Human steroidogenic cytochromes P450

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Biotransformation of drugs and biotechnological application

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This work is based on 3 original publications, which are reproduced in chapter 2 with permission of BioMed Central (2.1 Schiffer *et al.* 2015a; 2.2 Brixius-Anderko *et al.* 2015) and The American Society for Pharmacology and Experimental Therapeutics (2.3 Schiffer *et al.* 2015b). A forth manuscript (2.4 Müller and Schiffer *et al.* 2015) was submitted to the *Journal of Steroid Biochemistry and Molecular Biology* (Elsevier) and is under review during the release of this thesis. Additionally, the author contributed to the publication of a review article (2.5 Schiffer *et al*. 2015c, Review) during the time of the doctoral study, which is reproduced at the end of chapter 2 with permission of Elsevier.

2.1 Schiffer *et al.* 2015a

The author planned and carried out all the presented experiments and wrote the manuscript.

2.2 Brixius-Anderko *et al.* 2015

The author participated in the establishment of the expression of human CYP21A2 in *Escherichia coli* and purification.

2.3 Schiffer *et al.* 2015b

The author designed and conducted all experiments (protein purification, *in-vitro* reconstitution assays, HPLC measurements, determination of kinetic parameters, difference spectroscopy, whole-cell bioconversions, product purification), except for NMR and mass spectrometry measurements. The author wrote the manuscript, except for the sections about NMR spectroscopy and mass spectrometry.

2.4 Müller and Schiffer *et al.* 2015, under review

The author planned all of the presented experiments and contributed to the performance of difference spectroscopy. The author wrote the manuscript with contributions of J. Zapp.

2.5 Schiffer *et al*. 2015, Review article

The author performed literature search and drafted the major part of the manuscript.

Content

Abstract

In humans, monooxygenases from the cytochrome P450 superfamily contribute to the biosynthesis of steroid hormones by catalyzing the side-chain cleavage of cholesterol and subsequent regio- and stereo-selective hydroxylations, which determine the bioactivity of the hormones. In this work, the additional contribution of these steroidogenic cytochromes P450s to the metabolism of xenobiotics was established and their employment as highly selective and efficient biocatalysts in recombinant bacteria for the production of commercially relevant glucocorticoids, which are important anti-inflammatory and immunosuppressive drugs, was realized.

The capability of CYP11A1, CYP11B1 and CYP11B2 to metabolize synthetic anabolic steroids, mineralocorticoid receptor antagonists and synthetic gestangens was demonstrated. Products were identified and analyzed for an alteration of their pharmacodynamic properties.

For the biotechnological application of CYP11B1 in the production of cortisol from 11-deoxycortisol, a whole-cell biocatalyst was developed by reconstituting the functional CYP11B1 system, including its redox partners adrenodoxin reductase and adrenodoxin, in *Escherichia coli*. It was optimized by molecular evolution of CYP11B1 and by increasing the synthesis of the redox partner adrenodoxin.

Additionally, the author contributed to the establishment of an *Escherichia coli*-based system for the application of CYP21A2 in the production of the synthetic glucocorticoid premedrol.

Zusammenfassung

Beim Menschen tragen Monooxygenasen der Cytochrom P450 Superfamilie zur Steroidhormonbiosynthese bei, indem sie die Seitenkettenspaltung von Cholesterin sowie sich anschließende regio- und stereoselektive Hydroxylierungen katalysieren, die die Bioaktivität der Hormone bestimmen. In dieser Arbeit wurde die zusätzliche Beteiligung dieser Steroidhormon synthetisierenden Cytochrome P450 am Metabolismus von Xenobiotika festgestellt und ihre Anwendung als hoch selektive und effiziente Biokatalysatoren in rekombinanten Bakterien für die Produktion von kommerziell relevanten Glucocorticoiden, die wichtige anti-inflammatorische und immunsuppressive Medikamente darstellen, realisiert.

Das Potential von CYP11A1, CYP11B1 und CYP11B2, synthetische anabole Steroide, Mineralocorticoidrezeptor Antagonisten und Gestagene zu metabolisieren, wurde aufgezeigt. Produkte wurden identifiziert und auf Veränderungen ihrer pharmakodynamischen Eigenschaften hin untersucht.

Für die biotechnologische Anwendung von CYP11B1 bei der Produktion von Cortisol aus 11-Desoxycortisol wurde ein Ganzzellbiokatalysator in *Escherichia coli* durch die Widerherstellung des funktionellen CYP11B1 Systems mit seinen Redoxpartnern, Adrenodoxin Reduktase und Adrenodoxin, entwickelt. Das System wurde durch molekulare Evolution von CYP11B1 und eine Erhöhung der Synthese des Redoxpartners Adrenodoxin optimiert.

Zusätzlich wirkte die Autorin an der Etablierung eines *Escherichia coli*-basierten Systems für die Anwendung von CYP21A2 für die Produktion des synthetischen Glucocorticoids Premedrol mit.

