Metabolism of Oral Turinabol by Human Steroid Hormone–Synthesizing Cytochrome P450 Enzymes

Lina Schiffer, Simone Brixius-Anderko, Frank Hannemann, Josef Zapp, Jens Neunzig, Mario Thevis, and Rita Bernhardt

ABSTRACT

The human mitochondrial cytochrome P450 enzymes CYP11A1, CYP11B1, and CYP11B2 are involved in the biosynthesis of steroid hormones. CYP11A1 catalyzes the side-chain cleavage of cholesterol, and CYP11B1 and CYP11B2 catalyze the final steps in the biosynthesis of gluco- and mineralocorticoids, respectively. This study reveals their additional capability to metabolize the xenobiotic steroid oral turinabol (OT; 4-chlor-17β-hydroxy-17α-methylpregnan-3,20-dione-3,17[2F–2S]–), which is a common doping agent. By contrast, microsomal steroid hydroxylases did not convert OT. Spectroscopic binding assays revealed dissociation constants of 17.7 mM and 5.4 μM for CYP11B1 and CYP11B2, respectively, whereas no observable binding spectra emerged for CYP11A1. Catalytic efficiencies of OT conversion were determined to be 46 min⁻¹ mM⁻¹ for CYP11A1, 741 min⁻¹ mM⁻¹ for CYP11B1, and 3338 min⁻¹ mM⁻¹ for CYP11B2, which is in the same order of magnitude as for the natural substrates but shows a preference of CYP11B2 for OT conversion. Products of OT metabolism by the CYP11B subfamily members were produced at a milligram scale with a recombinant Escherichia coli–based whole-cell system. They were identified by nuclear magnetic resonance spectroscopy to be 11β-OH-OT for both CYP11B isomers, whereby CYP11B2 additionally formed 11β,18-diohex-OT and 11β-OH-OT-18-al, which rearranges to its tautomeric form 11β,18-exo-xylohex-18-OT. CYP11A1 produces six metabolites, which are proposed to include 2-OH-OT, 16-OH-OT, and 2,16-diohex-OT based on liquid chromatography–tandem mass spectrometry analyses. All three enzymes are shown to be inhibited by OT in their natural function. The extent of inhibition thereby depends on the affinity of the enzyme for OT and the strongest effect was demonstrated for CYP11B2. These findings suggest that steroidogenic cytochrome P450 enzymes can contribute to drug metabolism and should be considered in drug design and toxicity studies.

Introduction

In humans, most steps of steroid biosynthesis are catalyzed by monooxygenases from the cytochrome P450 (P450) superfamily, which synthesize glucocorticoids, mineralocorticoids, and sex hormones. Steroidogenesis is initiated by CYP11A1, which cleaves the side chain of cholesterol, thereby producing pregnenolone, the common precursor for all steroid hormones. In addition, CYP11A1 can convert a variety of other steroid derivatives, as well as vitamins D₃ and D₅ (Slominski et al., 2015) and a set of endogenous steroid intermediates (Mosa et al., 2015). CYP21A2 generates the substrates for gluco- and mineralocorticoid biosynthesis by its 21-hydroxylase activity. CYP11B1 and CYP11B2 subsequently synthesize gluco- and mineralocorticoids. CYP11B1 catalyzes the 11β-hydroxylation of 11-deoxycortisol yielding the glucocorticoid cortisol. CYP11B2 catalyzes hydroxylations of 11-deoxycorticosterone (DOC) in positions 11β and 18, followed by an 18-oxidation to give aldosterone, the major mineralocorticoid. CYP17A1 represents the branch point to the biosynthesis of sex hormones by its 17α-hydroxylase and 17,20-lyase activities. CYP19A1 can, finally, aromatize androgens to estrogens (Bernhardt and Waterman, 2007).

P450 catalysis requires the presence of a suitable electron transport system, which delivers the electrons necessary for the activation of molecular oxygen from the external electron donor NADPH. The mitochondrial P450 enzymes CYP11A1 and the two CYP11B isoforms depend on a class I redox system, which consists of the FAD containing NADPH-dependent ferredoxin reductase, adrenodoxin reductase (AdR), and an [2Fe–2S] ferredoxin, adrenodoxin (Adx). The microsomal P450 enzymes CYP17A1, CYP19A1, and CYP21A2 are supported by a single electron transfer partner (class II redox system), the NADPH-dependent cytochrome P450 oxidoreductase (CPR), which carries FMN and FAD centers (Hannemann et al., 2007).

Several studies have recently hinted at an involvement of steroidogenic P450 enzymes in the biotransformation of xenobiotic compounds, which is, according to traditional classifications of human P450 enzymes, believed to be solely conducted by microsomal isozymes from the liver (Guengerich, 2001). CYP21A2 and both CYP11B isoforms were shown to be involved in the metabolism of the synthetic anabolic androgenic steroid (AAS) metandienone (Zollner et al., 2010; Parr et al., 2012) and there are even indications for the contribution of CYP11B1 to the
bioactivation of the nonsteroidal environmental pollutant 3-methylsulfanyl-2,2-bis(4-chlorophenyl)-1,1-dichlorehene (Lund and Lund, 1995).

In this study, we aimed to characterize the putative metabolism of the AAS oral turinabol (OT; 4-chlor-17β-hydroxy-17α-methylandrost-1,4-dien-3-ox) by steroid hormone–synthesizing P450 enzymes to further expand our understanding of their substrate specificity and possible participation in biotransformation. Anabolic agents, particularly AASs, are widely misused for doping purposes in all sports. Among them, 17α-alkylated AASs such as OT are especially popular for their oral availability, which is attributed to a reduction of the first-pass effect in the liver as a result of 17α-alkylation (Fragkaki et al., 2009). However, AASs also frequently appear in adulterated nutritional supplements, leading to an unintentional intake of AASs (Geyer et al., 2008). In antidoping controls, AASs represent the most frequently detected class of substances prohibited by the World Anti-Doping Agency (2014). Numerous side effects—including physical phenomena such as cardiovascular risks (Angell et al., 2012; Deligiannis and Kouidi, 2012) and increased risks of breast and Leydig cell cancer (Chimento et al., 2012; Siriani et al., 2012) as well as psychiatric disorders (Palmié et al., 2013)—are attributed to AASs. Although several hypothetical models exist to describe the mechanisms behind the cardiovascular issues (Melchert and Welder, 1995; Deligiannis et al., 2006), their ability to explain all symptoms is still fragmentary. Increases in blood pressure up to hypertension are described to be secondary to increases in blood volume, which can result from a disruption of mineralocorticoid signaling (Rockhold, 1993). Mineralocorticoids, the most important of which are DOC and aldosterone in humans, regulate water and electrolyte homeostasis by controlling renal water and sodium retention as well as potassium secretion via the mineralocorticoid receptor (MR) signaling pathway (Funder, 1997). Ligand-induced activation of the cytosolic MR leads to the release of bound chaperones and nuclear localization, followed by DNA binding and the recruitment of specific coactivators, which subsequently initiates the transcription of specific target genes (Galigniana et al., 2004).

