Identification of the Cytochrome P450 and Other Enzymes Involved in the In Vitro Oxidative Metabolism of a Novel Antidepressant, Lu AA21004

Mette G. Hvenegaard, Benny Bang-Andersen, Henrik Pedersen, Morten Jørgensen, Ask Püschl, and Lars Dalgaard

Department of Drug Metabolism (M.G.H., L.D.) and Division of Discovery Chemistry and DMPK, H. Lundbeck A/S, Valby, Denmark (B.B.-A., H.P., M.J., A.P.)

Received January 19, 2012; accepted April 11, 2012

ABSTRACT:

1-[2-(2,4-Dimethyl-phenylsulfanyl)-phenyl]-piperazine (Lu AA21004) is a novel antidepressant that is currently in late-stage clinical development for major depressive disorder. In the present study, the metabolism of Lu AA21004 was investigated using human liver microsomes (HLM), human liver S9 fraction, and recombinant enzymes. Lu AA21004 was found in vitro to be oxidized to a 4-hydroxy-phenyl metabolite, a sulfoxide, an N-hydroxylated piperazine, and a benzyl alcohol, which was further oxidized to the corresponding benzoic acid [3-methyl-4-(2-piperazin-1-yl-phenylsulfanyl)-benzoic acid (Lu AA34443)]. The formation of the 4-hydroxy-phenyl metabolite was catalyzed by CYP2D6 with some contribution from CYP2C9, whereas the formation of the sulfoxide was mediated by CYP3A4/5 and CYP2A6. CYP2C9 and CYP2C19 were the primary enzymes responsible for the formation of the N-hydroxylated metabolite. The benzyl alcohol was formed by CYP2D6 only. The oxidation of the benzyl alcohol to the corresponding benzoic acid of Lu AA21004 was catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase, with some contribution from aldehyde oxidase. CYP2D6 was also capable of catalyzing the formation of the benzoic acid of Lu AA21004; however, its overall contribution to this pathway was negligible. Enzyme kinetic parameters revealed that the rate-limiting step in the formation of the benzoic acid from Lu AA21004 is the formation of the corresponding alcohol. Thus, the intrinsic clearance (Vmax/Km) in HLM for metabolism of Lu AA21004 to the benzyl alcohol was 1.13 × 10−8 l·min⁻¹·mg⁻¹, whereas the subsequent metabolism of the benzyl alcohol to the benzoic acid of Lu AA21004 is characterized by an intrinsic clearance (Vmax/Km) in S9 fraction of 922 × 10−8 l·min⁻¹·mg⁻¹.

Introduction

1-[2-(2,4-Dimethyl-phenylsulfanyl)-phenyl]-piperazine (Lu AA21004) is a multimodal antidepressant that is thought to work through a combination of two pharmacological modes of action: reuptake inhibition and receptor activity. In vitro studies indicate that Lu AA21004 is a 5-HT3 receptor antagonist, 5-HT1B receptor agonist, 5-HT7 (gene HTR7) receptor agonist, 5-HT1A (gene HTR1A) receptor agonist, and 5-HT1B receptor partial agonist and inhibitor of the 5-HT (gene SLC6A4) transporter. In vivo nonclinical studies have demonstrated that Lu AA21004 enhances levels of the neurotransmitters serotonin, noradrenaline, dopamine, acetylcholine, and histamine in specific areas of the brain. Data from the nonclinical studies suggest that the multimodal activity of Lu AA21004 is likely to contribute to its mechanism of action and therapeutic activity (Artigas et al., 2009; Bang-Andersen et al., 2011). Lu AA21004 is currently in clinical development for major depressive disorder. In the nonclinical absorption, distribution, metabolism, and excretion studies Lu AA21004 is eliminated by extensive metabolic conversion via multiple pathways and metabolites are excreted both via bile and urine (L. Bendahl and L. Dalgaard, unpublished data). In the human metabolism and excretion study using 14C-labeled Lu AA21004, six metabolites were quantified in plasma. The major metabolites in human plasma were the benzoic acid of Lu AA21004, M0 [3-methyl-4-(2-piperazin-1-yl-phenylsulfanyl)-benzoic acid (Lu AA34443)], and its glucuronide, M4(b). Other metabolites detected in human plasma were a 4-hydroxy-phenyl metabolite, M8, which is further conjugated to its glucuronide, M3, a sulfoxide, M4(a), and two glucuronides, M11 and M12, of the N-hydroxylated Lu AA21004 [L. Bendahl and L. Dalgaard, unpublished data; Uldam et al. (2011)]. The primary metabolites in excreta were the benzoic acid metabolite of Lu AA21004, M0, and its glucuronide conjugate, M4(b), accounting for the vast majority of the dose administered (L. Bendahl and L. Dalgaard, unpublished data). The aim of the present study was to identify the enzymes involved in the phase 1 metabolism of Lu AA21004 including the intermediates, namely the N-hydroxy piperazine and the benzyl alcohol of Lu AA21004. The enzymes responsible for the formation of the individual metabolites formed after incubation of Lu

ABBRIVIATIONS: Lu AA21004, 1-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazine; Lu AA34443, 3-methyl-4-(2-piperazin-1-yl-phenylsulfanyl)-benzoic acid; FMO3, flavin-containing monoxygenase 3; P450, cytochrome P450; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; DMSO, dimethyl sulfoxide; SRM, single reaction monitoring; MS, mass spectrometry; CV, coefficient of variation; HLM, human liver microsomes.
AA21004 was determined using human recombinant FMO3 and cytochrome 450 enzymes and phenotyped human liver microsomes. Furthermore, a detailed study with determination of enzyme kinetic parameters was performed on the transformation of Lu AA21004 via the benzylic alcohol to the benzoic acid, M0, to determine the most important enzymes catalyzing this pathway, i.e., determining the rate-limiting step. The in vitro studies described herein were conducted in accordance with the U.S. Food and Drug Administration draft guideline on the conduct of in vitro metabolism studies (Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072101.pdf, 2006) and the principles described by Tucker et al. (2001), Bjornsson et al. (2003), and Huang et al. (2008).

