measured using an EnVision plate reader (PerkinElmer).

CellTox Green Cytotoxicity Assay. The viability of HepG2 and HepG2-3A4 cells treated with LAP and OD-LAP was measured using the CellTox Green cytotoxicity assay (Promega). Cells (1 × 10^4 cells/mL) in 100 μL of phenol-red-free high-glucose growth medium were plated in a 96-well clear-bottom black plate, and the next day, the medium was exchanged with fresh media containing various concentrations of LAP (10, 30, and 50 μM), OD-LAP (10, 30, and 100 μM), vehicle control (0.1% DMSO), or toxic control. After 24 h, CellTox Green reagent (2×) was prepared according to the manufacturer’s instructions, and 100 μL was added to the cells. The
plate was incubated at room temperature for 15 min, and fluorescence was read at 485 nm and 520 nm using a Spectra Max M5e microplate reader (Molecular Devices).

Cellular Oxygen Consumption. Measurement of cellular respiration was performed using a Seahorse XF24 analyzer (Seahorse Biosciences, North Billerica, MA). HepG2 and HepG2-3A4 cells were seeded in Seahorse XF 24-well cell culture microplates at 15 000 cells/well in 100 μL of high-glucose growth medium and allowed to adhere overnight. Cells were treated with different concentrations of drugs for different time points depending on the experiment. Before the experiment, growth medium was replaced with 500 μL of unbuffered medium, and cells were equilibrated for 30 min at 37 °C in a CO2-free incubator before being transferred to the XF24 analyzer. The oxygen consumption rate (OCR) was measured prior to, during, and after injections with the ATP synthase inhibitor oligomycin, the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and a combined injection of the complex I and II inhibitors antimycin A and rotenone to yield final concentrations in the wells of 1 μg/μL, 1.2 μM, and 1.6 μM + 1.6 μM, respectively. Basal mitochondrial respiration was calculated by subtracting nonmitochondrial respiration (OCR following injection with antimycin A + rotenone) from the baseline respiration prior to oligomycin injection. Maximum respiration was calculated by subtracting nonmitochondrial respiration from the maximum respiration measured following FCCP injection.

Measurement of Nrf-2 Activation by LiveBLAzer β-Lactamase Reporter Assay. The LiveBLAzer ARE-bla β-lactamase reporter assay (Life Technologies) was used to measure the activation of ARE-regulated genes. The Cell Sensor ARE-bla HepG2 cell line contains a β-lactamase reporter gene under the control of an ARE stably integrated into HepG2 cells. ARE-bla HepG2, ARE-bla HepG2-3A4 (transduced with hP450 3A4), and ARE-bla HepG2-3A5 (transduced with hP450 3A5) were seeded in clear-bottom, black 384-well plates at 3.9 × 10⁵ cells/mL and cultured in high-glucose growth medium at 37 °C in a 5% CO2 humidified incubator overnight.

Figure 2. Effect of OD-LAP on OCR in HepG2 cells. (A) OCR after 6 and 24 h of exposure to 30 μM OD-LAP. (B) Basal respiration and maximum respiration (FCCP) after 6 and 24 h of exposure to 30 μM OD-LAP. (C) Concentration-dependent decrease in NAD(P)H levels after 24 h of OD-LAP treatment relative to DMSO control (set as 100%). (D) Cell viability after 24 h of exposure to OD-LAP, as measured by membrane integrity. (E) Concentration dependence of OD-LAP on OCR after 24 h of treatment. (F) Effect of a 24 h pretreatment of HepG2 cells with 50 μM BSO followed by a 24 h treatment with 20 μM OD-LAP. Data represent the mean ± SD of triplicate values. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with vehicle (unpaired t-test, two-tailed P values).
The next day, test compounds were added, and the cells were incubated in the incubator for 15 h. This was followed by the addition of the FRET substrate CCF4-AM. The substrate consists of two fluorophores that form a FRET pair, which in the absence of β-lactamase activity results in a green fluorescence signal. In the presence of β-lactamase activity, cleavage of the substrate disrupts FRET, resulting in a blue fluorescence signal. Fluorescent measurements were made using an EnVision plate reader (PerkinElmer). The β-lactamase activities were measured by the ratio of the blue product, monitored at λ = 460 nm, to the green substrate, monitored at λ = 535 nm (see the manufacturer’s protocol for detailed instructions).