Here, we analyzed the metabolism of the xenobiotic steroid OT by human steroidogenic P450 enzymes on the molecular level to explore their drug-metabolizing capabilities. Therefore, we took advantage of the recombiant, high-yield expression of these enzymes in the bacterial host Escherichia coli, which only recently became feasible for all of these P450 enzymes. Dissociation constants were determined by UV-visible spectroscopy to enable a comparison with affinities toward endogenous substrates, and kinetic studies on OT metabolism were carried out using a reconstituted in vitro system with purified enzymes combined with high-performance liquid chromatography (HPLC) analysis. Metabolites were characterized by tandem mass spectrometry (MS/MS) or produced with an HPLC analysis. Metabolites were characterized by tandem mass spectrometry (MS/MS) or produced with an HPLC analysis.

### Materials and Methods

**Chemicals**

All reagents were obtained from standard sources with the highest purity available. OT was kindly provided by the Center for Preventive Doping Research (German Sports University, Cologne, Germany). Other steroids were purchased from Sigma-Aldrich (St. Louis, MO). Bacterial media were purchased from Becton Dickinson (Heidelberg, Germany), and isopropyl β-n-1-thiogalactopyranoside and 5-aminolevulinic acid were from Carbolution Chemicals (Saarbrucken, Germany).

**Protein Expression and Purification**

**Expression and Purification of P450 Enzymes.** All P450 enzymes were expressed with a C-terminal polyhistidine tag from a pET-17b or pET-22b vector (Invitrogen/Life Technologies, Carlsbad, CA) in *E. coli* C43(DE) and purified as previously described with slight modifications, if necessary. The human CYP11A1 cDNA sequence (Chung et al., 1986) encoding I301 instead of M301 was modified as described by Woods et al. (1998) and was expressed and purified by immobilized metal ion affinity chromatography (IMAC) and ion exchange as presented for bovine CYP11A1 (Neunzig and Bernhardt, 2014), with slight modifications demanded for ion exchange because of differences in pl. After IMAC, the eluate was dialyzed overnight against buffer A [20 mM potassium phosphate, pH 6.8, 20% glycerol, 0.1 mM EDTA, 0.1 mM dithioerythritol (DTE), 1% sodium cholate, and 0.1% Tween 20] and applied to a SP Sepharose Fast Flow column (GE Healthcare Life Sciences, Freiburg, Germany) equilibrated with buffer A. The column was washed with buffer A, followed by buffer A containing 30 mM potassium phosphate (pH 6.8). CYP11A1 was subsequently eluted with buffer B (40 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTE, 1% sodium cholate, and 0.1% Tween 20), which was replaced with buffer C (50 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTE, 1% sodium cholate, and 0.05% Tween 20) by dialysis. Human CYP11B1, CYP11B2, and CYP21A2 were expressed and purified by IMAC and ion exchange as previously described by Zöller et al. (2008), Hobler et al. (2012), and Arase et al. (2006), respectively. Human CYP17A1, modified as described by Imai (1993), was purified by IMAC according to the methods of Khatri et al. (2014a) and Petrunak et al. (2014); human CYP19A1 was purified according to the methods of Khatri et al. (2014b). Final P450 concentrations were determined by CO-difference spectroscopy (Oumura and Sato, 1964) with a molar extinction coefficient (ε) of 91 mM⁻¹ cm⁻¹.

**Expression and Purification of Redox Partners.** AdR (Sugiyama and Yamano, 1975; Sagara et al., 1993) and Adx (Uhlmann et al., 1992; Schiffler et al., 2004) were expressed in *E. coli* and purified as previously described. Concentrations were determined with ε450 of 11.3 mM⁻¹ cm⁻¹ for AdR and ε414 of 9.8 mM⁻¹ cm⁻¹ for Adx. CPR was expressed in C34(DE) as an N-terminally truncated version with C-terminal 3-glycine-6-histidine tag and purified by IMAC as described (Sandee and Miller, 2011). The truncation of 27 amino acids at the N terminus enables high-yield expression of a soluble, catalytically active CPR. Slight modifications were made during the purification: Triton was replaced with 1% sodium cholate in all buffers and imidazole was used for washing and elution at 30 and 200 mM concentrations, respectively. The protein was finally dialyzed against buffer C described above for the removal of imidazole. The CPR concentration was determined using ε450 of 2.4 mM⁻¹ cm⁻¹ (Vermilion and Coon, 1978) for the semiquinone form. Bovine cytochrome b5 was purified as reported previously by others (Neunzig et al., 2014).

