CYP3A Specifically Catalyzes 1β-Hydroxylation of Deoxycholic Acid: Characterization and Enzymatic Synthesis of a Potential Novel Urinary Biomarker for CYP3A Activity

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ABSTRACT

The endogenous bile acid metabolite 1β-hydroxy-deoxycholic acid (1β-OH-DCA) excreted in human urine may be used as a sensitive CYP3A biomarker in drug development reflecting in vivo CYP3A activity. An efficient and stereospecific enzymatic synthesis of 1β-OH-DCA was developed using a Bacillus megaterium (BM3) cytochrome P450 (P450) 3A4 (P450) mutant, and its structure was confirmed by nuclear magnetic resonance (NMR) spectroscopy. A [4H4]-labeled analog of 1β-OH-DCA was also prepared. The major hydroxylated metabolite of deoxycholic acid (DCA) in human liver microsomal incubations was identified as 1β-OH-DCA by comparison with the synthesized reference analyzed by UPLC-HRMS. Its formation was strongly inhibited by CYP3A inhibitor ketoconazole. Screening of 21 recombinant human cytochrome P450 (P450) enzymes showed that, with the exception of extrahepatic CYP46A1, the most abundant liver P450 subfamily CYP3A, including CYP3A4, 3A5, and 3A7, specifically catalyzed 1β-OH-DCA formation. This indicated that 1β-hydroxylation of DCA may be a useful marker reaction for CYP3A activity in vitro. The metabolic pathways of DCA and 1β-OH-DCA in human hepatocytes were predominantly via glycoside and, to a lesser extent, via taurine and sulfate conjugation. The potential utility of 1β-hydroxylation of DCA as a urinary CYP3A biomarker was illustrated by comparing the ratio of 1β-OH-DCA:DCA in a pooled spot urine sample from six healthy control subjects to a sample from one patient treated with carbamazepine, a potent CYP3A inducer; 1β-OH-DCA:DCA was considerably higher in the patient versus controls (ratio 2.8 vs. 0.4). Our results highlight the potential of 1β-OH-DCA as a urinary biomarker in clinical CYP3A DDI studies.

Introduction

Drug-drug interactions (DDIs) are a major concern in drug development and clinical practice. The likelihood and magnitude of inhibition and/or induction of drug metabolizing enzymes are integral parts of the assessment of new drug candidates. When the potential for a DDI is identified in preclinical studies, further evaluation of the risk of DDI is often required in clinical investigations. Such studies are most often carried out using drug probe compounds, which are selectively metabolized by the affected enzyme. The vast majority of clinical DDI studies investigate the effect on cytochrome P450 (P450) 3A. A sensitive probe drug for CYP3A is midazolam, which is commonly used to assess inhibition or induction of this enzyme. Sensitive endogenous biomarkers, especially urinary biomarkers, if applicable, would have great utility, avoiding unnecessary drug exposure and invasive sampling. The 6β-hydroxycortisol to cortisol ratio (6β-hydroxycortisol ratio) in urine has been suggested as an endogenous marker for CYP3A4 activity (Galteau and Shamsa, 2003). The intraindividual and interfindividual variations of this ratio are large, however, and can be used only when the subjects are their own controls. Its specificity for CYP3A4 activity is also debated (Galteau and Shamsa, 2003). The urinary ratios of 6β-hydroxycortisone to cortisone and 7β-hydroxy-dehydroepiandrosterone [7β-hydroxy-dehydropiandrosterone (DHEA)] to DHEA have also been suggested to be useful indicators of CYP3A4 activity, but this observation needs to be validated in vivo (Shin et al., 2013). One newly developed endogenous biomarker for CYP3A3 is the blood level of 4β-hydroxycholesterol (Kanebratt et al., 2008; Bjorkhem-Bergman et al., 2013). The elimination half-life of 4β-hydroxycholesterol is about 17 days, resulting in stable plasma concentrations within subjects (Diczfalusy et al., 2009), but this excludes 4β-hydroxycholesterol as a marker for rapid changes in CYP3A4 activity. A sensitive new biomarker, preferably measurable in urine spot

ABBREVIATIONS: BM, Bacillus megaterium; CA, cholic acid; CDCA, chenodeoxycholic acid; CDCA-24-G, chenodeoxycholic acid-24-acyl-β-D-glucuronide; CE, collision energies; DCA, deoxycholic acid; DCA-3-S, deoxycholic acid 3-sulfate; DDI, drug-drug interaction; DHEA, dehydroepiandrosterone; G-CA, glycolic acid; G-CDCA, glycodeoxycholic acid; G-CDCA-24-G, glycodeoxycholic acid-24-acyl-β-D-glucuronide; G-CDCA-3-S, glycodeoxycholic acid-3-sulfate; G-DCA, glycodeoxycholic acid; G-1β-OH-DCA, 1β-hydroxy-deoxycholic acid glycine conjugate; [4H4]-1β-OH-DCA, (1β,3β,5α,12β)-1,3,12-trihydroxy-(2,2,4,4-4H4)-cholan-24-oic acid; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; MS, mass spectrometry; 1β-OH-DCA, 1β-hydroxy-deoxycholic acid, (1β,3β,5α,12β)-1,3,12-trihydroxycholan-24-oic acid; LC, liquid chromatography; NMR, nuclear magnetic resonance; P450, cytochrome P450; T-CA, taurocholic acid; T-CDCA, taurochenodeoxycholic acid; UPLC, ultra-high-performance liquid chromatography.
samples, with a fast response to change in CYP3A activity, would thus be of great benefit for the assessment of DDIs and phenotyping in humans.