Quantitative PCR To Measure mRNA Expression Levels of Nrf2 Activating Genes. Cells were plated in a 6-well plate (7.5 × 10^5 cells/well) in high-glucose growth medium, and the next day, the cells were incubated with various concentrations of LAP (dissolved in serum-free media) or OD-LAP. RNA was isolated using the RNAeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, and its purity was determined on a spectrophotometer (Thermo Scientific NanoDrop 1000). Pure RNA (2 μg) was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The cDNA was then used with the Power SYBR Green PCR master mix (Life Technologies) for real-time qPCR quantification using an Applied Biosystems 7900 HT fast real-time PCR system. All samples were analyzed in triplicate. Relative quantities of specifically amplified cDNA were determined using the comparative threshold cycle (Ct) method. GAPDH was used as an endogenous reference gene, and no-template and no-reverse-transcription controls were used to exclude nonspecific amplification.

The cycling conditions used were 95 °C for 10 min for initialization followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers for the PCR reactions (purchased from Integrated DNA Technologies) are listed in the Supporting Information, Table S1.

Animals and Experimental Protocols. Male C57BL/6 and Nrf2-KO (C57BL/6 background) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were 10 weeks old and weighed approximately 23 g. They were housed in the AALAC accredited Scripps vivarium and kept in a temperature-controlled room with a 12 h light/dark cycle and ad libitum access to food and water. All animal procedures were reviewed and approved by the Scripps Florida IACUC. The tested LAP dose was 400 mg/kg. This dose represents three times the allometric scaled human LAP dose (1250 mg/day). For in vivo studies, 400 mg/kg of LAP was formulated as a 20 mg/mL solution in 10:10:80 DMSO/Tween 80/water and injected intra-peritoneally (IP) daily for 5 days. On days 1 and 5 (8 h postdose), blood was drawn for the evaluation of alanine transaminase (ALT) and aspartate transaminase (AST) and drug level quantitation. A section of liver from each mouse was collected and flash frozen in liquid nitrogen at the end of the experiment on day 5 for quantitation of liver levels of LAP and OD-LAP.

LAP and OD-LAP Quantitation in Plasma and Liver Tissue. The tissue distribution of LAP was evaluated in C57BL/6 and Nrf2-KO mice (n = 4). Tissues were not perfused to reduce the risk that LAP would be eluted from the tissue during the perfusion process. Plasma was generated using standard centrifugation techniques, and the plasma and tissues were frozen at −80 °C. Plasma and tissues were mixed with ACN (1:5 v/v or 1:5 w/v, respectively). Tissues were sonicated with a probe tip sonicator and clarified by filtration through a 0.2 μm filter plate. Samples were analyzed by LC-MS/MS using an ABSciex 5400 triple quadrupole mass spectrometer. MRM was used to detect LAP (m/z 582.1 → 366.1) and OD-LAP (m/z 473.2 → 349.8), and carbamazepine was used as an internal standard (m/z 238 → 195.1). Plasma drug levels were determined against standards made in plasma and liver tissue matrix. See Tables S2–S4 of the Supporting Information for instrument settings and detailed analytical conditions.

Biomarker Analyses. Following the collection of blood from anesthetized animals, ALT and AST levels were determined using a clinical chemistry analyzer, Cobas C311, from Roche Diagnostics.

Statistical Analyses. All cell-based experiments were performed in triplicate in one to three independent experiments. The mean and standard deviation (SD) or standard error of the mean (SEM) for each experiment were determined using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). Treatment groups were compared with their respective controls using an unpaired t-test. P values were calculated by two-tailed analysis, with P < 0.05 considered significant.