**In Vitro Conversion and Enzyme Activity Assay.** In vitro substrate conversion was carried out at 37°C with a reconstituted system in 50 mM HEPES (pH 7.4) supplemented with 20% glycerol and 100 µM 1,2-dilauroyl-sn-glycero-3-phosphocholine. Prior to use, the buffer was sonicated in a sonication bath for 5 minutes for the reconstitution of 1,2-dilauroyl-sn-glycero-3-phosphocholine vesicles. The system contained 0.5 µM P450, 1 mM MgCl₂, and 1 mM NADPH as well as a NADPH-regenerating system composed of 5 mM glucose-6-phosphate and 4 U/ml glucose-6-phosphate dehydrogenase. For mitochondrial P450 enzymes, 0.5 µM AdR and 10 µM Adx were added; 1 µM CPR was added for microsomal P450 enzymes. For CYP17A1, reactions including 2 µM bovine cytochrome b5 were additionally performed. Substrate was added in the respective concentration from a stock solution in ethanol or in 2-hydroxypropyl-β-cyclodextrin with a final concentration of 0.225% for cholesterol. The final ethanol concentration was adjusted within each set of reactions. It was kept between 2% and 3% (2% for the kinetic studies with CYP11B1 and CYP11B2, 3% for the kinetic studies with CYP11A1 due to the higher OT concentrations required for saturation, 3% for CYP11B inhibition experiments, and 2% for CYP11A1 inhibition experiments, because cholesterol was added from a solution in cyclodextrines), whereby no effect on reaction kinetics was observed in that range. Steroids were extracted twice with chloroform, evaporated, and suspended in acetonitrile for HPLC analysis. For product quantification, progesterone was added as an internal standard prior to extraction and quantification was performed by HPLC using a calibration curve.
For OT turnover, product formation was calculated from the applied OT concentration deducting OT consumption, which was determined with a calibration curve. To monitor CYP11A1-dependent conversion of cholesterol to pregnenolone at 240 nm, the samples were boiled for 5 minutes in a water bath after the respective reaction time and a subsequent cholesterol oxidase reaction was performed for 1 hour at 37°C, which enables detection of the steroids as cholestenol and progesterone. Cortisol was used for quantification of product formation in this case.

For the determination of kinetic parameters of substrate conversion, enzyme concentrations were scaled down to 0.25 μM P450, 0.25 μM Adr, and 4 μM Adx for CYP11B1 and to 0.1 μM P450, 0.1 μM Adr, and 2 μM Adx for CYP11B2. For CYP11A1, the conditions described above were maintained. Reactions were stopped under steady-state conditions by freezing in liquid nitrogen. Reaction times were between 2 and 15 minutes for CYP11B1 and CYP11B2 and between 20 and 25 minutes for CYP11A1.

**HPLC/UV-Visible Detection**

Steroids were separated on a Jasco reversed-phase HPLC system (Jasco, Gross-Umstadt, Germany) using a 4.6 × 125 mm Nucleosil C18 Iris reversed-phase column (Macherey-Nagel, Düren, Germany) with an acetonitrile/water gradient at 40°C and a flow rate of 0.8 ml/min. The steroid pattern was monitored by an UV-visible detector (UV-2075 Plus; Jasco) at 240 nm.

**Spectroscopic Binding Assay**

The determination of dissociation constants was performed by difference spectroscopy using tandem cuvettes as described (Schenkman, 1970) with a Jasco V-630 spectrophotometer. CYP11B1 or CYP11B2 (1 μM) diluted in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 20% glycerol, 0.5% sodium cholate, and 0.05% Tween20 was titrated with increasing concentrations of the steroid from stock solutions in dimethylsulfoxide and difference spectra were recorded from 350 to 500 nm. Titurations were performed three times. To determine the binding dissociation constant (Kd), the averaged ΔA (peak-to-trough absorbance difference) was plotted against the ligand concentration. Plots were fitted with Origin 8.6 software (OriginLab Corporation, Northampton, MA) by either hyperbolic regression [ΔA = (Amax[S]/Kd) + [S]] or a tight binding quadratic equation [ΔA = (Amax2[E])/(KΔ + [E] + [S])], whereby ΔA represents the peak-to-trough absorbance difference at every ligand concentration, Amax is the maximum absorbance difference at saturation, [E] is the enzyme concentration (1 μM), and [S] is the substrate concentration. To measure binding spectra of CYP11A1, a 5-μM Adx was added to enhance the spectroscopic signal.

**E. coli-Based Whole-Cell Product Formation and Product Purification**

Large-scale substrate conversion by human CYP11B2 was conducted with a recombinant E. coli whole-cell system as previously described for CYP11B1 (Schiffer et al., 2015b). Briefly, E. coli C43(DE3) [F- ompT gal hsdSB (r-b–m-b–) dcm ion ] cells were transformed by electroporation with the pET-17b-based plasmid Twin_11B2 encoding human CYP11B2, bovine Adr, and bovine Adx1-108 and the plasmid pGro12 for the cosynthesis of the molecular chaperones GroEL/ES (Nishihara et al., 1998). Terrific broth media (150 ml; 24 g yeast extract technical, 12 g peptone, 4 ml glycerol, 4.62 g KH2PO4, 25 g K2HPO4, 1 liter of distilled water) were supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin in a 2-liter Erlenmeyer flask and were inoculated from an overnight culture. Cultures were incubated at 37°C and 210 rpm until an optical density at 600 nm of 0.5 was reached. Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside, 1 mM β-aminolevulinic acid, 4 mg/ml arabinose, and 50 μg/ml ampicillin and took place at 275°C and 200 rpm for 24 hours. For subsequent substrate conversion, cells were harvested by centrifugation (3200 x g, 10 minutes, 18°C), washed in 50 mM potassium phosphate buffer (pH 7.4), and suspended in 150 mM phosphate buffer supplemented with 1 mM isopropyl β-D-thiogalactopyranoside, 4 mg/ml arabinose, 1 mM β-aminolevulinic acid, 50 μg/ml ampicillin, and 2% glycerol in a 2-liter Erlenmeyer flask. OT was added from a stock solution in ethanol to a final concentration of 100 μM and conversion was performed at 27.5°C and 200 rpm for 24 hours. Steroids were extracted twice with one culture volume of ethyl acetate and the organic phase was evaporated to dryness. Steroids were suspended in acetonitrile and separated on a Jasco reversed-phase HPLC system with a NucleoDur 100-5.5 × 250 mm C18 EC column (Macherey-Nagel) and an acetonitrile/water gradient at a flow rate of 3 ml/min and 40°C. Steroids were monitored at 240 nm and fractions containing the desired products were collected, evaporated to dryness, and analyzed by NMR and liquid chromatography (LC)/mass spectrometry (MS).