Concentrations of the bile acid metabolite 1β-hydroxy-deoxycholic acid (1β-OH-DCA) increased significantly in the urine of patients taking the known CYP3A4 inducer carbamazepine (Bodin et al., 2005). This study also showed that the conversion of deoxycholic acid (DCA) to 1β-OH-DCA was catalyzed in vitro by recombinant CYP3A4 and by human liver microsomes, although no data were presented on the specificity of this transformation in terms of the contribution of additional enyzmes from the CYP3A family or other cytochromes (Bodin et al., 2005). We reasoned that it may be possible to use 1β-OH-DCA as a urinary biomarker to measure CYP3A induction and inhibition in clinical DDI studies rather than using a conventional invasive plasma biomarker or probe drug.

The metabolite 1β-OH-DCA is not available commercially. A nine-step chemical synthesis (Tohma et al., 1986) and a microbial synthesis (Carlström et al., 1981) of 1β-OH-DCA have been reported previously. Bacterial P450s have shown promise in the synthesis of drug metabolites (Gilliam and Hayes, 2013), and we thought it might be possible to synthesize 1β-OH-DCA enzymatically using a commercially available bacterial mutant P450.

The aim of the present study was to investigate the 1β-hydroxylation of DCA by human liver microsomes and to assess the contribution of individual cytochromes using human recombinant P450 enzymes and chemical inhibitors. To facilitate this work, a library of Bacillus megaterium P450 mutants was screened using DCA as a substrate, and a suitable enzyme was identified that produced the major hydroxylated metabolite formed in human liver microsomes (HLMs). The aim was to generate sufficient quantities of this hydroxylated metabolite for full structural characterization by nuclear magnetic resonance (NMR) spectroscopy. A stable isotope, labeled analog [2H4]-1β-OH-DCA, required for biochemical method development, was also synthesized using similar methods. An ultra-high-performance liquid chromatography/high-resolution mass spectrometry (UPLC/HRMS)–based assay was used for metabolite identification and quantification. The potential application of 1β-hydroxylation of DCA as a urinary CYP3A biomarker was illustrated by comparison of two spot human urine samples, one pooled sample from healthy subjects as a control and one from a patient treated with carbamazepine, a CYP3A inducer.

Materials and Methods

Chemicals and Reagents. DCA, cholic acid (CA), chenodeoxycholic acid (CDCA), glycodeoxycholic acid (G-CDCA), glycodeoxycholic acid (G-CA), taurochenodeoxycholic acid (T-CDCA), taurocholic acid (T-CA), NADPH, β-glucuronidase/arylsulfatase (Helix pomatia), and cholyglycine hydrolyase from Clostridium perfringens (Clostridium welchi) were obtained from Sigma-Aldrich (Dorset, UK). Deoxycholic acid (2,2,4,4,7,7,12,12,13,13,15,15,17,17,19,19,20,20-d20-DCA, 99 atom % D) was purchased from CDN isopipes (Quebec, QC, Canada). Deoxycholic acid 3-sulfate (DCA-3-S) and ketoconazole were obtained from Compound Management, AstraZeneca (Gothenburg, Sweden). Chenoxycholic acid-24-acetyl-β-D-glucuronide (CDCA-24-G) was purchased from Toronto Research Chemicals (Toronto, Canada). Purified HLMs were purchased from BD Gentest (Woburn, MA), and recombinant human cytochromes 1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 3A7, 4A11, 4F2, 4F3, 17A1, and 46A1 were obtained from Cyrex Ltd. (Dundee, Scotland). Pooled human hepatocytes were purchased from Celsis-In Vitro Technologies (Baltimore, MD). Micropost screening plates (MCPY-0343), purified P450 enzyme (MCPY0029), and glucose dehydrogenase were purchased from Codexis (Redwood City, CA). Acetonitrile (LC-MS grade) was obtained from Fisher Scientific (Fair Lawn, NJ). Formic acid (purity 98%–100%) was purchased from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents were of the highest quality available commercially.

Spot Human Urine Samples. A spot human urine sample originated from a reported clinical study in which all patients were receiving monotherapy with carbamazepine for at least 1 year and, apart from epilepsy, were healthy (Bodin et al., 2005) (approved by the local human ethics committee). Control urine was prepared by pooling 2 ml of spot urine samples collected from six healthy subjects.