RESULTS
Effect of OD-LAP on OCR in HepG2 Cells. To directly examine the response of HepG2 cells to OD-LAP-induced mitochondrial dysfunction, cellular metabolism was assessed using a Seahorse XF24 analyzer, which measures the mitochondrial respiration (oxygen consumption) of cells. Addition of oligomycin inhibits mitochondrial F1F0-ATPase, resulting in a reduction of electron flow through the electron transport chain and thereby decreasing oxygen consumption. Addition of carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone (FCCP), on the other hand, accelerates cellular oxygen consumption, allowing the maximal rate of oxygen consumption by the electron transport chain to be observed. Finally, the addition of antimycin/rotenone (inhibits complexes III and I) allows extra mitochondrial oxygen consumption to be quantitated. Figure 2A,B shows the oxygen consumption of HepG2 cells after treatment with vehicle (0.1% DMSO, 24 h) or 30 μM OD-LAP (6 and 24 h). Oxygen consumption was measured in real time for 106 min, as described in the Experimental Procedures. The oxygen consumption rate in HepG2 cells, prior to the addition of any of the above-mentioned effectors of the electron transport chain, decreased in the presence of 30 μM OD-LAP at 6 and 24 h. Once a correction for background oxygen consumption was made, basal mitochondrial oxygen consumption was minimally affected after 6 h, but the maximal mitochondrial respiratory capacity was significantly reduced. By 24 h, both basal and maximal mitochondrial respirations were decreased (Figure 2B). Most Seahorse experiments were repeated three to four times. Replicate experiments showed consistent trends, but variability was observed in the magnitude of the OCR changes between experiments. OD-LAP incubated at concentrations of 30 and 100 μM for 24 h reduced NAD(P)H levels to ~60 and ~40% of the DMSO control, respectively, in HepG2 cells, as measured by the MTT assay (Figure 2C). This decrease does not involve cell viability, as demonstrated by a Cell-Tox Green viability assay (Figure 2D), suggesting that the decrease observed in the MTT assay was due to mitochondrial impairment and not from reduced cell viability via cellular necrosis.

As shown in Figure 2E, OD-LAP decreased both the basal and maximal respiratory capacities of HepG2 cells in a concentration-dependent manner. Since OD-LAP can be further metabolized to LAPQI and LAPQII can be trapped with GSH,25 we tested to see whether depletion of intracellular GSH increased OD-LAP-induced mitochondrial damage. GSH, with or without glutathione S-transferase, quenches electrophiles by coupling them with GSH. i-Buthionine sulfoximine (BSO) depletes intracellular GSH by inhibiting GCL, the enzyme involved in the first step of GSH synthesis. Pretreatment with BSO (50 μM) for 24 h was used to deplete GSH in HepG2 cells. This was followed by the addition of 20 μM OD-LAP for another 24 h. In control incubations, BSO alone (50 μM) and treatment with OD-LAP (20 μM) decreased basal and maximal uncoupled respirations only slightly compared to that of vehicle treated cells (Figure 2F). However, a combination of BSO (50 μM) and OD-LAP (20 μM) drastically diminished the
OCR in HepG2 cells. These results indicate that the presence of GSH protects against OD-LAP-induced mitochondrial damage.

**Effect of LAP on OCR in HepG2 Cells.** There was no significant difference in the oxygen consumption rate between vehicle control HepG2 cells and cells treated with 10 μM LAP (Figure 3A). Note that LAP precipitation was visible in serum-containing assay media at concentrations above 10 μM, precluding the ability to match LAP and OD-LAP concentrations in all assays. However, precipitation was not observed in serum-free media at concentrations up to 50 μM. As seen in Figure 3B, there was also no significant difference in MTT reduction between cells treated with vehicle and 50 μM LAP (dissolved in serum-free media), which implies that LAP did not affect cellular NADH/NADPH in HepG2 cells.