**LC-MS/MS Quadrupole Time-of-Flight Setup**

High-resolution/high-accuracy LC-MS/MS measurements were conducted using an Agilent 6520 iFunneL quadrupole time-of-flight LC-MS/MS instrument (Waldbronn, Germany) equipped with a dual Agilent Jet Stream electrospray ionization (ESI) source operated at a gas temperature of 290°C and an ionization voltage of 3500 V in positive mode. The mass spectrometer was calibrated using the manufacturer’s protocol allowing for mass errors < 5 ppm for the period of analysis. The mass analyzer acquired data from mass-to-charge (m/z) ratios of 50 to 600 with an acquisition time of 200 milliseconds per spectrum, and collision energies of MS/MS experiments were adjusted between 15 and 25 eV. LC was accomplished by means of an Agilent 1290 Infinity LC system equipped with an Agilent Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm) protected by a guard column of the same material. The eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) run at a flow rate of 1 ml/min, enabling gradient elution of the analytes starting at 98% A, decreasing to 0% A within 14 minutes, followed by a 4-minute re-equilibration period at starting conditions.

**NMR Characterization of the Major Metabolites**

The NMR spectra were recorded in CDCl3 with a Bruker DRX 500 or a Bruker Avance 500 NMR spectrometer at 298 K (Bruker, Billerica, MA). The chemical shifts were relative to CHCl3 at δ 7.26 (1H NMR) and CDCl3 at δ 7.70 (13C NMR), respectively, using the standard δ notation in parts per million. The one-dimensional NMR spectra (1H and 13C NMR, Distortionless Enhancement by Polarization Transfer measurements with 135°) and the two-dimensional NMR spectra (gradient selected (gs)-HH-Correlated Spectroscopy, gs-Nuclear Overhauser Enhancement Spectroscopy, gs-Heteronuclear Single Quantum Correlation, and gs-Heteronuclear Multiple Bond Correlation) were recorded using the Bruker pulse program library. All assignments were based on extensive NMR spectral evidence.

**MR Transactivation Assay**

Steroids were analyzed for their ability to activate the human MR applying the Human Mineralocorticoid Receptor Reported Assay System (INDIGO Biosciences, State College, PA) following the manufacturer’s protocol in a dose-dependent manner with concentrations from 2 to 20,000 pM. Aldosterone, which was used as positive control, was supplied with the assay.

**Results**

In Vitro Metabolism Assay of OT by Human Steroidogenic P450 Enzymes

Conversion of OT by the human steroid hormone–synthesizing P450 enzymes was assayed with recombinant proteins purified from E. coli as previously described (for references, see the Materials and Methods). The natural electron transfer chain from NADPH to P450 was reconstituted with human Adx and Adr for mitochondrial P450 enzymes and CPR for the microsomal P450 enzymes, and the reaction was supported by an NADPH-regenerating system. Putative product formation was analyzed by HPLC and is summarized in Table 1. It demonstrated OT metabolism by CYP11A1 as well as by the two isozymes of the CYP11B subfamily. By contrast, the microsomal P450 enzymes CYP17A1, CYP19A1, and CYP21A2, did not show any conversion of this AAS. CYP11B1- and CYP11B2-dependent conversion of OT showed distinct but partially overlapping product patterns (Fig. 1). Metabolites with the same retention times in the HPLC...
measurements are assumed to be the same OT derivatives. Whereas CYP11B1 forms one main metabolite (1) as well as two side products (2 and 3) in minor amounts, CYP11B2 produces three main products (1, 4, and 5) and three intermediate (2) or side products (3 and 6). For the CYP11B2-catalyzed reaction, time dependence of the product pattern could be observed. Peak area portions of metabolites 1 and 2 are reduced over the time, whereas those of metabolites 4 and 5 increase (Fig. 1, C and D), which suggests that metabolites 4 and 5 are formed from 1 and 2 in a follow-up reaction. CYP11A1 converted OT rather unselectively to one main product (metabolite 9) and several side products (metabolites 7, 8, and 10; HPLC chromatogram not shown). Because product formation was weak compared with CYP11B1 and CYP11B2 and a preparative setup did not seem to be feasible, we subsequently used an LC-MS/MS approach to obtain the maximal information about the metabolites of the CYP11A1-catalyzed conversion of OT.

**LC-MS/MS Analysis of CYP11A1-Dependent OT Metabolism**

The mixture of in vitro CYP11A1-derived metabolites was analyzed by LC-MS/MS using full-scan and product ion scan experiments. By means of the accurate masses of mono- and dihydroxylated analogs to OT as well as the consideration of diagnostic product ions generated from protonated molecules of the observed analytes, several different metabolic products were identified. The extracted ion chromatograms of mono- and dihydroxylated OT are illustrated in Fig. 2, suggesting the formation of at least six metabolites (metabolites 7–12). The product ion mass spectrum of metabolite 7 is depicted in Fig. 3A, presenting several product ions indicative for an unmodified steroidal A/B-ring system such as m/z 155, 169, and 181 in accordance to literature data (Thevis and Schänzer, 2005; Pozo et al., 2008), which support assigning metabolite 7 to C- or D-ring hydroxylated OT. Metabolite 8 also yielded product ions at m/z 155, 169, and 181 similar to metabolite 7 but at substantially different abundances (Fig. 3B). Moreover, intense product ions at m/z 205

**TABLE 1**

<table>
<thead>
<tr>
<th>P450 Enzyme</th>
<th>Redox System</th>
<th>OT Conversion</th>
<th>Control Reaction</th>
</tr>
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<tbody>
<tr>
<td>CYP11A1</td>
<td>Mitochondrial</td>
<td>+ Cholesterol → pregnenolone</td>
<td></td>
</tr>
<tr>
<td>CYP11B1</td>
<td>Mitochondrial</td>
<td>+ DOC → corticosterone</td>
<td></td>
</tr>
<tr>
<td>CYP11B2</td>
<td>Mitochondrial</td>
<td>+ DOC → corticosterone</td>
<td></td>
</tr>
<tr>
<td>CYP17A1</td>
<td>Microsomal</td>
<td>− Progesterone → 17-hydroxyprogesterone</td>
<td></td>
</tr>
<tr>
<td>Microsomal plus cytochrome b5</td>
<td>− 17-hydroxyprogesterone → androstenedione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP19</td>
<td>Microsomal</td>
<td>− Androstenedione → estrone</td>
<td></td>
</tr>
<tr>
<td>CYP21A2</td>
<td>Microsomal</td>
<td>− Progesterone → DOC</td>
<td></td>
</tr>
</tbody>
</table>

Product formation is indicated by a plus sign, and no product forming activity is indicated by a minus sign. A positive control was performed with a natural substrate.