1. Introduction

1.1 Cytochromes P450

1.1.1 Structure, nomenclature and functions

Cytochromes P450 (P450, CYP) are an evolutionary highly conserved superfamily of heme thiolate monooxygenases. They carry a protoporphyrin IX, whose central iron is coordinated with the thiolate of a cysteine residue as fifth ligand. This unique structural feature of P450s leads to their eponymous spectral characteristic: Their reduced CO-complex exhibits an absorbance maximum at ~450 nm (Omura and Sato, 1962; Omura and Sato, 1964a; Omura and Sato, 1964b). Despite a high diversity of their primary sequences, all P450s share a conserved folding and topology due to structurally conserved regions (Sirim et al., 2010), which is shown exemplarily in **Figure 1.1 A** for the human aldosterone synthase, CYP11B2. In general, P450s are built of 12 main α-helices (A-L) and 4 β-sheets (Peterson and Graham, 1998; Sirim et al., 2010). Thereby, 4 α-helices (D, E, I and L) form a bundle constituting the conserved structural core, which incorporates the heme between the I- and L-helices (Mestres, 2005). The axial **cysteine** ligand of the heme is part of the conserved sequence motif FxxGx(H/R)x**C**xG in the heme ligand loop. However, several highly variable regions including 6 substrate recognition sites (Gotoh, 1992), which are flexible and regulate substrate binding and access to the active site, enable the acceptance of a large variety of substrates by the different P450 isoforms. P450 substrates include without limitations fatty acids, terpenes, steroids, organic solvents, alkylaryl hydrocarbon products, peptides and different types of drugs and pesticides (Bernhardt, 2006).

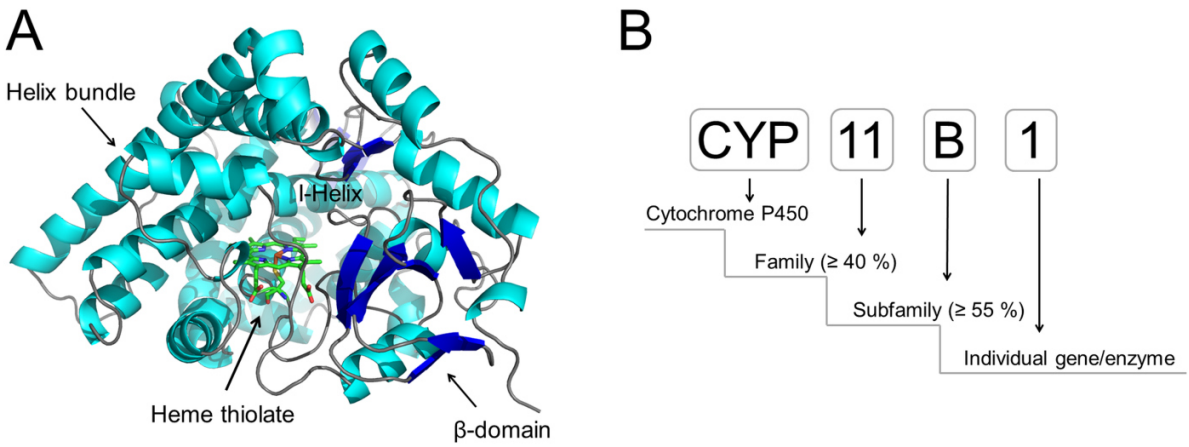

Fig. 1.1: Tertiary structure and nomenclature of cytochromes P450. (**A**) Crystal structure of human CYP11B2 (PDB-ID: 4DVQ) with α-helices in cyan and β-sheets in blue. The heme thiolate is shown as stick model with color coded atoms. The central I-helix is labelled. (**B**) Nomenclature of cytochromes P450 as proposed by Neybert *et al.* (1989). Primary sequence identities underlying the classification in families and subfamilies are indicated in brackets.

Cytochromes P450 can be found in all domains of life and have even been discovered in viruses (Lamb et al., 2009; Nelson, 2013). Nevertheless, the number of P450s per organism varies between different species. In humans, 57 individual P450 genes exist, while the gut bacteria *Escherichia coli* does not possess any P450, but the rice plant *Oryza sativa* owns 457 ones (Ortiz de Montellano, 2010). Today more than 21,000 P450 genes are known (Nelson, 2009; Nelson, 2013). The high and still increasing number of P450s requires a coherent classification and nomenclature, which was introduced for the first time in 1987 (Nebert et al., 1987) and is based on sequence identities on protein level. The name for each P450 is composed of the abbreviation "CYP", denoting the affiliation to the cytochrome P450 superfamily, an Arabic number designating its family and a letter designating its subfamily followed by another Arabic number to indicate the individual P450 gene. Thereby, sequences which exhibit ≥ 40 % identity are grouped as a family and sequences with ≥ 55 % identity are defined as a subfamily. This classification is summarized for the steroid 11β-hydroxylase, CYP11B1, in **Figure 1.1 B**.