**Materials and Methods**

**Chemical and Reagents.** Lu AA21004-piperazine HBr (Lu AA21004 HBr), benzoic acid of Lu AA21004, M0 (benzoic acid of Lu AA21004; Lu AA34443), M4a (sulfoxide of Lu AA21004), M8 (4-hydroxy-phenyl of Lu AA21004 sodium salt), and the N-hydroxylated intermediate of Lu AA21004 were prepared at H. Lundbeck A/S as described in supplemental data and by Uldam et al. (2011). [14C]-Lu AA21004 HBr was purchased from (Quotient Biorsearch, Cambridgeshire, UK), NADPH, NAD+, NADP+, isocitric acid, isocitric dehydrogenase, KCl, MgCl2, Tris-HC1, KH2PO4, raloxifene, alfuzopurinol, and disulfiram were purchased from Sigma-Aldrich (St. Louis, MO) and 4-methylpyrazole was purchased from ACROS (Geel, Belgium). Microsomes from human B-lymphoblastoid AHH-1 cell line expressing human CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, control microsomes from human B-lymphoblastoid AHH-1 cell line containing only the vector, microsomes from baculovirus-infected insect cells (BTI-TN-5B1–4) expressing human FMO3, and control microsomes from insect cells (BTI-TN-5B1–4) infected with wild-type baculovirus (Autographa californica), and pooled human liver S9 (pool of 50 donors) were all purchased from Gentest Corporation (Woburn, MA). HepatoSomes control pool (lot 2.0) (pooled human liver microsomes) and Hepatocore Test Kit with 15 donors (phenotyped human liver microsomes) was purchased from Human Biologics International (Scottsdale, AZ). Each donor was enzymatically characterized by the supplier with respect to P450 isoform-selective reactions as follows (isoenzyme in parentheses): caffeine N3-demethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), S-mephenytoin N-deethylation (CYP2B6), tolbutamide methyl hydroxylation (CYP2C9), S-mephenytoin-4′-hydroxylation (CYP2C19), dextromethorphan O-deethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), dextromethorphan N-deethylation (CYP3A), testosterone 6β-hydroxylation (CYP3A), and lauric acid 12-hydroxylation (CYP4A11). Pooled human liver microsomes (pool of 50 donors) and a Reaction Phenotyping Kit with 16 donors (phenotyped human liver microsomes) were purchased from XenoTech, LLC (Lafayette, KS). Each donor was enzymatically characterized by the supplier with respect to P450 isoform-selective reactions as follows (isoenzyme in parentheses): 7-ethoxyresorufin O-dealkylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), S-mephenytoin N-deethylation (CYP2B6), pento- laxel (Taxol) 6 α-hydroxylation (CYP2C8), diclofenac 4′-hydroxylation (CYP2C9), S-mephenytoin-4′-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), testosterone 6β-hydroxylation (CYP3A), lauric acid 12-hydroxylation (CYP4A11), and benzydamine N-oxidation (FMO3).

**Metabolite Identification after Incubation with Lu AA21004.** To investigate which metabolites were formed after incubation of Lu AA21004 in pooled human liver microsomes, 0.5 and 1.0 mg protein/ml microsomes were incubated at 37°C in 50 mM Tris-HCl (pH 7.4), 154 mM KCl, 5 mM MgCl2, and an NADPH-regenerating system (1 mM NADP+, 5 mM isocitric acid, and 1 U/ml isocitric dehydrogenase) in a final volume of 0.5 ml. After a 5-min preincubation, the reaction was initiated by adding the substrate (100 μM final concentration), which was dissolved in water-acetonitrile (1:1), resulting in a final concentration of acetonitrile of 0.5% in the incubation. The incubation times were 0, 30, and 180 min. The reaction was terminated by the addition of 1 ml of ice-cold acetonitrile containing internal standard. The samples were then centrifuged at approximately 4000g, and the supernatant was evaporated to dryness under N2 at 25°C. The residue was dissolved in 125 μl of 10 mM ammonium acetate (pH 5)-acetonitrile (9:1), and the ~18,000g supernatant was analyzed by HPLC-MS/MS. The samples were analyzed using method A (see Analysis).

**NADPH Dependence after Incubation with Lu AA21004.** The NADPH dependence of the metabolism of Lu AA21004 was examined by incubating 0.5 mg of protein/ml pooled human liver microsomes for 0 and 180 min 1) with an NADPH-regenerating system, 2) with an NADPH-regenerating system without NADP+, and 3) without an NADPH-regenerating system. The incubation conditions and sample preparation was the same as described under Metabolite Identification after Incubation with Lu AA21004. The samples were analyzed using method A (see Analysis).

**Enzyme Kinetics after Incubation of Lu AA21004.** The incubation conditions were the same as described under Metabolite Identification after Incubation with Lu AA21004. The protein concentration was 0.5 mg/ml, and the final volume was 0.5 ml. After a 10-min preincubation, the reaction was initiated by addition of [14C]-Lu AA21004 (1–500 μM), which was dissolved in ethanol, resulting in a final concentration of ethanol of 1%. The level of radioactivity was dependent on the specific activity and was in the range of 0.07 to 0.4 μCi/ml. The incubation time was 30 min. The reaction was terminated by the addition of 0.5 ml of ice-cold acetonitrile containing internal standard. The samples were then centrifuged at ~4000g, and the supernatant was evaporated to dryness under N2 at 20°C overnight. The residue was dissolved in 250 μl of reconstitution buffer (methanol-50:50 nm ammonium formate, pH 3.5; 6:4) and analyzed by HPLC-MS/MS and an on-line radioactivity detector using method B (see Analysis). The enzyme kinetic investigations were performed within the range of linearity with respect to protein concentration and incubation time, which were determined in a preliminary experiment.