**Fig. 1.** Reversed-phase HPLC chromatograms of the in vitro conversion assays of OT by human CYP11B1 and CYP11B2. OT (100 μM) was incubated at 37°C with a reconstituted P450 system consisting of 0.5 μM P450, 10 μM Adx, 0.5 μM ADR, 1 mM NADPH, and an NADPH-regenerating system. (A) Incubation without NADPH for 30 minutes as the negative control. (B) CYP11B1-dependent conversion of OT for 30 minutes. (C and D) CYP11B2-dependent conversion of OT for 10 minutes and 30 minutes, respectively.
and 207 complemented this spectrum, which were suggested to also originate from the steroidal A/B-ring based on the observed accurate masses and corresponding elemental compositions (C₁₂H₁₀ClO and C₁₂H₁₂ClO, respectively). However, in the absence of further information, a structural assignment of this analyte was not possible. The product ion mass spectrum of metabolite 9 contained a characteristic ion at $m/z$ 171 (Fig. 3C), representing the counterpart to $m/z$ 155 after hydroxylation. Hence, the location of the hydroxyl function is postulated to be within the A/B-ring moiety of the steroid. Noteworthy, both metabolites 7 and 8 showed two subsequent water eliminations to yield a product ion at $m/z$ 315.149, whereas metabolite 9 predominantly generated a product ion at $m/z$ 315.195 as a result of a HCl elimination (Fig. 3C, inset), which further corroborated an A/B-ring modification of this analyte.

In addition to the aforementioned monohydroxylated metabolites of OT, dihydroxylated species were also observed by means of the accurate mass of the protonated molecules (Fig. 2, compounds 10–12). Because of the comparably low abundance of these analytes and the limited amount of features identifiable by means of ESI-MS/MS experiments, further studies into the structural compositions were conducted only for metabolite 11 (Fig. 3D). Here, the same product ion as observed for product 9 was observed at $m/z$ 171, suggesting the location of one hydroxyl function at the A/B-ring of the metabolite. In addition, the protonated molecule at $m/z$ 367 was found to release HCl (36 Da), followed by two consecutive water losses to yield $m/z$ 331, 313, and 295, respectively. Assuming that the significant proton affinity of the conjugated $\pi$-electron system of the A-ring leads to charge-remote elimination processes, a location of the second hydroxyl function at the steroidal C/D-ring system is likely.

**Determination of Dissociation Constants and Kinetic Parameters**

To assess the efficiency of OT metabolism by the human CYP11 family members, in vitro characterization of substrate affinity and reaction kinetics was performed. For CYP11B1 and CYP11B2, a high-spin shift of the heme iron was observed upon OT binding. This feature was used to determine dissociation constants ($K_d$) from the type I difference spectra recorded during the titration of P450 with increasing concentrations of OT (Fig. 4). CYP11B2 showed a $K_d$ value of 5.4 ± 0.4 μM, whereas CYP11B1 has less affinity for OT with a $K_d$ of 17.7 ± 2.2 μM. CYP11A1, however, showed a putative type II–like difference spectrum with a minimum around 405 nm and a maximum between 422 and 424 nm, which was not quantifiable even under high P450 concentrations and in the presence of Adx (Fig. 4C). The observation of a type II difference spectrum with OT is unexpected because as all other type II ligands described in the literature, to our knowledge, possess a nitrogen atom, whose association with the heme iron induces a low spin shift. However, the spin-state equilibrium can also be influenced by distortions of the porphyrin molecule (Groenhof, 2007). Because of the small size of OT compared with cholesterol, two molecules of OT might bind in the active site, resulting in a very close position of one OT molecule above the heme. Putative emerging substrate-induced heme deformation could then lead to a spin-state crossover toward the low-spin state.

Moreover, we determined kinetic parameters of OT conversion by performing in vitro reactions under steady-state conditions and quantification of OT consumption (Fig. 5). Molar ratios of P450 and its redox partners were 1:20:1 P450/Adx/AdR, because the excess of Adx excludes the electron transfer to P450 as a limiting step and maximum activities of

![Fig. 2.](https://example.com/fig2.png) Extracted ion chromatograms of CYP11A1-derived mono- and dihydroxylated OT. OT (100 μM) was incubated at 37°C with a reconstituted P450 system consisting of 0.5 μM CY11A1, 10 μM Adx, 0.5 μM AdR, 1 mM NADPH, and an NADPH-regenerating system. (A and B) Protonated molecules of steroids [M+H]$^+$ as measured by LC-ESI-MS are shown with metabolites 7, 8, and 9 representing monohydroxylated OT (m/z 351) (A) and metabolites 10, 11, and 12 representing dihydroxylated OT (m/z 367) (B).
CYP11 systems are observed at P450/AdR ratios \( \approx 1 \) upon Adx excess (Seybert et al., 1978, 1979; Lambeth et al., 1982). For CYP11B2, formation of the downstream products was approximately 20% under these reaction conditions, so that the parameters primarily describe the first hydroxylation reaction. The resulting parameters \( K_M \), \( k_{cat} \), and the catalytic efficiency \( k_{cat}/K_M \) are summarized in Table 2.