Screening for BM3 P450 Catalyzed Conversion of Deoxycholic Acid. The screening of DCA against the Codexis Microcyt 96-well screening plate A (MCYP-0343) was performed as follows: DCA (20 mg, 0.05 mmol) was dissolved in acetonitrile (1.0 ml) and added to a solution of MCYP-RXN buffer (46.6 ml, 0.802 g in deionized water (28.1 ml). The MCYP-RXN buffer contained glucose (30 mM), NADP+ (12 mM), glucose dehydrogenase (0.6 nmol) in potassium phosphate buffer (120 mM). The solution was adjusted to pH 8.0 with NaOH (0.1 M). Aliquots (0.25 ml) were pipetted into each enzyme well, and the solution was shaken at 30°C at 250 rpm in an orbital shaker. An aliquot of reaction mixture (10 μl) was removed from each well of the 96-well screening plate and diluted with 90 μl of mobile phase A (0.1% formic acid in water). The mixture was centrifuged using a benchtop centrifuge at 1200g and then transferred to either a 96-well Nunc plate or to glass injection vials (Waters Corp, Milford, MA) before UPLC/HRMS analysis. LC/MS data were processed using Metabolynx (Waters) software. Hit enzymes were identified on the basis of forming a +16-Da (mono-oxidized) metabolite of DCA with a retention time of 3.22 minutes.

Enzymatic Synthesis of 1β-OH-DCA and [4H4]-1β-OH-DCA Using MCYP0029. Preparation of 1β-OH-DCA (1β,3α,5β,12α)-1,3,12-trihydroxychol-24-oxic acid. To a 250-ml conical flask cooled in an ice/water bath was added phosphate buffer (0.12 M, 95 ml, pH 8), n-glucose (0.45 g, 2.5 mmol), NADP+ (74.3 mg, 0.1 mmol), and glucose dehydrogenase (50 mg). Deoxycholic acid (39.2 mg, 0.1 mmol) was dissolved in MeOH (100 μl) and added to the reaction flask. MicroCYP0029 (151 mg, 100 nmol) was dissolved in phosphate buffer (0.12 M, 5 ml, pH 8), and the solution was added to the reaction flask. The flask was shaken in an orbital shaker at 200 rpm, 30°C, and the reaction was monitored by UPLC/HRMS. After 16 hours, the reaction was quenched with cold acetonitrile (100 ml), and the solution was allowed to stand in a refrigerator at 4°C for 30 minutes, after which the contents were transferred to Falcon tubes (4 x 50 ml) and centrifuged (4000g, 4°C) for 15 minutes. The supernatants were decanted off, pooled, and concentrated on a rotary evaporator. After removal of the organic, the aqueous was split between two Oasis HLB SPE cartridges (2 x 6 ml, 200 mg). Each cartridge was washed with H2O (1 x 5 ml) and eluted with acetonitrile (1 x 5 ml). The organic eluates were pooled and concentrated to dryness under an N2 stream, and the crude residue was purified by mass-directed preparative high-performance liquid chromatography (HPLC).

Preparation of [4H4]-1β-OH-deoxycholic acid (1β,3α,5β,12α)-1,3,12-trihydroxy(2,2,4,4,7,7,12,12,13,13,15,15,17,17,19,19,20,20-d20)-chol-24-oxic acid. The [4H4]-1β-OH-deoxycholic acid was prepared using the same method as that used for the unlabeled analog. Deoxycholic acid-2,2,4,4,7,7,12,12,13,13,15,15,17,17,19,19,20,20-d20 (39.3 mg, 0.1 mmol) was used as substrate.

Mass-Directed Preparative HPLC Purification of 1β-OH-DCA and [4H4]-1β-OH-DCA. The residue from the concentrated SPE plate was dissolved in DMSO (1 ml) and purified by mass-directed preparative HPLC using a Waters Sunfire C18 3 OD(50 mm, 5 μm). Mobile phases were 0.1 M NH4OAc (solvent A) and acetonitrile (solvent B). Gradient was 10% B increasing to 50% over 20 minutes. Flow rate was 60 ml/min. A Waters ZQ mass spectrometer was used for mass detection. 1β-OH-DCA eluted between 11.0 and 11.8 minutes. Fractions containing the targeted mass of m/z 407, corresponding to mono-oxidized DCA, were pooled, the organs were removed on a rotary evaporator, and the aqueous residue was subjected to SPE on a Waters Oasis HLB cartridge (500 mg). The cartridge was washed with water (3 ml) and allowed to dry under vacuum and then eluted with MeOH (3 ml). The MeOH eluate was concentrated to dryness, yielding a white solid (10.5 mg) with mass of m/z 407.2799 (Fig. 1). The [4H4]-1β-OH-DCA was purified using an identical method and yielded (13.4 mg) as a white solid with a mass of m/z 411.3060 (Fig. 1).