**Effect of LAP on OCR in Transduced 3A4-HepG2 Cells.** On the basis of the findings described above, we stably expressed human P450 3A4 in HepG2 cells to investigate the mechanism by which P450 3A4 contributes to the toxicity of LAP. HepG2 cells are largely metabolically incompetent and cannot be used to look at the biotransformation of LAP into OD-LAP, so to examine P450 3A4-mediated mitochondrial dysfunction caused by LAP, HepG2 cells were virally transduced with enzyme hP450 3A4. The success of the transduction was assessed using midazolam as a P450 3A4 probe to monitor the formation of 1′-OH midazolam in HepG2-3A4- and HepG2-3A5-transduced cell lines. HepG2 cells that were nontransduced or those transduced with GFP showed no P450 3A4 activity (Figure 4A). The observed rate of midazolam hydroxylation was approximately 3-fold higher than what we have previously reported for human cryopreserved hepatocytes from multiple individual donors.21

To investigate the role of P450 3A4-mediated metabolism as a mechanism of LAP hepatotoxicity, LAP was incubated with transduced HepG2-3A4 cells (note that serum-free media was used to allow for higher LAP concentrations). As discussed earlier in the results of Figure 3A, there was no significant change in OCR caused by LAP (10 μM) treatment in metabolically incompetent HepG2 cells and in vehicle-treated cells. Treatment with 10 μM LAP for 24 h caused a significant decrease in OCR when it was tested with transduced HepG2-3A4 cells, and treatment with 30 μM for 24 h drastically reduced the OCR (Figure 4B). As observed in Figure 4C, both 10 and 50 μM LAP decreased NAD(P)H levels in the transduced cells but did not affect viability (Figure 4D). These results indicate that the P450 3A4-catalyzed O-dealkylation of physiologically relevant concentrations of LAP is sufficient to impair mitochondrial function but not viability. Preliminary experiments were conducted with either P450 3A4- or 3A5-transduced cells, and both P450 enzymes appeared to have the same effect on mitochondrial OCR (data not shown). Later experiments utilized only HepG2-3A4 cells because 3A4 expression in humans is ubiquitous, whereas only about 20–30% of patients have functional 3A5.

**Effects of LAP and OD-LAP Treatments on Cellular ATP Levels in HepG2-3A4 Cells.** To increase the magnitude of LAP- and OD-LAP-induced mitochondrial dysfunction, transduced HepG2 cells were forced to increase their reliance on oxidative phosphorylation for ATP production rather than glycolysis by reducing the concentration of glucose in the growth media and supplementing with galactose. Susceptibility to mitochondrial toxicants increases in galactose-grown HepG2 cells.22 Figure 4E shows a concentration-dependent decrease in ATP in the presence of LAP and OD-LAP after 16 h of drug treatment in galactose-containing media. Neither LAP nor OD-LAP affected cell viability at the concentrations tested (Figure 4D,F).

**SOD1 and SOD2 as Markers of Cytoplasmic and Mitochondrial Stresses.** To test cytoplasmic versus mitochondrial-related stresses, the mRNA expression of SOD1 and SOD2, which differentially increase in response to cytoplasmic and mitochondrial oxidative stress, respectively, were measured in HepG2 cells treated for 2 and 4 h with 40 μM LAP as well as for the same amounts of time with 40 μM OD-LAP. As seen in Figure 5A,B, the mRNA expression of mitochondrial SOD2 increased in cells treated with 40 μM OD-LAP, whereas the expression of cytoplasmic SOD1 remained unchanged. This further indicated that OD-LAP affects the mitochondria. In transduced HepG2-3A4 cells treated with LAP for 4 h, there was a significant increase in the mRNA levels of both cytoplasmic SOD1 and mitochondrial SOD2 (Figure 5C,D), indicating increased cytosolic and mitochondrial stresses.

**Detection of LAPQI.** Because LAPQI is highly reactive and short lived, detection of covalent adducts of glutathione with LAPQI (GS-LAPQI) was taken as evidence of LAPQI formation in cells. GS-LAPQ was detected when 40 μM OD-LAP was added to HepG2 and HepG2-3A4 cells. However, only HepG2-3A4 cells were capable of generating GS-LAPQI in incubations containing 40 μM LAP, consistent with earlier data demonstrating that the toxic metabolite LAPQI can be generated by mitochondria through oxidation of OD-LAP but that P450 3A enzymes are required for the formation of OD-
LAP from LAP. Methods and spectra were consistent with earlier published data for GS-LAP formation in HepaRG cells (data not shown).23