Influence of OT on Natural Substrate Conversion

The binding and metabolism of OT at relevant catalytic rates by the three enzymes suggests possible interference with the conversion of their natural substrates. In vitro conversions using cholesterol as substrate for CYP11A1 and DOC for CYP11B1 and CYP11B2 in the presence of increasing OT concentrations demonstrate the capability of

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**Fig. 3.** Product ion mass spectra of protonated CYP11A1-derived OT products at \( m/z \) 351 and \( m/z \) 367. (A–D) Product ion mass spectra of product 7 (\( m/z \) 351, collision energy: 15 eV; RT = 7.5 minutes) (A), product 8 (\( m/z \) 351, collision energy: 15 eV; RT = 8.0 minutes) (B), product 9 (\( m/z \) 351, collision energy: 25 eV; RT = 9.3 minutes) (C), and product 11 (\( m/z \) 367, collision energy: 25 eV; RT = 7.7 minutes) (D). Proposed structures of the respective OT metabolites are shown. Fragmentation and the resulting fragment ion masses are indicated. RT, retention time.
OT to inhibit steroid biosynthesis on an enzymatic level (Fig. 6). Product formation was inhibited for all three enzymes in a concentration-dependent manner, with a stronger effect on CYP11B2 than on CYP11B1 and CYP11A1, which are influenced to a comparable extent. With a 5-fold excess of OT over the respective natural substrate, CYP11A1- and CYP11B1-dependent product formation was reduced to 42% and 49%, respectively, whereas CYP11B2-dependent product formation was reduced as low as 12%. The regulation of steroid biosynthesis is also the result of signaling on multiple levels (hypothalamic-pituitary-adrenal axis, renin-angiotensin system, etc.). A deeper characterization of the inhibitory effects of OT was thus not considered as conducive for the interpretation of their relevance.

E. coli–Based Whole-Cell OT Conversion

Preparative scale production of the OT metabolites formed by the CYP11B isoforms for NMR characterization and further investigation of the major metabolites was performed with an E. coli–based whole-cell conversion system as previously reported for CYP11B1-dependent transformation of 11-deoxycortisol to cortisol (Schiffer et al., 2015b). Because CYP11B1 products are also formed by CYP11B2, application of CYP11B2 was chosen. The complete redox chain was transferred into E. coli by coexpression of CYP11B2, bovine AdR, and truncated bovine Adx1 from a tricistronic plasmid. Functional folding was supported by the overexpression of the molecular chaperones GroEL/ES (Nishihara et al., 1998). The CYP11B2 whole-cell biocatalyst was applied for the conversion of 100 μM OT under nongrowing conditions to maximize the availability of NADPH for the CYP11B2 reaction. After 24 hours, approximately 90% of OT was converted with the same product pattern as observed in vitro (data not shown). Individual yields for the three major products as estimated by peak area portions were about 30%, 33%, and 23% for metabolites 1, 4, and 5, respectively. All three products were purified in milligram amounts by preparative HPLC.

NMR Characterization of the OT Metabolites

Purified products were structurally characterized by NMR spectroscopy. Elucidated structures are illustrated in Fig. 7.

Metabolite 1. Compared with OT, the 1H and 13C NMR spectra of its conversion metabolite 1-[11β-OH-OT (4-chlor-11β,17β-dihydroxy-17α-methylandrost-1,4-dien-3-one)] showed signals for an additional secondary hydroxyl group (δH 4.44 td; δC 70.36), which could be located at C-11 by means of two-dimensional NMR. For example, its proton showed correlations to H-9 (δ = 3.7 Hz) and to H-12 (J = 2.5 and 3.7 Hz) and is therefore in an equatorial position because of its small coupling constants to H-9 (J = 3.7 Hz) and to H-12 (J = 2.5 and 3.7 Hz) and is therefore in α orientation. Consequently, the hydroxyl at C-11 is β oriented. 1H NMR (CDCl3, 500 MHz) data were as follows: δ 1.07 (dd, 11.0 and 3.7 Hz, H-9), 1.08 (m, H-7a), 1.13 (m, H-14), 1.15 (s, 3xH-20), 1.18 (s, 3xH-18), 1.44 (qd, 12.0 and 6.5 Hz, H-15a), 1.53 (s, 3xH-19), 1.57 (dd, 14.2 and 3.7 Hz, H-12a), 1.64 (dd, 14.2 and 2.5 Hz, H-12b), 1.65 (m, H-15b), 1.76 (ddd, 14.0, 9.5, and 6.5 Hz, H-16a), 1.85 (ddd, 14.0, 12.0, and 3.5 Hz, H-16b), 2.11 (m, H-7b), 2.14 (m, H-8), 2.39 (td, 13.8 and 5.3 Hz, H-6a), 3.24 (ddd, 13.8, 4.8, and 2.3 Hz, H-6b), 4.44 (td, 3.7 and 2.5 Hz, H-11), 6.39 (d, 10.0 Hz, H-2),...
and 7.32 (d, 10.0 Hz, H-1). 13C NMR (CDCl3, 500 MHz) data were as follows: δ 16.42 (CH3, C-18), 21.42 (CH3, C-19), 23.43 (CH2, C-15), 25.87 (CH3, C-20), 28.23 (CH2, C-6), 32.05 (CH, C-8), 32.40 (CH2, C-7), 32.70 (CH2, C-7), 34.19 (CH2, C-12), 39.21 (CH2, C-16), 46.90 (C, C-10), 48.31 (C, C-13), 50.38 (CH, C-14), 56.50 (CH, C-9), 63.49 (CH, C-18), 69.12 (C, C-17), 81.76 (C, C-17), 112.66 (CH, C-2), 112.73 (CH, C-4), 155.84 (CH, C-1), 163.37 (C, C-5), and 178.57 (C, C-3).

Metabolite 5. The NMR spectra of metabolite 5 [11β, 18-epoxy-18-CH3, 18-trihydroxy-17α-methyl-androdien-3-one] revealed a (11α,18)-hemiacetal function. This was obvious by the characteristic acetal resonance for C-18 (δC 99.40, CH) and its correlation with the hydroxyl proton H-11 (δH 4.77 d) in the HMBC. Thus, the structure of metabolite 5 represented the tautomeric form of the 11β-hydroxy-18-aldehyde derivat of OT. 13C NMR (CDCl3, 500 MHz) data were as follows: δ 1.04 (d, 10.7 Hz, H-9), 1.10 (m, H-7a), 1.28 (s, 3xH-20), 1.49 (m, H-14), 1.28 (d, 11.5 Hz, H-12a), 1.53 (m, H-15a), 1.36 (s, 3xH-19), 1.85 (m, H-15b), 1.95 (m, H-16a and H-16b), 2.07 (m, H-7b), 1.85 (m, H-8), 2.30 (m, 13.7 and 4.8 Hz, H-6a), 2.42 (dd, 11.5 and 6.5 Hz, H-12b), 3.34 (dt, 13.7 and 3.5 Hz, H-6b), 5.32 (s, H-18), 4.77 (d, 6.5 Hz, H-11), 6.40 (d, 10.0 Hz, H-2), and 7.14 (d, 10.0 Hz, H-1). 13C NMR (CDCl3, 125 MHz) data were as follows: δ 20.40 (CH3, C-19), 23.34 (CH2, C-15), 27.40 (CH3, C-20), 28.83 (CH2, C-6), 31.07 (CH3, C-7), 35.27 (CH2, C-12), 36.92 (CH, C-8), 39.51 (CH2, C-16), 45.64 (C, C-10), 48.01 (CH, C-14), 55.32 (CH, C-9), 57.19 (CH, C-13), 75.65 (CH, C-11), 78.64 (C, C-17), 99.40 (CH, C-18), 126.63 (CH, C-2), 128.94 (CH, C-4), 154.46 (CH, C-1), 161.42 (C, C-5), and 178.13 (C, C-3).