NMR Characterization of 1β-OH-DCA and [4H4]-1β-OH-DCA. NMR spectra were recorded on the Bruker 600 MHz AVANCE III system equipped with a 5-mm QCi Cryoprobe using standard Bruker pulse sequences. Experiments were run in deuterated methanol (CD3OD) at 25°C. Chemical shifts are referenced relative to the residual methyl signal in CD3OD set to 3.31 ppm (H) or 47.8 ppm (13C). NMR assignments of 1β-OH-DCA and [4H4]-1β-OH-DCA were based on 1H and 13C chemical shifts and two-dimensional correlations via 1H–1H COSY, ROESY and 1H–13C HSQC, 1H–13C HMBC experiments.
enzymatic hydrolysis of glucuronides and amino acid conjugates, an aliquot of 150 μl of sodium phosphate buffer (pH 5) was added, followed by the addition of acetonitrile after 60 minutes. The reaction mixture was then centrifuged at 4000 g for 20 minutes. An aliquot of 50 μl of the supernatant was separated and evaporated under nitrogen to a reduced volume of less than 50 μl. The remaining liquid was acidified by adding formic acid (0.1% in H2O, 50 μl). The resulting mixture was loaded onto an Oasis MAX 96-well μElution Plate (2 mg of sorbent per well, 30 μm particle size, Waters) preconditioned with methanol and water. Loaded cartridges were then successively washed with 0.2 ml of water and eluted with 150 μl of isopropanol-acetonitrile (64:36, v/v). The eluate containing bile acids was then solvolysed. To the eluate, an aliquot of 100 μl of acetone and 40 μl of hydrochloric acid (1 M) was added and incubated for 1 hour at 37°C. After 1 hour, the solvent was evaporated and the residue reconstituted in 0.5 ml NaOH (4 M) and 1.2 ml isopropanol and incubated overnight at 70°C. The incubated mixtures were then neutralized with the addition of formic acid (200 μl, 98%) at the end of the solvolysis and the solvent evaporated to a volume of less than 300 μl. The remaining aqueous phase was diluted with 200 μl of 0.1% formic acid, and a second extraction was performed on an Oasis MAX 96-well μElution Plate using the same method described previously. The eluate was then evaporated under nitrogen, and the residue was dissolved in 100 μl of 50% acetonitrile solution before UPLC/HRMS analysis. Calibration standards were constructed by spiking serial concentrations (16–5000 nM) of DCA and 1β-OH-DCA into 0.1 M sodium phosphate buffer (pH 8). The standard solutions were then subjected to sample preparation in parallel with urine samples, including enzyme hydrolysis and solvolysis. Reference solutions in sodium phosphate buffer (pH 5) containing 2 μM of G-CDCA, T-CDCA, G-CA, T-CA, CDCA-24-G, or DCA-3-S were subjected to enzymatic hydrolysis using a mixture of β-glucuronidase/arylsulfatase and choloylglycine hydrolase to monitor the hydrolysis of bile acid amide, glucuronide, and/or sulfate conjugates.

**UPLC/HRMS Analysis.** To separate and identify the hydroxylated metabolites of DCA, a Synapt G2 Q-TOF mass spectrometer equipped with an ACQUITY UPLC system (Waters Corp.) was used. The UPLC/HRMS system was operated by MassLynx software (version 4.1, Waters Corp.). UPLC separations were performed on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters, Milford, MA). Mobile phase A was 0.1% formic acid aqueous solution, and mobile phase B was acetonitrile. The initial mobile phase was 90:10 A-B and was transitioned via a linear gradient to 10:90 B-A over 6 minutes. The flow rate was 0.5 ml/min, and the total run time was 7 minutes. The column oven and autosampler were set at 45°C and 8°C, respectively. The UPLC eluent was introduced into the Q-TOF mass spectrometer with an electrospray ionization source in negative mode. Specific mass spectrometric source conditions were as follows: capillary voltage of 0.5 kV, sample cone voltage of 40 V, desolvation temperature, and source temperature were 550 and 150°C, respectively. HRMS and MS3 scan functions were programmed with two independent collision energies (CE). The HRMS spectra were obtained with a mass accuracy of 50 ppm, where the transfer CE and trap CE were 0 eV and 4 eV, respectively. MS3 spectra were acquired with a high collision energy, where the transfer CE and trap CE were 30 eV and were ramped from 15 to 35 eV, respectively. Data were acquired over the range m/z 100–1200. In the extraction, extracted ion chromatograms (XIC) at m/z 391.285 (DCA), m/z 407.280 (1β-OH-DCA), and m/z 411.305 ([2H4]-1β-OH-DCA) were generated with a mass tolerance of 10 mDa. Reference standards were analyzed in parallel for definitive confirmation. Quantification of the 1β-OH-DCA and DCA was performed using TargetLynx. The concentrations of bile acids were calculated based on the peak area ratio of each compound to the internal standard and compared with the calibration curves. Mass responses for standards of DCA, 1β-OH-DCA, CA, G-CA, T-CA, CDCA, G-CDCA, and T-CDCA were similar by the UPLC/HRMS method used, which allowed a semiquantitative assessment of the relative abundance of DCA and 1β-OH-DCA and their conjugated metabolites in hepatocytes to be made by comparison of peak areas. The metabolite identification of DCA and 1β-OH-DCA in different samples was performed using Metabolynx (Waters) software.

**Results**

**Enzyme Synthesis and Structural Characterization of 1β-OH-DCA.** Deoxycorticolic acid was screened against a library of commercially available BM3 P450 mutants looking for formation of its major oxidized
metabolite (M3), which had been identified in HLM and rCYP3A4 incubations. Chromatographic retention time and mass spectral data for M3 were known, allowing a focused screen to be performed. Only BM3 mutants forming M3 in good conversion and ideally as a single metabolic product were of interest. Of the 90 mutants screened, only MCYP0027 and MCYP0029 gave 35% conversion to a +16-Da metabolite, which cochromatographed (retention time, 3.22 minutes) with the desired major HLM metabolite based on semiquantitative estimates of parent compound consumed and M3 formed (Fig. 2). The enzyme MCYP0029 was selected for scale-up because of its better conversion (about 50%–60%) and a simpler metabolite profile. Interestingly, a wide range of mono-oxidized metabolites of DCA was also produced by the enzyme library with retention times of 3.06, 3.59, 3.68, 3.73, and 3.98 minutes. The identity of these metabolites has not been investigated further.