**Incubation of Lu AA21004 with Recombinant Enzymes.** Recombinant human liver FMO3 and P450 isoenzymes (CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, and 3A4) were incubated with 8 and 50 μM Lu AA21004. The experimental procedure was the same as for the metabolism experiment. The protein content was 1 mg/ml as suggested by the supplier, and the incubation time was 120 min. As a control, microsomes from the same cell line containing only the vector were used. The samples were analyzed by method A (see Analysis).

**Incubation of Phenotyped Human Liver Microsomes with Lu AA21004.** Microsomes from each of the 15 phenotyped donors were incubated under the same conditions as those for the metabolism identification experiment. The protein concentration was 0.5 mg/ml and the incubation time was 0 and 60 min. The concentrations of Lu AA21004 were 8 and 50 μM. The samples were analyzed by method A (see Analysis). The incubation conditions were within the range of linearity with respect to protein concentration and incubation time for pooled human liver microsomes, which were determined in a preliminary experiment. The results obtained were subjected to correlation analysis.

**NADPH Dependence of the Metabolism of the Benzyl Alcohol of Lu AA21004 to the Benzoic Acid of Lu AA21004 in Human Liver Microsomes.** The NADPH dependence of the metabolism of the benzylic alcohol of Lu AA21004 to the corresponding benzoic acid was examined by incubating 50 μM benzyl alcohol with 0.5 mg of protein/ml pooled human liver microsomes for 0 and 180 min 1) with an NADPH-regenerating system, 2) with an NADPH-regenerating system without NADP+, 3) without an NADPH-regenerating system, and 4) with Tris buffer only (no microsomes). The incubation conditions and sample preparation were according to the method described for the kinetics experiment with the benzylic alcohol in the same conditions as those for the metabolism identification experiment. The protein concentration was 0.5 mg/ml and the incubation time was 0 and 60 min. The concentrations of Lu AA21004 were 8 and 50 μM. The samples were analyzed by method A (see Analysis). The incubation conditions were within the range of linearity with respect to protein concentration and incubation time for pooled human liver microsomes, which were determined in a preliminary experiment. The results obtained were subjected to correlation analysis.

**Enzyme Kinetics in Human Liver Microsomes after Incubation with the Benzyl Alcohol of Lu AA21004.** Pooled human liver microsomes were incubated at 37°C in Tris buffer containing 50 mM Tris-HCl, 154 mM KCl, 5 mM MgCl2, and an NADPH-regenerating system (1 mM NADP+, 5 mM isocitric acid, and 1 U/ml isocitric dehydrogenase). The protein concentration was 0.5 mg/ml, and the final volume was 0.5 ml. After a 10-min preincubation, the reaction was initiated by adding the benzyl alcohol of Lu AA21004 (1–500 μM), which was dissolved in water-acetonitrile, resulting in a final concentration of acetonitrile of 0.5% in the incubation. The incubation time...
was 20 min. The reaction was terminated by the addition of 0.5 ml of ice-cold acetonitrile containing internal standard. The samples were then centrifuged at 4000g, and the supernatant was evaporated to dryness under N2 at 20°C over night. The residue was dissolved in 250 µl of reconstitution buffer (methanol:50 mM ammonium formate, pH 3.5; 6:4) and analyzed by HPLC-MS/MS using HPLC method B (see Analysis). The enzyme kinetic investigations were performed within the range of linearity with respect to protein concentration and incubation time, which were determined in a preliminary experiment.  

**Incubation of Recombinant Enzymes with the Benzyl Alcohol of Lu AA21004.** Recombinant human liver FMO3 and P450 isoenzymes (CYP1A2, CYP2C9, and CYP3A4) were incubated with 50 mM benzylic alcohol of Lu AA21004. The experimental procedure was the same as for the kinetics experiment of the benzylic alcohol of Lu AA21004 with human liver microsomes. The incubation time was 120 min. As a control, microsomes from the same cell line containing only the vector were used. The samples were analyzed by HPLC-MS/MS using method B (see Analysis).

**Incubation of Phenotyped Human Liver Microsomes with the Benzyl Alcohol of Lu AA21004.** Human liver microsomes from each of the phenotyped 15 donors were incubated under the same conditions as those for the kinetic experiment with the benzylic alcohol of Lu AA21004 in human liver microsomes. The incubation time was 15 min. The concentrations of the benzylic alcohol of Lu AA21004 were 8 and 50 µM. The samples were analyzed by method B (see Analysis). The incubation conditions were performed within the range of linearity with respect to protein concentration and incubation time for pooled human liver microsomes, which were determined in a preliminary experiment. The results obtained were subjected to correlation analysis.

**Cofactor Dependence of the Metabolism of the Benzyl Alcohol to the Benzoic Acid of Lu AA21004 in Human Liver S9 Fraction.** The NAD+ and NADPH dependence of the metabolism of the benzylic alcohol of Lu AA21004 to the corresponding acid in human liver S9 fraction was examined by incubating 8 and 50 µM benzylic alcohol of Lu AA21004 with 0.02 mg protein/ml for 15 min. In addition to the substrate, the incubations contained 1) S9 fraction and NADPH, 2) S9 fraction and NAD+, 3) S9 fraction only, and 4) potassium phosphate buffer. The incubation conditions and sample preparation were according to the method described for the kinetics experiment in human liver S9 fraction. The samples were analyzed by HPLC-MS/MS using HPLC method B (see Analysis).