Activation of Nrf2 in HepG2 and HepG2-3A4 Cells. In order to test the hypothesis that the reactive metabolite LAPQI may be involved in the activation of the Nrf2 defense pathway, the ability of the OD-LAP metabolite to induce Nrf2 target genes in HepG2 cells was assessed using the LiveBLAzer ARE-β-lactamase FRET-based reporter assay. This assay measures Nrf2 activation in CellSensor ARE-β-lactamase reporter gene containing HepG2 cells that contain a β-lactamase reporter gene under the control of an ARE that is stably integrated in HepG2 cells. Figure 6A shows Nrf2 activation dose−response curves in the presence of various concentrations of LAP (10, 5, 2.5, and 1.25 μM and 625 nM) or OD-LAP (50, 25, 12.5, 6.25, and 3.125 μM, and 781 nM) in HepG2-3A4 cells in galactose-containing media. (F) Cell viability of HepG2-3A4 cells after 24 h of exposure to OD-LAP. Data represent the mean ± SD of triplicate values. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with vehicle (unpaired t-test, two-tailed P values).

Figure 4. Effects of LAP and OD-LAP treatment in HepG2-3A4 cells. (A) 1′-OH midazolam formation in HepG2 with or without viral transduction with GFP, P450 3A4, or P450 3A5. (B) OCR after 24 h of exposure to 10 or 30 μM OD-LAP in HepG2-3A4 cells. (C) Concentration-dependent decrease in NAD(P)H levels after 24 h of LAP treatment in P450 3A4-transduced HepG2 cells relative to the DMSO control. (D) Cell viability of HepG2-3A4 cells after 24 h of exposure to LAP. (E) Cellular ATP depletion in the presence of various concentrations of LAP (10, 5, 2.5, and 1.25 μM and 625 nM) or OD-LAP (50, 25, 12.5, 6.25, and 3.125 μM, and 781 nM) in HepG2-3A4 cells in galactose-containing media. (F) Cell viability of HepG2-3A4 cells after 24 h of exposure to OD-LAP. Data represent the mean ± SD of triplicate values. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with vehicle (unpaired t-test, two-tailed P values).
serum-containing media, LAP was dissolved in serum-free media. In order to observe LAP-induced Nrf2 activation, CellSensor ARE-bla HepG2 cells were virally transduced with hP450 3A4 and 3A5. As seen in Figure 6B, the cells were successfully transduced, as demonstrated by the increased rate of midazolam hydroxylation to 1′-OH midazolam. The ARE-bla β-lactamase reporter assay was carried out again using the transduced cells (Figure 6C). LAP activation of Nrf2 in these cells is consistent with the earlier Seahorse data indicating that LAP needs the metabolic activity of P450 3A4/5 in order to activate the Nrf2 pathway in HepG2 cells.

**mRNA Expression Levels of Nrf2 Target Genes in HepG2 and HepG2-3A4 Cells.** In order to test the hypothesis that LAPQI, the reactive metabolite of LAP, activates Nrf2, the induction of two cytoprotective Nrf2 genes (HO-1 and NQO-1) was measured by quantifying their mRNA levels after treatment with LAP and OD-LAP in HepG2 cells (Figure 7A,C). LAP activation of Nrf2 in these cells is consistent with the earlier Seahorse data indicating that LAP needs the metabolic activity of P450 3A4/5 in order to activate the Nrf2 pathway in HepG2 cells.