A weighable quantity of M3 (which we reasoned was 1β-OH-DCA) was required for structural confirmation by NMR. Larger quantities of both 1β-OH-DCA and [2H4]-1β-OH-DCA were also required for use as bioanalytical standards in forthcoming clinical DDI studies. Therefore, scale-up experiments, using MCYP0029 and either DCA or deoxycholic-2,2,4,4-2H4 acid as substrate, were performed at 0.1-mmol scale and monitored by UPLC/HRMS. After workup and purification by mass directed preparative LC, the colorless solids were characterized by NMR spectroscopy.

NMR spectroscopy confirmed that the identity of M3 was indeed 1β-OH-DCA. The NMR assignments of 1β-OH-DCA and [2H4]-1β-OH-DCA were compared with the fully assigned1Ha n d13C spectra of DCA, as shown in Table 1. Numbering follows the IUPAC-approved atom numbering of steroids. Some marker groups of DCA are the OH substituted positions CH(12); 3.96, 72.8 (1H,13C chemical shift in ppm) and CH(3); 3.53, 71.3, methyl groups CH3(18); 0.71, 12.0, CH3(19); 0.93, 22.5, and CH3(21); 1.01, 16.4. CH2(1) is found at 0.98(dt, J = 14.2, 14.2, 3.4)/1.78, 35.2 ppm. The equatorial (β) 1H signal from CH(12) appeared as a triplet with two small J-couplings (1.8, 1.8 Hz) to the axial and equatorial CH2(11) protons. The axial (α) 1H signals from CH(3) appeared as a triplet of triplets with two large trans-couplings and two smaller cis-couplings (11.1, 11.1, 4.6, 4.6 Hz). 1H NMR spectra of

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Extracted MS ion chromatograms of DCA and its hydroxylated metabolites formed after incubation of DCA in HLMs, recombinant CYP3A4, and Codexis MCYP00029. Mass tolerance was 10 mDa, and MS was operated under negative ionization mode. The start concentrations of DCA in incubations were 2 μM in HLM and CYP3A4, and 10 μM in Codexis MCYP00029. XIC – extracted ion chromatogram.

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<td>1.01 (d, J = 6.6 Hz)</td>
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<tr>
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<td>30.9</td>
<td>1.33, 1.79</td>
<td>30.8</td>
<td>1.33, 1.79</td>
<td>31.1</td>
<td>1.33, 1.78</td>
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<tr>
<td>23</td>
<td>30.7</td>
<td>2.20, 2.34</td>
<td>30.8</td>
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<td>30.8</td>
<td>2.21, 2.34</td>
</tr>
<tr>
<td>24</td>
<td>176.9</td>
<td>n.a.</td>
<td>177.0</td>
<td>n.a.</td>
<td>177.0</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

br, broad; d, doublet; dt, doublet of triplet; m, multiplet; n.a., not applicable; n.d., not determined; s, singlet; t, triplet; t, triplet of triplet.

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**TABLE 1**

1H and 13C nuclear magnetic resonance data for 1β-OH-DCA, [2H4]-1β-OH-DCA, and DCA

Chemical shifts are reported relative to the residual methanol signal set to 3.31 ppm (1H) and 48.7 ppm (13C). Chemical shifts for overlapping 1H signals are taken from relevant two-dimensional experiments. 1H-Coupling Constants for selected signals are obtained from one-dimensional 1H- spectra (multiplicity, J Hz).
1β-OH-DCA and [3H₂]-1β-OH-DCA are shown in Fig. 3. ¹³C NMR spectra of 1β-OH-DCA and [3H₂]-1β-OH-DCA are shown in Supplemental Fig. S1.

In 1β-OH-DCA, the signals assigned to CH₂(1) in DCA are missing, and a new CH signal appeared at δ 3.82, 72.9 ppm, indicating hydroxylation at this position. The assignment of this signal to CH(1) was supported by COSY cross-peaks to CH₃(2), CH₃(19) (Supplemental Fig. S2), ROESY cross-peaks indicated its close proximity to CH₃(2), CH₃(19), and CH₂(11eq (α)) at 1.43 ppm (Fig. 4), as well as C-H long-range correlations to C(10), CH₂(2), CH₃(19). The ¹H coupling constants (t, J = 2.6, 2.6 Hz) to CH₂(2) were consistent with an equatorial configuration of the proton [i.e., OH in the axial (β)-position].

It should be noted that the axial(β) ¹H signal from CH(3), which appeared in DCA as a well resolved triplet of triplets, were not as well resolved in 1β-OH-DCA, although, again, two large and two small couplings were observed. This difference was explained by the fact that, in 1β-OH-DCA, the resonances from the axial(α) CH(4) and CH(5) appear at approximately the same shift, resulting in second-order J-coupling effects on CH(3).