**Enzyme Kinetics of the Metabolism of the Benzyl Alcohol to the Benzoic Acid of Lu AA21004 in Human Liver S9 Fraction.** Pooled human liver S9 fraction was incubated at 37°C in potassium phosphate buffer containing 50 mM KH2PO4 (pH 7.4), and 1 mM NAD+. The protein concentration was 0.02 mg/ml, and the final volume was 0.5 ml. After a 10-min preincubation, the reaction was initiated by adding the benzylic alcohol of Lu AA21004 (1–500 µM), which was dissolved in water-acetonitrile, resulting in a final concentration of acetonitrile of 0.5% in the incubation. The incubation time was 20 min. The reaction was terminated by the addition of 0.5 ml of ice-cold acetonitrile containing internal standard. The samples were then centrifuged at 4000g, and the supernatant was evaporated to dryness under N2 at 25°C. The residue was dissolved in 250 µl of reconstitution buffer (methanol:50 mM ammonium formate, pH 3.5; 6:4) and analyzed by HPLC-MS/MS using HPLC method B (see Analysis). The enzyme kinetic investigations were performed within the range of linearity with respect to protein concentration and incubation time.

**Incubation of Pooled Human Liver S9 Fraction with the Benzyl Alcohol of Lu AA21004 and Chemical Inhibitors.** Pooled human liver S9 fraction was incubated with one of the following enzyme inhibitors (concentration and enzyme in parentheses): raloxifene (100 nM, aldehyde oxidase), allopurinol (100 µM, xanthine oxidase), disulfiram (40, 100, and 500 µM, aldehyde dehydrogenase), and 4-methylpyrazole (600 µM, alcohol dehydrogenase) (Kassam et al., 1989; Klysovy et al., 1996; Lam et al., 1997; Dawidek-Pietryka et al., 1998; Walsh et al., 2002; Okamoto et al., 2003; Obach 2004). The protein content was 0.02 mg/ml, and incubation time was 15 min. The inhibitors were dissolved in DMSO, resulting in a final DMSO concentration of 1% in the incubations. Controls contained only the solvent (1% DMSO) and were preincubated for 10 min except for incubations containing allopurinol and disulfiram for which the preincubation time was 30 min to allow time-dependent inhibition. The concentration of substrate was 2 µM (equal to Km), 8 µM, and 50 µM. The procedure was the same as that for the kinetics experiment in human liver S9 fraction. The samples were analyzed by HPLC-MS/MS using method B (see Analysis).

**Analysis.** Method A. Chromatographic separation was obtained using a Waters 2790 HPLC system and Waters Symmetry C8 guard (Waters, Milford, MA) column and column (3.9 × 20 and 3.9 × 50 mm, respectively). Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0), whereas mobile phase B consisted of 10 ml of 10 mM ammonium acetate buffer (pH 5.0) in 1 liter of acetonitrile. The temperature of the column oven was 40°C, and the flow rate was 1 ml/min. The column volume was 20% for each standard. The suitability test was also injected several times during the analysis of the samples the acceptance criteria of the run being as above.

Method B. Chromatographic separation was obtained using a Surveyor HPLC system and Waters Xterra guard column (4.6 × 10 mm) and Waters Luna 5-µm phenyl-hexyl column (4.6 × 250 mm). Mobile phase A consisted of 50 mM ammonium formate (pH 3.5)/acetonitrile (1:9, v/v), whereas mobile phase B consisted of 50 mM ammonium formate (pH 3.5)/acetonitrile (1:9, v/v). The temperature of the column oven was 40°C, and the flow rate was 1 ml/min. A linear gradient (1 ml/min) was applied (0–11.0 min) from 88 to 70% mobile phase A and was followed by 70% mobile A from 11.0 to 15.0 min. Then a linear gradient (15.0–15.1 min) from 70 to 60% mobile phase A was applied followed by another linear gradient (15.1–20 min) from 60 to 50% mobile phase A. The column was then washed for 5 min with 5% mobile phase A and re-equilibrated for 5.4 min (total run time 31 min). The eluent was coupled to a Fle One radiodetector and a Finnigan TSQuantum mass spectrometer. A positive electrospray ionization method was used. The single reaction monitoring channels were as in method A except for the benzylic alcohol of Lu AA21004, for which the SRM were 315 > 150. This method was further modified to quantify the benzoic acid of Lu AA21004 after incubation with the benzylic alcohol of Lu AA21004, resulting in a total run time of 14 min. Samples were analyzed quantitatively for the benzylic acid of Lu AA21004 by HPLC-MS/MS. The calibration curve was prepared by spiking microsomes or S9 fractions with increasing concentrations of the benzoic acid of Lu AA21004 followed by the same sample preparation as for the authentic samples. The MS response was linear in the range of 5 nM to 20 µM for the benzylic alcohol of Lu AA21004. Repeatability was tested before each set of analysis by injecting a suitability test [Lu AA21004 and available metabolite standards (see above)]. The suitability test was also injected several times during the analysis of the samples the acceptance criteria of the run being as above.
alcohol, and the sulfoxide of Lu AA21004 was analyzed quantitatively using radioactivity. The amount of benzoic acid of Lu AA21004 formed after incubation with the benzylic alcohol was determined using a calibration curve as described in method B.

The apparent kinetic parameters, $K_m$ and $V_{max}$, were calculated by nonlinear regression using GraphPad Prism (version 4.01; GraphPad Software Inc., San Diego, CA). The data were fitted to a one-enzyme Michaelis-Menten equation $[V = V_{max} \times ([S]/(K_m + [S]))]$ or a Michaelis-Menten model with uncompetitive substrate inhibition $[V = V_{max} \times ([S]/(K_m + [S] + ([S]^2/K_c)))]$ (Venkatakrishnan et al., 2001). Edadie-Hofsteet transformations were made to investigate any indications of involvement of multiple enzymes.