In *Vivo* Evaluation of LAP-Induced Hepatic Toxicity. The experimental dose of LAP used in the mouse studies was set to 3 times the human-equivalent dose, as determined by allometric scaling of the AUC values. The mouse equivalent of 3 times the 1250 mg human dose was determined to be approximately 400 mg/kg. Preliminary experiments demonstrated a high level of variability after oral dosing in nonfasted mice, likely due to LAP solubility and the known effect of food on LAP absorption, so LAP dosing was switched from oral gavage to IP injection in order to reduce variability and to preclude daily fasting of the mice in a multiday study. As seen in Figure 9A,B, LAP (400 mg/kg, IP) administration to Nrf2-knockout (KO) and C57BL/6 wild-type (wt) male mice caused
an increase in the liver enzymes ALT and AST to more than 3 times their normal levels in both sets of mice on the first day. With continual dosing, C57BL/6 appeared to be robust and did not show any obvious impairment. The Nrf2-KO mice became dehydrated, had reduced mobility, showed hunched posture, and demonstrated reduced grooming. For this reason, the multiday dosing study was ended after 5 days. ALT and AST levels in C57BL/6 mice reverted back to normal levels by day 5. In contrast, the Nrf2-KO mice had a further elevation of both enzymes (∼8 × normal ALT and ∼38 × normal AST). These results imply that the liver is protected from LAP-induced toxicity by the Nrf2 detoxification pathway. Plasma levels of LAP and OD-LAP were determined 8 h after the initial dose on days 1 and 5 (Table 1). At the conclusion of the study, liver was collected to determine tissue exposure. While there was not a statistically significant change in plasma levels of the analytes tested between C57BL/6 and Nrf2-KO mice, there was a similar trend of decreasing LAP levels between days 1 and 5 for both strains of mice.

**DISCUSSION**

Even though LAP has a satisfactory safety profile for use in breast cancer treatment, elevation in liver enzymes, ALT > 3 times upper limit of normal (ULN) and total bilirubin >2 times ULN, have been reported in clinical trials in around 1% of patients. If Hy’s rule is correct for LAP, this would result in an expected rate of liver failure, resulting in death or liver transplantation in approximately 1:1000 patients. Ascertaining the true incidence of LAP-induced hepatotoxicity is likely complicated due to coadministered drugs, disease progression, and the likelihood of metastatic breast cancer to metastasize to the liver. Identifying the mechanisms and risk factors that cause treatment-related hepatotoxicity could improve the management of drug-induced toxicity in breast cancer patients. HLA-DQA1*02:01 was found to be associated with an increased incidence of LAP-induced hypersensitivity reactions after pharmacogenetic investigation. This might be due to the covalent binding of reactive LAP metabolites to cellular proteins to form haptenes that are presented as antigens by DQA1*02:01 and result in an adaptive immune response.

Reactive metabolites of LAP were previously shown to interact with P4503A enzymes (Figure 1). OD-LAP was demonstrated to be a mechanism-based inhibitor of P450 3A5 via addition of LAPQI to the apoprotein. Hydroxyl amine and N-dealkylated metabolites have been reported, but their in vivo concentrations appear to be minimal. Additionally, an aldehyde metabolite has been reported. We found an aldehyde contaminant in two commercial sources of LAP, but we did not observe a time-dependent formation of the aldehyde in microsomal incubations (data not shown). Induction of P450 3A4 by dexamethasone and rifampicin was found to enhance the cytoxicity caused by LAP, and it correlated with the formation of LAPQI cysteine adducts in HepaRG cells. As part of our investigation of LAP-induced toxicity, we found LAP adducts to cysteine-containing model peptides containing nucleophilic amino acids, but these were unstable. The intensity of LAP–peptide adducts, as detected by LC-MS/MS, diminished rapidly at room temperature. Stability was increased when LAPQI was generated from OD-LAP in an electrochemical cell and directly added to peptides, but the addition of
denatured cellular extract enhanced the instability and prevented further investigation of lapatinib adducts in cells (data not shown). This phenomenon of instability of protein adducts in cells has been seen before, and it may affect the appearance of toxic effects of LAP in vitro.  

The exact mechanism leading to the hepatotoxicity of LAP is not well understood, nor are the toxicological implications of the OD-LAP metabolite. While there is an immune component to LAP toxicity, this does not explain the totality of the observed toxicity, and we hypothesized that mitochondrial dysfunction might play an additive role to the reported immune-mediated toxicity of LAP. Mitochondrial toxicity has been implicated in drug-induced organ toxicities, especially hepatotoxicity; however, mitochondrial toxicity can be difficult to ascertain from in vitro studies because the ultimate toxic insult may be due to metabolites of the parent molecule. For example, the HER2 tyrosine kinase inhibitor CP-724,714 was discontinued from clinical development due to unexpected hepatotoxicity in cancer patients, and it was later found to cause mitochondrial impairment in human hepatocytes that had previously gone undetected in other cell types.  