The NMR data of [2H₄]-1β-OH-DCA, deuterated at positions C(2) and C(4), confirmed the findings of 1β-OH-DCA discussed already. As expected, ¹H signals from CH(1): 3.81, 72.8, and CH(3): 3.96, 65.6 now lacked J-couplings and appeared as singlets (Table 1). Notably, the ¹³C signals of C(2) and C(4) broadened and disappeared in the spectrum as a result of quadrupolar coupling to ²H and lack of Overhauser effect to the directly bonded ¹H. Also notable were the ²H-induced isotope shifts, δᵰ, observed in the ¹³C spectrum of [2H₄]-1β-OH-DCA compared with 1β-OH-DCA, which decreased with the number of bonds from the deuterated carbons: δᵰ, C(3):0.22, C(5):0.18, C(6):0.04, C(1):0.09, C(10):0.06, C(9):0.01 ppm (Supplemental Fig. S3).

1β-OH-DCA in Human Liver Microsomes and the Inhibitory Effect of CYP3A Inhibitor Ketoconazole. Incubations of DCA with HLM in the presence of NADPH resulted in four hydroxylated metabolites (M₁–M₄, m/z 407.280) with M₃ as the most abundant one (Fig. 2). A further oxidized metabolite (−2 Da) was also found in the incubation. The profiles of the hydroxylated metabolites of DCA, when incubated at 20 μM (data not shown), were similar to those incubated with 2 μM of DCA (Fig. 2). Metabolites M₃ and M₄ were identified as 1β-OH-DCA and CA, respectively, by comparison with the synthesized and commercially available reference standards. Compared with the control samples, the formation of 1β-OH-DCA formation was significantly inhibited by ketoconazole by 37%, 93%, and 100% at ketoconazole concentrations of 0.1, 1, and 10 μM, respectively, when incubated at 2 μM of DCA (Fig. 5), and by 22%, 89%, and 98%, respectively, when incubated at 20 μM of DCA.

P450 Enzymes Involved in the 1β-Hydroxylation of DCA. To identify the specific P450s that catalyzed DCA 1β-hydroxylation, a panel of 21 recombinant human cytochrome isozymes was screened and compared with the profiles obtained in human liver microsomes. The same conclusion was drawn from incubations at both 2 and 20 μM of DCA.
DCA. Of the 21 recombinant human cytochromes tested, CYP3A4 and 3A7 showed the highest catalytic activity of 1β-OH-DCA formation and CYP3A5 and CYP46A1 showed much less activity toward this reaction. Only trace amounts of 1β-OH-DCA were detected in CYP2C8 incubations and none was detected in the incubation of the other CYPs investigated (Fig. 6).

**Metabolite Profiles of DCA and 1β-OH-DCA in Human Hepatocytes.** Both DCA and 1β-OH-DCA were rapidly metabolized in human hepatocytes. No parent DCA was detected after a 40-minute incubation, and the glycine conjugate (G-DCA) was the predominant metabolite formed. Additional minor metabolic pathways were also detected, including direct taurine conjugation and the combination of G-DCA with oxidation and sulfation (Fig. 7). Similar metabolic pathways were also detected in incubations of 1β-OH-DCA. After 40-minute incubation, the remaining parent 1β-OH-DCA accounted for 44% of the 0-minute sample, and the direct glycine conjugate (G-1β-OH-DCA) was

**Fig. 4.** Two-dimensional ROESY spectrum of 1β-OH-DCA showing correlations from CH(1) to CH2(2’, 2’’), CH2(11’(α), 11’’(β)) and CH3(19).

**Fig. 5.** Effect of ketoconazole on the formation of 1β-OH-DCA after incubation of DCA (2 μM) in HLMs for 60 minutes.

**Fig. 6.** Catalytic activities of DCA 1β-hydroxylation in a panel of 21 recombinant human cytochromes and in HLMs after 60-minute incubation. Results are the average of duplicates.
the only metabolite detected. With a longer incubation time of up to 2 hours, low levels of the direct taurine conjugate and the metabolites derived from the combination of desaturation and sulfation of G-1β-OH-DCA were also detected. It is noteworthy that the MS peak corresponding to the G-1β-OH-DCA in 1β-OH-DCA incubations was also detected in hepatocyte incubations of DCA, where the MS peak area of the G-1β-OH-DCA was approximately 11% of the peak area of G-DCA after 2-hour incubation of DCA. Furthermore, the formation of the G-1β-OH-DCA was totally abolished in the coincubation of DCA with ketoconazole in human hepatocytes; however, no significant differences were observed on the formation of amidated conjugates (glycine and taurine conjugates) with or without coincubation with ketoconazole.