Data obtained from phenotyped human liver microsomes were subjected to linear regression analysis using Microsoft Excel 2000. The data used was the average of triplicate determinations. An F-test was used to test whether the slope was significantly different from 0. Data from the experiment using inhibitors were analyzed by F-tests to assure homogeneity of variance followed by unpaired t tests. When variances were different, an unpaired t test with Welch correction was used. All analysis was performed using GraphPad Prism.

Results

Metabolite Identification after Incubation with Lu AA21004. Incubation of pooled human liver microsomes with Lu AA21004 resulted in the formation of benzylic alcohol, benzoic acid (M0), sulfoxide (M4a), 4-hydroxy-phenyl metabolite (M8), and an N-hydroxylated piperazine of Lu AA21004. Furthermore, three metabolites with $m/z = 331$ were also formed, indicative of the dihydroxylated metabolites of Lu AA21004 or monohydroxylated sulfoxides. Metabolites with $m/z = 331$ were not detected in the in vivo human metabolite radioprofiling study (L. Bendahl and L. Dalgaard, unpublished data) and are therefore not further discussed. An overview of the in vitro metabolic pathway is shown in Fig. 1. No metabolism was observed in the absence of any of the metabolites (data not shown). Thus, all of the metabolites formed were dependent on the presence of NADPH.

Incubation of Lu AA21004 with Recombinant Enzymes. Lu AA21004, after incubation with recombinant enzymes, was oxidized to the benzylic alcohol of Lu AA21004, its corresponding benzoic acid, a sulfoxide, an N-hydroxylated piperazine, and a 4-hydroxy-phenyl metabolite. CYP2D6 showed the highest catalytic activity with respect to the formation of the benzylic alcohol of Lu AA21004 (normalized with respect to the amount of CYP540). CYP2C9 and CYP2C19 also had the capability to form the benzylic alcohol of Lu AA21004, although with a lower catalytic activity (Fig. 2). CYP2D6 was the only P450 isozyme catalyzing the formation of the benzoic acid of Lu AA21004 (Fig. 2). With respect to the sulfoxide of Lu AA21004, CYP3A4 as well as CYP2A6 and CYP2C8 was capable of catalyzing the formation of this metabolite, with CYP3A4 having the highest catalytic activity (Fig. 2). The formation of the N-hydroxylated piperazine was catalyzed by CYP2C9 and CYP2C19 and to a much lower extent by CYP2B6 (Fig. 2). Recombinantly expressed CYP2D6 showed the highest catalytic activity, whereas CYP2C9 and CYP2C19 showed some catalytic activity with respect to the formation of the 4-hydroxy-phenyl metabolite (Fig. 2). None of the other recombinant enzymes were able to metabolize Lu AA21004 to the 4-hydroxy-phenyl metabolite under the conditions used. FM03 showed no or very little catalytic activity with respect to the formation of any of the metabolites (data not shown).

Incubation of Phenotyped Human Liver Microsomes with Lu AA21004. Lu AA21004, after incubation with phenotyped human liver microsomes, was oxidized to a benzylic alcohol of Lu AA21004, its corresponding benzoic acid, a sulfoxide, an N-hydroxylated piperazine, and 4-hydroxy-phenyl metabolite under the conditions used. CYP2A6, CYP2B6, and CYP3A activities. Furthermore, at a substrate concentration of 8 μM, the formation correlated with CYP2E1 activity. At a substrate concentration of 8 μM, the formation of the N-hydroxylated piperazine correlated with CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP3A activities. However, use of a substrate concentration of 50 μM resulted in correlation only with CYP2A6, CYP2B6, and CYP3A activities. The metabolism of Lu AA21004 to the 4-hydroxy-phenyl metabolite correlated with CYP2D6 enzyme activity. Some correlation was obtained with...
CYP2C9 enzyme activity but only at a substrate concentration of 8 μM (Table 1). Intercorrelation existed between the following enzyme activities: CYP2A6 and CYP2B6, CYP2A6 and CYP3A, CYP2B6 and CYP3A, CYP2C9 and CYP3A, and CYP2C19 and CYP3A (Table 1).

**Formation of the Benzoic Acid of Lu AA21004 from the Benzylic Alcohol of Lu AA21004.** The second step in the biotransformation of Lu AA21004 to the benzoic acid metabolite was investigated using the benzylic alcohol of Lu AA21004 as a substrate. The benzoic acid of Lu AA21004 was formed after incubation of the benzylic alcohol with human liver microsomes and human liver S9 fraction (data not shown). NADPH was not required for the metabolism of the benzylic alcohol to its corresponding benzoic acid in human liver microsomes. Thus, the metabolism of the benzylic alcohol to the benzoic acid of Lu AA21004 in human liver microsomes occurred in the absence of NADPH. However, the amount of benzoic acid of Lu AA21004 formed increased with a factor of 2 with the presence of NADPH, indicating that the formation of this metabolite is catalyzed by NADPH-dependent as well as NADPH-independent enzymes (data not shown). In human liver S9 fraction, the benzylic alcohol of Lu AA21004 was metabolized to its corresponding benzoic acid metabolite to some extent in the absence of cofactors. The amount of benzoic acid of Lu AA21004 formed was not affected by the presence of NADPH, indicating that the contribution of CYP450 under those conditions is negligible. The presence of NADPH increased the formation of the benzoic acid by a factor of 9 and 7 at substrate concentrations of 8 and 50 μM, respectively (data not shown), indicating that alcohol dehydrogenase and aldehyde dehydrogenase are probably the major enzymes responsible for the formation of the benzoic acid.