HepG2 cells were compared to HepG2 cells transduced with hP450 3A4. The cellular oxygen consumption rate, which primarily reflects oxygen utilization by the mitochondrial electron transport chain, was clearly lower in HepG2-3A4 cells compared to that in HepG2 cells after treatment with LAP. The involvement of the O-dealkylated metabolite (OD-LAP) was demonstrated by the clear reduction in OCR in HepG2 cells when OD-LAP was directly added to the cells. A further demonstration of impaired mitochondrial function was evidenced by the dose-dependent decrease in both NAD(P)H and ATP. To ensure that decreased mitochondrial function was not due to a reduction in the number of live cells in the incubations, cytotoxicity was evaluated by measuring membrane integrity. Immortalized cell lines such as HepG2 tend to grow rapidly under hypoxic and high-glucose conditions, so they derive most of their ATP from glycolysis rather than mitochondrial oxidative phosphorylation. This condition is termed the Warburg and/or Crabtree effect. In order to make the transduced HepG2-3A4 cells more reliant on oxidative phosphorylation to observe the effect that LAP has on the mitochondria, we dosed LAP and OD-LAP in galactose-containing media. These conditions reduce the cells’ ability to get their ATP from glycolysis, thereby increasing ATP production from oxidative phosphorylation. Such conditions are conducive for assessing drug-induced mitochondrial dysfunction, and in our experiments, we were indeed able to see a dose-dependent decrease in ATP production when

Figure 7. Quantitative RT-PCR: HepG2 cells. mRNA expression of (A) HO-1 and (B) NQO-1 in HepG2 cells after exposure to LAP and OD-LAP for 2 and 4 h. Data represent the mean ± SD of triplicate values. *P < 0.05 and **P < 0.01 compared with vehicle (unpaired t-test, two-tailed P values).

Figure 8. Quantitative RT-PCR: HepG2-3A4 cells. mRNA expression of (A) HO-1 and (B) NQO-1 in HepG2 cells transduced with hP450 3A4 after exposure to LAP for 4 h. Data represent the mean ± SD of triplicate values. *P < 0.05 and **P < 0.01 compared with vehicle (unpaired t-test, two-tailed P values).
HepG2-3A4 cells were dosed in galactose-containing media with LAP and OD-LAP.

**In vitro** studies by Teng et al. showed that OD-LAP is further oxidized to LAPQI and can covalently adduct to GSH.** We tested to see if depletion of GSH in HepG2 cells would increase LAPQI-mediated toxicity and make the cells more susceptible to mitochondrial dysfunction. Treatment of HepG2 cells with BSO depletes GSH. When dosed with OD-LAP at 20 μM in addition to BSO, there was a profound decrease in the OCR of HepG2 cells as compared to that of the control.

LAP metabolism to OD-LAP, and further oxidation to the electrophile LAPQI, appears to be responsible for much of the observed mitochondrial toxicity. However, there may be additional factors yet to be elucidated. This is implicated in the decreased OCR and NAD(P)H levels depicted in Figures 2 and 4, where 30 μM OD-LAP was effective at reducing OCR and NAD(P)H levels but 10 μM OD-LAP had a minimal effect. However, in P450 3A4-transduced HepG2 cells, 10 μM LAP significantly decreased both OCR and NAD(P)H levels and LAP appeared to be a more potent mitochondrial toxin than when the identical concentration of OD-LAP was directly added to the cell. The increased expression of both SOD1 and SOD2 in LAP-treated HepG2-3A4 cells implies that there is a cytosolic stress that was not present in incubations of HepG2 with OD-LAP. SOD1 and SOD2 are located in the cytoplasm and mitochondrial matrix, respectively.**

We believe that the concentrations of LAP tested in the reported studies are physiologically relevant and that some level of mitochondrial impairment in LAP-treated patients is likely. While LAP concentrations in human liver under the standard dosing schedule are not known, imaging studies using **[14C]**LAP in male rats demonstrated a high degree of LAP accumulation in the liver and reduced concentrations in the brain when analyzed by whole-body autoradiography.** Similarly, the activity of P450 3A4 in the transduced HepG2 cells used in this study was only 3 times the average level seen previously in cryopreserved hepatocytes.** Given the high degree of variability in P450 3A4 activity between individuals,** the HepG2-3A4 cells are a reasonable model to study the metabolic activation and associated toxicity of LAP in cells.