**Determination of 1β-OH-DCA and DCA in Human Urine Samples.** Urine samples from healthy subjects and from a patient on monotherapy with carbamazepine were prepared by enzymatic deconjugation, followed by chemical solvolysis. Table 2 shows the concentrations of 1β-OH-DCA, DCA, and their ratios expressed as 1β-OH-DCA/DCA in urine samples after enzyme hydrolysis with and without solvolysis. After enzyme hydrolysis and chemical solvolysis, the ratio of 1β-OH-DCA to DCA in the urine (ratio of 2.8) from a carbamazepine-treated patient was 7-fold higher than that in the control urine (ratio of 0.4). Urine samples after only enzymatic hydrolysis also showed that the ratio of 1β-OH-DCA/DCA was 7-fold higher in patient urine (ratio of 1.3) than that in the control sample (ratio of 0.2) (Fig. 8). The efficiency of the enzyme catalyzed deconjugation was estimated by monitoring the disappearance of the reference conjugates and the formation of their corresponding free bile acids after incubation with the enzymes. The β-glucuronidase/arylsulfatase catalyzed hydrolysis of glucuronides and choloylglycine hydrolyase catalyzed hydrolysis of the amidated conjugates (glycine and taurine conjugates) of bile acids was determined to be complete by UPLC/HRMS analysis; however, desulfation of the reference compound DCA-3-S was poor, with only low levels of deconjugated DCA formed, indicating the poor catalytic activity of the arylsulfatase to the desulfation reaction of these sulfated bile acids under the experimental conditions.

**TABLE 2**

<table>
<thead>
<tr>
<th>Urine</th>
<th>Enzymatic Hydrolysis</th>
<th>Enzymatic Hydrolysis followed by Solvolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>nM</td>
</tr>
<tr>
<td>Control</td>
<td>1β-OH-DCA</td>
<td>406</td>
</tr>
<tr>
<td></td>
<td>DCA</td>
<td>2048</td>
</tr>
<tr>
<td></td>
<td>Ratio (1β-OH-DCA/DCA)</td>
<td>0.2</td>
</tr>
<tr>
<td>Patient</td>
<td>1β-OH-DCA</td>
<td>1653</td>
</tr>
<tr>
<td></td>
<td>DCA</td>
<td>1249</td>
</tr>
<tr>
<td></td>
<td>Ratio (1β-OH-DCA/DCA)</td>
<td>1.3</td>
</tr>
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</table>
Discussion

A prerequisite for using an endogenous metabolite as a sensitive biomarker for probing a particular enzyme activity in humans is that the metabolite is formed selectively by the enzyme of interest. The current study clearly shows that the formation of 1β-OH-DCA from DCA is predominantly catalyzed by CYP3A enzymes. The only other cytochrome enzyme investigated that produced a significant amount of this metabolite was CYP46A1, which is a cholesterol metabolizing enzyme located in the brain (Dutheil et al., 2009). Since the level of bile acids in the brain is very low (Bron et al., 1977), the contribution of CYP46A1 to the overall production of 1β-OH-DCA in humans should be minor, thereby making the formation of 1β-OH-DCA from DCA a specific biomarker for CYP3A activity.

The finding that CYP3A7 has a high capacity to produce 1β-OH-DCA is intriguing. CYP3A7 is mainly a fetal form of CYP3A that is downregulated after birth (Kitada et al., 1985). The high capacity of the fetal liver to metabolize DCA and specifically form its 1β-hydroxylated metabolite was described by Gustafsson et al. (1985). CYP3A7 is very active in the fetal liver, peaks during the first weeks after birth, and then progressively declines. At the same time, CYP3A4 levels begin to rise, reaching 30%–40% of adult levels after 1 month (Lacroix et al., 1997). There are known substrate specificity differences between CYP3A7 and CYP3A4 (e.g., the catalytic efficiency of CYP3A7 to metabolize midazolam is much lower than for CYP3A4 and CYP3A5) (Williams et al., 2002). The specificity of CYP3A to form 1β-OH-DCA from DCA could also be developed into a useful in vitro tool for phenotyping in human liver microsomes and human hepatocytes, keeping in mind the extensive glycine conjugation of both DCA and 1β-OH-DCA observed in human hepatocytes.

Human P450s metabolize most current small-molecule therapeutics (Rendic and Guengerich, 2015). Their use for preparative biotransformation is limited primarily because of poor stability. Bacterial P450s continue to show promise for the synthesis of drug metabolites (Gilham and Hayes, 2013) with mutant enzymes from B. megaterium being particularly thoroughly investigated (Sawayama et al., 2009; Reinen et al., 2015). Both protein and process engineering approaches continue to enhance the possibilities of using these diverse catalysts at scale (Kiss et al., 2015; Venkataraman et al., 2015). The synthesis of rare and unusual endogenous metabolites with engineered P450s is less well explored. In this study, we have successfully synthesized at milligram scale, both unlabeled 1β-OH-DCA and [2H4]-1β-OH-DCA using a BM3 mutant P450, in one step with high stereoselectivity and regioselectivity.