**Incubation of Recombinant Enzymes with the Benzylic Alcohol of Lu AA21004.** The benzylic alcohol of Lu AA21004 was oxidized to its corresponding benzoic acid after incubation with recombinant enzymes. CYP2D6 was the only enzyme catalyzing the formation of the benzoic acid metabolite (data not shown).

**Incubation of Phenotyped Human Liver Microsomes with Benzylic Alcohol of Lu AA21004.** The benzylic alcohol of Lu AA21004 was also oxidized to its corresponding acid after incubation with phenotyped human liver microsomes. However, no correlation be-
between the formation of the benzoic acid metabolite and any of the P450 isozyme activities was obtained (data not shown). Thus, enzymes other than CYP450 seem to participate in this biotransformation, because a high correlation with CYP2D6 activity would be expected if only CYP450 was involved (on the basis of the results from the recombinant enzymes, in which only CYP2D6 showed catalytic activity). However, it should be kept in mind that the microsomes are contaminated with other organelles.

Incubation of Pooled Human Liver S9 Fraction with Benzylic Alcohol of Lu AA21004 and Chemical Inhibitors. Pooled human liver S9 fraction was incubated with four different inhibitors at three substrate concentrations. In addition to the 8 and 50 μM used for the other assays, 2 μM benzylic alcohol of Lu AA21004 was also included in this inhibitor assay to include a substrate concentration equal to K_m. Allopurinol did not inhibit the metabolism of the benzylic alcohol of Lu AA21004 to its corresponding acid at any of the substrate concentrations used (Fig. 3). At the substrate concentration equal to K_m, the metabolism was inhibited approximately 40% by disulfiram and raloxifene, whereas 4-methylpyrazole inhibited the reaction by approximately 60%. At substrate concentrations of 8 and

### TABLE 1

Correlation analysis between formation of selective enzyme activities and the formation of metabolites at 8 μM Lu AA21004

Phenotyped human liver microsomes were incubated with 8 μM Lu AA21004 for 60 min. The formation of the individual metabolites was correlated to form selective activities. Data shown are the correlation coefficients. Similar data were obtained after incubation with 50 μM Lu AA21004. Inter correlation (r value): CYP2A6 and CYP2B6: 0.8081**; CYP2A6 and CYP3A: 0.8460**; CYP2B6 and CYP3A: 0.9925**; CYP2C9 and CYP4A11: 0.6798**; CYP2C9 and CYP3A: 0.7054**; CYP2C9 and CYP2C19: 0.6892**; CYP2C19 and CYP3A: 0.7243**; CYP1A2 and CYP2C19: 0.0884.

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Sulfoxide</th>
<th>Benzylic Alcohol of Lu AA21004</th>
<th>Benzoic acid of Lu AA21004</th>
<th>N-Hydroxy-piperazine</th>
<th>4-Hydroxy-phenyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>0.5068</td>
<td>0.3190</td>
<td>0.2183</td>
<td>0.3556</td>
<td>0.360</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>0.8401**</td>
<td>0.1410</td>
<td>0.2004</td>
<td>0.7556**</td>
<td>0.031</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>0.8196**</td>
<td>0.3098</td>
<td>0.0718</td>
<td>0.4764**</td>
<td>0.152</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.4806</td>
<td>0.6110*</td>
<td>0.4080</td>
<td>0.7277**</td>
<td>0.546*</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.3882</td>
<td>0.1681</td>
<td>0.1547</td>
<td>0.7039**</td>
<td>0.096</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>0.1693</td>
<td>0.7639**</td>
<td>0.7445**</td>
<td>0.2024</td>
<td>0.788**</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>0.5091</td>
<td>0.1905</td>
<td>0.0374</td>
<td>0.3448</td>
<td>0.044</td>
</tr>
<tr>
<td>CYP3A*</td>
<td>0.8714**</td>
<td>0.2846</td>
<td>0.1174</td>
<td>0.8662**</td>
<td>0.115</td>
</tr>
<tr>
<td>CYP3A*</td>
<td>0.9048**</td>
<td>0.3401</td>
<td>0.0464</td>
<td>0.8690**</td>
<td>0.171</td>
</tr>
</tbody>
</table>

* p < 0.05.
** p < 0.01.
* Dextromethorphan N-demethylation.
* Testosterone-6β-hydroxylation.

*Fig. 3. Incubation of the benzylic alcohol with chemical inhibitors. Pooled human liver S9 fraction was incubated with four different inhibitors at three substrate concentrations. In addition to the 8 and 50 μM used for the other assays, 2 μM benzylic alcohol of Lu AA21004 was also included in this inhibitor assay to include a substrate concentration equal to K_m. Allopurinol did not inhibit the metabolism of the benzylic alcohol of Lu AA21004 to its corresponding acid at any of the substrate concentrations used (Fig. 3). At the substrate concentration equal to K_m, the metabolism was inhibited approximately 40% by disulfiram and raloxifene, whereas 4-methylpyrazole inhibited the reaction by approximately 60%. At substrate concentrations of 8 and
50 μM, disulfiram inhibited the metabolism more than 80%. Raloxifene inhibited the metabolism by approximately 45 and 35% at substrate concentrations of 8 and 50 μM, respectively, whereas 4-methylpyrazole inhibited approximately 55 and 35% at substrate concentrations of 8 and 50 μM, respectively. This result indicates that at low substrate concentrations, alcohol dehydrogenase, aldehyde dehydrogenase, and aldehyde oxidase are involved to the same extent in the metabolism of the benzylic alcohol of Lu AA21004 to the benzoic acid metabolite, whereas at higher substrate concentrations, aldehyde dehydrogenase is the major enzyme responsible for this metabolism, with some contribution from alcohol dehydrogenase and aldehyde oxidase. Xanthine oxidase is not involved in the metabolism of the benzylic alcohol of Lu AA21004 to its corresponding benzoic acid under the conditions used in this study, because allopurinol did not inhibit this biotransformation.