Despite evidence of significant mitochondrial impairment and elevated liver enzymes after a single dose in mouse, LAP demonstrated an acceptable safety profile and sufficient therapeutic benefit for approval as a cancer therapy. The potential of LAP or its metabolite (OD-LAP) to upregulate cytoprotective pathways through Nrf2 activation was evaluated. The Nrf2—ARE pathway can sense and respond to chemical and oxidative stresses, detoxifying electrophiles and ameliorating their effect. OD-LAP was shown to activate Nrf2 in a cell-based reporter assay and by quantitative RT-PCR, whereas LAP activated Nrf2 only in cells transfected with P450 3A4. Increased mRNA for Nrf2 target genes was observed after 4 h in the cell-based assays, but this was not observed at earlier time points. Additional time would be expected for the translation of Nrf2 targets to take effect. This delay may explain why significant signs of hepatotoxicity were observed after a single LAP dose, but during multiday dosing in which Nrf2 target genes are likely upregulated prior to the daily dose, LAP did not appear to cause hepatic necrosis, despite very high liver concentrations of both LAP and OD-LAP.

The decreased plasma levels of both LAP and OD-LAP between days 1 and 5 suggest the additional possibility of non-Nrf2-mediated enzyme induction. Because the decrease in the concentration of LAP and LAPQI is similar between days 1 and 5 in the Nrf2-KO and control mice, but only the control mice exhibit reduced hepatic damage upon repeat dosing, we do not believe that the decreased drug concentrations are significant factors in hepatic protection.

After the initial dose, Nrf2 target genes are likely to decrease cellular damage caused by subsequent doses. This may have an important connection with immune-mediated hepatotoxicity through reducing the intensity of the danger-associated molecular patterns required to produce an immune response. Nrf2 would not be expected to play a role in the development of immune tolerance, which appears to be a driving factor in immune-mediated hepatotoxicity.**
The results of the current investigation support the hypothesis that LAP is a mitochondrial toxin and that its hepatocellular toxicity is mainly linked to P450 3A4-catalyzed generation of OD-LAP, which is further oxidized to form the reactive electrophile LAPQI. These findings may explain the liver-related toxicity observed in LAP-treated breast cancer patients. The study illustrates the usefulness of HepG2 cells transduced with P450 3A4 for generating P450 3A4-dependent metabolites to investigate metabolism-induced toxicity. In addition, we have highlighted the importance of Nrf2 activation in the protection against LAP-induced toxicity.

■ ASSOCIATED CONTENT

 Supporting Information

 The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00524.

 PCR primers used and a detailed analytical method for quantitation of LAP (PDF)

 ■ AUTHOR INFORMATION

 Corresponding Author

 *E-mail: cameron@scripps.edu.

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 ■ ABBREVIATIONS

 LAP, lapatinib; OD-LAP, O-dealkylated lapatinib; Nrf2, nuclear factor erythroid 2-related factor c2; NQO-1, NAD(P)H quinone oxidoreductase 1; GST, glutathione S-transferase; UGT, uridine 5’-diphospho-glucuronosyltransferases; GS, glutathione synthetase; GCL, glutamate-cysteine ligase; FRET, fluorescence resonance energy transfer; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; FDA, Food and Drug Administration; HLA, human leukocyte antigen; LAPQI, lapatinib-quinone imine; ARE, antioxidant response element; Nrfl-KO, Nrfl knockout; wt, wild type; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenyllydrazone; BSO, l-buthionine sulfoximine; OCR, oxygen consumption rate; GFP, green fluorescent protein; SOD, superoxide dismutase; ULN, upper limit of normal

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