MR Activation Assay with the CYP11B-Derived OT Metabolites

CYP11B-derived OT metabolites carry the same o xo-functionalizations as are introduced into the stear scaffold during the biosynthesis of natural mineralocorticoids. Therefore, we evaluated their potential to activate the human MR to investigate putative new or altered functions of the OT metabolites. The assay was performed with a commercially available cell culture–based system, which gives a luminescence signal upon activation of the MR. Purity of the test compounds was verified by LC-MS prior to the assay. Test concentrations ranged from 2 to 20,000 pM, which resulted in full dose response for the natural MR ligand aldosterone. The respective EC50 value of 42 pM is consistent with the range of the value given in the manufacturer’s protocol. A detectable MR activation by OT and its metabolites was observed only with the highest assay concentration and represented only 50% of the maximum aldosterone response (Fig. 8). The modifications at C11 and C18 introduced by CYP11B2 did not alter the agonist potential of OT.

Discussion

Human P450 enzymes are traditionally classified into a group of drug-metabolizing P450 enzymes expressed in the liver and those that carry out the biosynthesis of endogenous compounds such as steroid hormones. In this study, we examined whether the second group might additionally contribute to the metabolism of xenobiotics by investigating the synthetic steroidal drug OT, which is a common doping agent. All six steroidogenic P450 enzymes were tested for their activity toward OT. Although no conversion of OT was found using CYP17A1, CYP19A1, and CYP21A2, the three mitochondrial P450 enzymes (CYP11A1 and both isoforms of the CYP11B subfamily) efficiently catalyze conversion of OT with an affinity and catalytic efficiency in the same order of magnitude as for their natural substrates. CYP11B2 binds OT with a Kd only approximately 4-fold higher than that for the natural substrate DOC (reported to be 1.34 μM; Hobler et al., 2012), and the catalytic efficiency (kcat/Km) of OT conversion by CYP11B1 and CYP11B2 is even slightly higher than previously determined for the natural substrates (Zölßner et al., 2008; Hobler et al., 2012). It is striking
that CYP11B2 shows a higher activity and affinity for OT than CYP11B1. It was postulated that the extended functional spectrum of CYP11B2 is enabled by an increase in retention time of the intermediates in the active site due to higher intrinsic flexibility compared with CYP11B1 (Strushkevich et al., 2013). A reduced flexibility of CYP11B1 might impede the access of the non-natural substrate to the active site and thus matches the lower binding affinity and catalytic efficiency of CYP11B1 toward OT.

As revealed by NMR characterization of the products, both CYP11B isoforms catalyze the same reactions with OT as with their natural substrates. An 11β-hydroxylation is performed by both enzymes and an additional subsequent 18-hydroxylation and 18-oxidation by CYP11B2. To the best of our knowledge, the respective products have not been described in the literature thus far. We can hypothesize the structure of metabolites based on studies on natural substrates (Kawainoto et al., 1990; Bureik et al., 2002a; Hobler et al., 2012). CYP11B1 and CYP11B2 generate the intermediate product 2 that is hypothesized to be 18-OH-OT. Metabolite 3 can be assumed to be a 19-hydroxylated OT derivative (Schiffer et al., 2015a). OT is thus the first exogenous substrate that is described to undergo all three reactions catalyzed by CYP11B2 with the same regio- and stereoselectivity as the endogenous substrate. The CYP11B2 crystal structure in complex with DOC (Strushkevich et al., 2013) revealed that it is bound to the active site via its 3-keto and 21-hydroxy groups. The absence of a 21-hydroxy group in OT does conclusively not negatively influence the selectivity or advancement of the catalytic reaction. However, the 3-keto-Δ4 motif, which was already supposed to be conserved among all CYP11B substrates using endogenous steroids (Strushkevich et al., 2013), is preserved and seems to be sufficient for proper binding. Interestingly, CYP11B2 metabolism of metandienone, which is structurally identical to OT except for the 4-chloro group, comprises only monohydroxylations in positions 11β and 18 (Parr et al., 2012). Compared with the metabolites formed from OT by CYP11B2, the differences between OT and metandienone metabolism lead to the suggestion that the C₄ substitution of OT determines the processivity of the reaction.

It is worth mentioning that the conducted bioconversion of OT by CYP11B2 at the preparative scale represents the first application of human CYP11B2 for substrate conversion in E. coli, which has thus far only been used for biotechnological purposes in recombinant yeast strains (Bureik et al., 2002b). Despite working under nonoptimized conditions with protein synthesis and substrate conversion taking place in shake flasks, the volumetric productivity of OT consumption of approximately 30 mg/l per day reaches the minimum requirements for potential application in industrial pharmaceutical production (Julsing et al., 2008). CYP11B2-dependent aldosterone biosynthesis plays a crucial role in the regulation of blood pressure and related diseases (Ardhani et al., 2015), and researchers are regaining interest in the use of selective CYP11B2 inhibitors in treatment of these diseases (Andersen, 2013; Hargovan and Ferro, 2014; Namsolleck and Unger, 2014). The presented system might alternatively serve as simple, economic prescreening for the effectiveness of potential inhibitors upon downscaling to a multiwell format.