**Enzyme Kinetics after Incubation of Lu AA21004.** The enzyme kinetics after incubation of Lu AA21004 was investigated in human liver microsomes. The benzylic alcohol and the sulfoxide of Lu AA21004 were detected by on-line radioactivity detection over the entire concentration range of Lu AA21004 (1–500 μM), whereas the sensitivity for the two other metabolites, 4-hydroxy-phenyl and the N-hydroxylated piperazine, was too low. Therefore, the \( K_m \) for the two latter metabolites was estimated using MS/MS. The best fit of data was obtained using the simple Michaelis-Menten equation. Under the incubation conditions used in this study, the \( K_m \) and \( V_{\text{max}} \) for the formation of the benzylic alcohol of Lu AA21004 in pooled human liver microsomes was estimated to 231 μM and 262 pmol of benzylic alcohol of Lu AA21004 · min\(^{-1} \) · mg protein\(^{-1} \) (Fig. 4). Eadie-Hofstee transformation did not reveal any evidence of two-enzyme kinetics. \( V_{\text{max}} \) with respect to the formation of the sulfoxide of Lu AA21004 was not reached under the conditions used in this study with substrate concentrations up to 500 μM. The data set was therefore subjected to nonlinear regression, setting \( V_{\text{max}} \) to greater than or equal to velocity obtained at 500 μM, resulting in \( K_m \approx 208 \) μM (Fig. 4). The Michaelis-Menten constants \( K_m \) for the formation of 4-hydroxy-phenyl metabolite and the N-hydroxylated piperazine of Lu AA21004 were 323 and 300 μM, respectively (Fig. 4).

**Enzyme Kinetics after Incubation with Benzylic Alcohol of Lu AA21004.** The enzyme kinetics of the formation of the benzoic acid of Lu AA21004 from its corresponding alcohol, the benzylic alcohol, was investigated in human liver microsomes and human liver S9 fraction to determine the rate-limiting step in the biotransformation of Lu AA21004 to the benzoic acid metabolite, the major metabolic pathway in humans. Data obtained after incubation of pooled human liver microsomes with increasing concentrations of the benzylic alcohol of Lu AA21004 were fitted to a Michaelis-Menten model with uncompetitive substrate inhibition using nonlinear regression to obtain the best fit. The resulting \( K_m \), \( K_s \), and \( V_{\text{max}} \) estimates were 95 μM, 275 μM, and 59 pmol · min\(^{-1} \) · mg protein\(^{-1} \), respectively, for the formation of the benzoic acid of Lu AA21004 (Fig. 5). Data obtained after incubation of pooled human liver S9 fraction with increasing concentrations of the benzylic alcohol were fitted to the Michaelis-Menten equation using nonlinear regression, resulting in \( K_m \) and \( V_{\text{max}} \) estimates of 1.7 μM and 1567 pmol · min\(^{-1} \) · mg protein\(^{-1} \), respectively, for the formation of the benzoic acid metabolite (Fig. 5). Eadie-Hofstee transformation did not reveal any evidence of more than one enzyme involved in the biotransformation, indicating that the enzymes involved in this biotransformation have similar \( K_m \) values toward the benzylic alcohol of Lu AA21004. An overview of enzyme kinetic parameters is shown in Table 2.

**Discussion**

The enzymes involved in the in vitro metabolism of Lu AA21004 were investigated. Four phase 1 metabolites are of clinical relevance because of the observation of the metabolites or their glucuronide conjugates in human plasma after oral administration of Lu AA21004. The four metabolites are the sulfoxide, the N-hydroxylated piperazine, the 4-hydroxy phenyl-metabolite, and the benzoic acid. In addition, the benzylic alcohol was shown in this study to be a precursor for the benzoic
The benzylic alcohol (precursor for the benzoic acid metabolite) is CYP2D6, whereas CYP2C9 seems to contribute to a minor extent.

The benzoic acid metabolite was also detected after incubation of Lu AA21004 with human liver microsomes as well as recombinant enzymes. Under those incubation conditions, CYP2D6 seems to be the only enzyme catalyzing the formation of this metabolite. To investigate the capability of CYP450 and FMO3 to catalyze the metabolism of the benzylic alcohol to the corresponding benzoic acid, the benzylic alcohol was incubated with recombinant enzymes as well as CYP450 and FMO3 phenotyped human liver microsomes. Recombinant CYP2D6 was the only enzyme catalyzing the formation of the benzoic acid metabolite, and, as expected, recombinant FMO3 did not show any catalytic capability toward this biotransformation. With respect to the phenotyped human liver microsomes, no correlation was obtained probably because of the contribution from NADPH-independent enzymes (and thus non-P450). To investigate whether enzymes other than CYP450 were involved in the metabolism of the benzylic alcohol to its corresponding benzoic acid, the benzylic alcohol was incubated with human liver S9 fraction, and the dependence of the cofactors NADPH and NAD+ was investigated. Some metabolism occurred without any cofactor present, which could indicate the involvement of enzymes such as aldehyde oxidase and xanthine oxidase. The amount of the benzoic acid formed was independent of the presence of NADPH, indicating that the contribution of CYP450 to the metabolism of the benzylic alcohol to corresponding acid is negligible in the S9 fraction. When NAD+ was added to the incubation mixture, the metabolism of the benzylic alcohol to the benzoic acid metabolite was increased by a factor of 7 to 9. Thus, NAD+-dependent enzymes were the major enzymes responsible for the metabolism of the benzylic alcohol to its corresponding acid in vitro. Alcohol dehydrogenase and aldehyde dehydrogenase are NAD+-dependent enzymes.