Bile acids constitute a large family of steroids carrying a carboxyl group in the side chain. DCA is a secondary bile acid and is excreted mainly in urine as conjugates, the sulfated and amidated metabolites, and their combinations being the major ones (Alme et al., 1977; Bathena et al., 2015a,b). In this study, we have established that the 1β-hydroxylation of DCA is specifically catalyzed by CYP3A and therefore has potential to be used in the clinic as a biomarker reflecting CYP3A activity in DDI investigations. To be able to evaluate the applicability of this metabolic reaction as a urinary CYP3A biomarker, establishing a robust and sensitive analytical method to determine total 1β-OH-DCA and DCA concentrations was critical. One approach to achieve this goal was to cleave the conjugates of these two bile acids, enzymatically and chemically, releasing the free bile acids for quantification (Bodin et al., 2005; Kakiyama et al., 2014). Using this approach, the quantification of the nonconjugated 1β-OH-DCA and DCA by LC/MS is rather straightforward with access to the reference standards. The disadvantage of this method is that the sample preparation steps are time consuming and the chemical cleavage reaction may be incomplete or give side reactions. Another approach is to directly measure concentrations of free bile acids and their corresponding amidated and sulfated conjugates to obtain the total concentrations of bile acids; however, this approach suffers from the
lack of access to some of the reference standards for accurate quantification. In this study, we modified a reported sample preparation method (Bodin et al., 2005). In addition to the reported enzyme hydrolysis by β-glucuronidase, we have used chlorylglycine hydrolyse to deconjugate the predominant amidated conjugates of 1β-OH-DCA and DCA in urine, followed by solvolysis to complete desulfation. The urinary concentrations of DCA after enzymatic hydrolysis and solvolysis were in the range of that reported for total DCA concentrations in urine (Bathena et al., 2015a). The concentration ratio of 1β-OH-DCA/DCA was used as a marker for CYP3A activity instead the individual concentration. Using this method, the impact of variable DCA levels, as a substrate for 1β-hydroxylation, can be minimized. Our results showed the ratio of 1β-OH-DCA/DCA was 7-fold higher in the urine from a patient with elevated CYP3A activity than that in the control sample. It is interesting to note that in performing only the mild enzymatic hydrolysis without solvolysis, the ratio of 1β-OH-DCA/DCA in the patient urine was again 7-fold higher than that in the control, although the concentrations of DCA and 1β-OH-DCA obtained were lower than those in samples treated by an additional chemical solvolysis step. This observation indicated that the improved enzymatic deconjugation method may be robust enough to provide data that differentiate between ratios of 1β-OH-DCA/DCA observed with differing levels of CYP3A activity.

In this study, the relative contribution of CYP3A4 versus CYP3A5 to DCA 1β-hydroxylation has not been studied in detail and needs further investigation; however, screening data from 21 rCYPs showed that 1β-OH-DCA formation by rCYP3A4 was approximately 50 and 10 times higher than rCYP3A5 at DCA concentrations of 2 and 20 μM, respectively, and highlights the major contribution of CYP3A4 compared with CYP3A5. The role of active transport of 1β-OH-DCA and its conjugates into urine has not been fully explored. Future studies validating 1β-OH-DCA as a CYP3A biomarker need to consider possible effects of inhibitors and inducers on transporter functions as possible confounding factors.

The value of using 1β-OH-DCA as a urine biomarker for CYP3A activity needs to be further studied in a large set of clinical samples to understand its variability over time and intersubject differences. It is clear that if the analysis of spot urine samples reveals human CYP3A activity, this would provide an obvious advantage in clinical DDI studies. Urine sampling over an extended period could be avoided, as is the case when using the biomarker 6β-hydroxycortisol. The 6β-hydroxycortisol/cortisol ratios exhibit large diurnal variability in urinary levels, thereby necessitating urine collection over a long period to obtain samples representing CYP3A activity (Shin et al., 2013). The dynamic range of a biomarker should be large enough to clearly display effects on CYP3A activity both by enzyme inhibitors and inducers. Ideally, the change in 1β-OH-DCA in spot urine should reflect the changes in CYP3A activity in subjects treated with inhibitors or inducers. A carefully designed clinical study is needed to optimize treatment and sampling schedules to evaluate this biomarker. Generally, potent inhibitors and inducers of cytochrome CYP3A are excluded from further development based on preclinical evaluation; however, some compounds with the potential for clinically relevant drug interactions do advance into human clinical studies, and some are eventually approved for use in patients. A useful biomarker should be able to reliably detect and estimate the level of changes in CYP3A activity by weak to moderate inhibitors and inducers. A disadvantage of using 4β-hydroxycholesterol as a biomarker is the small dynamic range, which is affected by CYP3A inhibition. After 14 days of treatment by the strong CYP3A inhibitor ketoconazole, plasma 4β-hydroxycholesterol levels decreased by a maximum of 13%; oral midazolam levels increased 11-fold (Kasichayanula et al., 2014), highlighting the low dynamic range of this biomarker.

The plasma biomarker 4β-hydroxycholesterol was previously found to be sensitive to the CYP3A5 genotype (Diczfalusy et al., 2008). Based on the results from expressed cytochrome enzymes, the contribution of CYP3A5 to the formation of 1β-OH-DCA is probably minor but should be investigated in subjects representing the CYP3A5 slow and extensive metabolizing genotypes.

In conclusion we have shown that 1β-hydroxydeoxycholic acid is specifically formed by CYP3A4/5/7 and may have potential as an in vivo CYP3A biomarker, replacing drug probes that are currently used. A sensitive UPLC/HRMS method was established, and an efficient BM3 P450 mutant catalyzed synthesis generated sufficient amounts of the metabolite required for ongoing clinical studies.

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Authorship Contributions

Participated in research design: Hayes, Li, Diczfalusy, Andersson.

Conducted experiment: Hayes, Grönberg, Li.

Performed data analysis: Hayes, Grönberg, Li.

Wrote or contributed to the writing of the manuscript: Hayes, Li, Grönberg, Andersson.

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