Compared with the two CYP11B enzymes, CYP11A1 exhibits a lower efficiency of OT conversion (46 min⁻¹ mM⁻¹ compared with 741 min⁻¹ mM⁻² and 3338 min⁻¹ mM⁻³), which is also lower than for the natural substrate cholesterol. Bovine CYP11A1 shows an efficiency of approximately 85 min⁻¹ nM⁻¹ for cholesterol (Neunzig and Bernhardt, 2014; Mosa et al., 2015). The low catalytic efficiency for OT is, however, consistent with the observed activities toward endogenous steroids (Mosa et al., 2015) and the preference of CYP11A1 for longer side chains (Morisaki et al., 1980). Because of low productivity, structural assignments for the emerging OT metabolites were proposed for three of the six metabolites from MS/MS product ion mass spectra and previous studies that describe 2β-, 6β-, and 16β-hydroxylase activity of CYP11A1 for steroids (Mosa et al., 2015) (Fig. 3). Metabolite 7 shows an unmodified A/B-ring and an OH-group that can be assigned to the C- or D-ring. It is thus proposed to be 16-OH-OT. The product ion mass spectrum of metabolite 9 provides strong indications for an A-ring hydroxylation and as positions 3 and 4 are occupied, we suggest 2-OH-OT as a putative structure. Metabolite 11 seems to carry a combination of these two hydroxylation and is assumed to be 2,16-diOH-OT. 6- and 16-Hydroxylated OT species are already known from previous metabolism and excretion studies. In humans, OT is mainly transformed by reduction of the A-ring double bonds and keto group; by hydroxylation in positions 6β, 12, and 16β by CYP3A4, among others; and by rearrangement to 18-nor-17β-hydroxy-methyl derivaties (Schänzer, 1996; Schänzer et al., 1996; Rendic et al., 1999; Sobolevsky and Rodchenkov, 2012). 2-OH-OT would hence be another new OT metabolite discovered in this study.
After studying the biotransformation of OT by human mitochondrial P450 enzymes, we were also interested to examine the effect of OT on the activity of these P450 enzymes toward their endogenous substrates. An inhibition of the natural function of OT-metabolizing P450 enzymes seemed likely, because both OT and natural substrates compete for binding to the active site with comparable affinities. In fact, an inhibitory effect of OT on the conversion of natural substrates was demonstrated by our in vitro data. Inhibition thereby depends on the concentration and mirrors the affinity of P450 enzymes for OT, resulting in the highest inhibitory effect on CYP11B2. Potential physiologic relevance is, however, the result of concentrations in the microenvironment of steroid-synthesizing enzymes and can hardly be assessed at this time. The cholesterol level in the inner mitochondrial membrane is controlled by STAR protein activity, which varies upon signaling via the hypothalamic-pituitary-adrenal axis and other steroidogenic stimuli. Plasma levels of the prohormone 19-norandronostenediol and its active metabolite nandrolone were reported to be in the range of several hundreds of nanograms per 100 ml after a single dose of 25–100 mg (Schrader et al., 2006), which is in excess of DOC and 11-deoxycortisol plasma levels (Mason and Fraser, 1975). Exact 19-norandronostenediol and metabolite plasma levels were, however, also dependent on the route of administration. DOC levels are generally lower than 11-deoxycortisol levels and CYP11B2 is expressed at low levels compared with other steroidogenic P450 enzymes. These facts and the observation of the strongest inhibitory effect of OT on CYP11B2 indicate that the biggest effect in vivo of OT can be expected for aldosterone synthesis from DOC. This hypothesis of a relevant inhibition of CYP11B2-dependent aldosterone synthesis by OT coincides with AAS-induced increases in DOC levels in rats, whose MR-agonist properties induce increases in blood pressure (Colby et al., 1970). However, this phenomenon has thus far only been explained on a transcriptional level (Brownie et al., 1970; Colby et al., 1970; Brownie et al., 1988; Gallant et al., 1991). Our new insights suggest additional direct effects on an enzymatic level.

The identification of metabolites and characterization of their bioactivity is a crucial step in drug design. The elucidation of the MR crystal structure with various ligands (Bledsoe et al., 2005; Li et al., 2005) displayed that a hydrogend bond network between the steroid ligand C11 and C18 positions and the ligand binding domain is crucial for its activation. The alteration of the hydrogen bond–forming properties of these positions can thus modulate the agonist activity of a steroid. We therefore tested OT and the new metabolites, which are produced by the CYP11B enzymes, for their potential to activate the MR. It turned out that OT acts only as a very weak MR agonist. The new metabolites also do not show a relevant activation of the MR, although they carry oxy-functionalization at C11 and C18. An endocrine-disrupting potential of OT and its metabolites at the MR is thus unlikely. It can be hypothesized that OT and its metabolites do not induce all conformational changes required for activation, because they lack the C9–C10-cyanoxylin and C11-hydroxyl functions, which interact with the receptor in presence of the native ligands (Bledsoe et al., 2005). In addition, it is assumed that the length of the ligand also determines the events leading to activation (Bledsoe et al., 2005). This assumption is consistent with the weak binding of the C19-steroid 1-testosterone to the MR (Friedel et al., 2006a) and the higher affinity of tetrahydrogestrinnone, which has a C19-ethyl group (Friedel et al., 2006b).

In summary, these results clearly demonstrated that OT is the second xenobiotic steroid whose metabolism by steroidogenic P450 enzymes has been observed, in addition to metandienone (Parr et al., 2012). Our detailed in vitro studies hint at a potentially systematic contribution of human steroidogenic P450 enzymes to the metabolism of xenobiotics, which suggests their consideration as drug-metabolizing enzymes during drug design and toxicity evaluation. Their metabolic potential and a need for their involvement in drug testing appears especially important in the case of steroidal drugs, which are widely applied to treat a variety of anti-inflammatory and contraceptive issues as well as for disease- or cancer-conditioned (postoperative) steroid replacement purposes. Product properties might differ from those of the parental compound, and the interference with the endogenous steroid bio-activation can cause severe adverse effects.

Acknowledgments
The authors thank Birgit Heider-Lips for purification of AdR and Adx.

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Participated in research design: Schiffer, Thewis, Bernhardt.
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References
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