The metabolism of the benzylic alcohol to the benzoic acid was inhibited after incubation with 4-methylpyrazole and disulfiram, inhibitors of alcohol dehydrogenase and aldehyde dehydrogenase, respectively. Furthermore, the aldehyde oxidase inhibitor, raloxifene, also inhibited the metabolism, whereas the xanthine oxidase inhibitor, 

<table>
<thead>
<tr>
<th>Enzyme Source and Reaction</th>
<th>(K_m)</th>
<th>(V_{\text{max}})</th>
<th>(V_{\text{max}}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HLM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lu AA21004 → benzylic alcohol</td>
<td>231</td>
<td>262</td>
<td>1.13 \times 10^{-6}</td>
</tr>
<tr>
<td>Lu AA21004 → sulfoxide</td>
<td>≥208</td>
<td>≥227</td>
<td></td>
</tr>
<tr>
<td>Lu AA21004 → 4-hydroxy-phenyl</td>
<td>333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lu AA21004 → N-hydroxylated piperazine</td>
<td>300[6]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylic alcohol → benzoic acid</td>
<td>95</td>
<td>59</td>
<td>0.621 \times 10^{-6}</td>
</tr>
<tr>
<td>S9 fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylic alcohol → benzoic acid</td>
<td>1.7</td>
<td>1567</td>
<td>922 \times 10^{-6}</td>
</tr>
</tbody>
</table>

\[ V_{\text{max}} \] was not reached under the conditions used in this study with substrate concentrations up to 500 \(\mu\)M. The data set was therefore subjected to nonlinear regression setting \(V_{\text{max}}\) to greater than or equal to the velocity obtained at 500 \(\mu\)M resulting in \(K_m\) ≥208 \(\mu\)M.

\[ \text{The } K_m \text{ for the } 4\text{-hydroxy-phenyl metabolite and the } \text{N-hydroxylated piperazine was estimated using areas obtained by MS/MS because of inadequate sensitivity using radiodetection. The } V_{\text{max}} \text{ is therefore expressed in terms of area per internal standard per minute per milligram.} \]
allopurinol, showed no inhibitory potency toward the metabolism of the benzylic alcohol to the benzoic acid. On the basis of those results together with the cofactor requirements, alcohol dehydrogenase and aldehyde dehydrogenase are the major enzymes responsible for the metabolism of the benzylic alcohol to its corresponding acid, with some contribution from aldehyde oxidase. Furthermore, CYP2D6 is also capable of catalyzing this reaction; however, the contribution is expected to be less important on the basis of the results mentioned above. This conclusion is also supported by the intrinsic clearance ($V_{\text{max}}/K_m$) which in HLM was $0.621 \times 10^{-6} \text{ l \cdot min}^{-1} \cdot \text{mg}^{-1}$ protein, whereas in human liver S9 fraction $V_{\text{max}}/K_m$ was $922 \times 10^{-6} \text{ l \cdot min}^{-1} \cdot \text{mg}^{-1}$ protein. The subcellular fractions are not directly comparable; however, the enzymes in HLM are more enriched, compared with those in the S9 fraction. This observation adds to the argument that the contribution of CYP2D6 can be considered negligible.

Moreover, the metabolism of the benzylic alcohol to its corresponding acid seems to be relatively fast compared with the metabolism of Lu AA21004 to the benzoic acid using the same arguments as above. Thus, the intrinsic clearance ($V_{\text{max}}/K_m$) in HLM for metabolism of Lu AA21004 to the benzylic alcohol was $1.13 \times 10^{-6} \text{ l \cdot min}^{-1} \cdot \text{mg}^{-1}$, whereas the subsequent metabolism of the benzylic alcohol to the benzoic acid is characterized by an intrinsic clearance ($V_{\text{max}}/K_m$) in the S9 fraction of $922 \times 10^{-6} \text{ l \cdot min}^{-1} \cdot \text{mg}^{-1}$. Thus, the latter is approximately at 800 times higher, and bearing in mind that the human liver S9 fraction is more diluted than the human liver microsomes, we believe that the oxidation of Lu AA21004 to the benzylic alcohol is the rate-limiting step in the formation of the benzoic acid. In conclusion, at least five cytochrome P450 isoenzymes are involved in the metabolism of Lu AA21004 in vitro. Furthermore, the benzylic alcohol of Lu AA21004 is the precursor of the benzoic acid metabolite, a step that was catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase, with some contribution from aldehyde oxidase. In addition, CYP2D6 was also capable of catalyzing the formation of the benzoic acid metabolite; however, the contribution is expected to be of less importance.

Acknowledgments

We thank K. G. Jensen for reviewing this article and for fruitful scientific discussions.

Authorship Contributions

*Participated in research design:* Hvenegaard and Dalgaard.
*Conducted experiments:* Hvenegaard, Bang-Andersen, Pedersen, Jørgensen, and Püschl.

**Contributed new reagents or analytic tools:** Bang-Andersen, Jørgensen, and Püschl.

**Performed data analysis:** Hvenegaard, Pedersen, Jørgensen, and Püschl.

**Wrote or contributed to the writing of the manuscript:** Hvenegaard, Bang-Andersen, Pedersen, Jørgensen, and Püschl.

**References**


**Address correspondence to:** Dr. Mette G. Hvenegaard, Department of Drug Metabolism, H. Lundbeck A/S, Ottiliavej 9, 2500 Valby, Denmark. E-mail: mgh@lundbeck.com