P450 biodiversity regarding their primary sequence and distribution is accompanied by a large functional diversity. In general, P450 functions can be divided into 2 major groups. The first group comprises the central functions of P450s in the biosynthesis of essential and secondary metabolites, while the second one summarizes their key contributions to the biodegradation of xenobiotics, whereby they enhance polarity of the xenobiotic compound by their oxygenase activity and thus support its solubility and excretion. In plants, P450 activity is essential for the biosynthesis of xanthophylles,

structural components as lignin and suberin, UV protective flavonoids, phytohormones and flavors, but also a typically species-dependent variety of secondary metabolites involved in, for example, defense and symbiosis (Mizutani, 2012; Renault et al., 2014). Additionally, plant P450s catalyze the degradation of pesticides (Kahn and Durst, 2000), which makes them an interesting target for the engineering of *i.e.* herbicide resistant crop plants (Morant et al., 2003). Human P450s contribute on the one hand to the biosynthesis of essential compounds like steroid hormones, fatty acids, eicosanoids and fat-soluble vitamins. On the other hand, they are the major enzymes responsible for drug metabolism and catalyze approximately 75 % of the metabolism of marketed-drugs (Guengerich, 2008). Nevertheless, all functions trace back to the potential of P450s to activate molecular oxygen and incorporate one oxygen atom into their respective substrate, while the other oxygen atom is reduced to water. This is enabled by a common catalytic mechanism catalyzed at the heme iron.

1.1.2 Reaction mechanism, redox systems and spectroscopic characteristics

The P450 catalysis exhibits a complex reaction mechanism, which includes according to current, general acceptance the 8 intermediates illustrated in **Figure 1.2 A** (Denisov et al., 2005). Some intermediate states represent, however, hardly measurable radicals. In the resting state of the enzyme, the ferric heme iron (Fe^{3+}) is bound to a water molecule as sixth ligand (**1**). Substrate binding (RH) perturbs the water coordination (**2**). Reduction by the first electron leads to the transition of the heme iron to the ferrous state (Fe^{2+}, **3**). Upon O_2 binding the oxy-P450 complex is formed (**4**), which represents the last relatively stable intermediate in the cycle. A reduction of the distal oxygen by the second electron initiates the formation of a peroxo-ferric intermediate (**5a**), also termed *compound 0*, and subsequent protonation forms a hydroperoxy-ferric intermediate (**5b**). After a second protonation of the distal oxygen, heterolysis of the O-O bond occurs yielding the formation of water and *compound I*, which is supposed to be a ferryl-oxo-π-cation radical (**6**). *Compound I* can oxygenate the substrate (ROH, **7**), whose dissociation restores the initial water-bound resting state of the enzyme (**1**). A common signature amino acid sequence (A/G)-Gx(E/D)**T** in the central kink of the I-helix carrying a highly conserved **threonine** together with a preceding acidic residue (acid/alcohol pair) thereby emerged as crucial for an efficient proton delivery, heterolytic O-O scission and the formation of *compound I* (Imai et al., 1989; Martinis et al., 1989; Kimata et al., 1995; Vidakovic et al., 1998). Nevertheless, a complete fulfillment of the cycle is not obligate. The peroxo-ferric intermediate (**5a**) can also intervene with proton independent catalysis, which has recently been demonstrated for the lyase activity of the mammalian CYP17A1 (Gregory et al., 2013) and for the oxidation of a hydroxyl to the corresponding ketone by the bacterial CYP106A1 (Kiss et al., 2015a). Additionally, 3 major side reactions or shunt pathways can abort the mechanism prior to product formation and convert the enzyme back to the ferric state, which is also indicated in

Figure 1.2 A. Besides simple carbon hydroxylations, the mechanism of oxygen activation allows additional versatile reactions, which include heteroatom oxygenation, aromatic hydroxylations, C-C bond cleavage and heteroatom dealkylation, epoxidation, oxidative deamination and dehalogenation. Furthermore, uncommon P450 reactions are reductions, desaturations, oxidative ester cleavage, ring formation and expansion, dehydration, rearrangements and isomerizations (Sono et al., 1996; Guengerich and Munro, 2013).

As described above, P450 oxygenase activity requires 2 electrons per introduction of one oxygen atom into the substrate. Electrons are obtained from NAD(P)H via specific electron transport chains which are constituted of additional proteins. Depending on the composition of these chains, P450 redox systems can be classified into 10 classes (Hannemann et al., 2007). The 2 classes occurring in humans are shown schematically in **Figure 1.2 B** and **C**. Their composition depends on the subcellular localization of the P450 system. Mitochondrial P450s are associated with the inner mitochondrial matrix and need 2 additional proteins, which obtain electrons from NADPH: The NADPH-dependent, FAD-containing ferredoxin reductase (FdR) and a [2Fe-2S]-ferredoxin (Fdx), which is a soluble protein of the mitochondrial matrix and sequentially transfers electrons from the FdR to the P450. This system is called *class I* system and also occurs in bacteria, whereby all 3 proteins are soluble in the cytoplasm. The other group of human P450s is located in the membrane of the endoplasmic reticulum on the cytoplasmic side and depends on a *class II* redox system. These microsomal P450s are supported by a single membrane-bound electron transfer partner, cytochrome P450 reductase (CPR), which directly transfers electrons to the P450 via its FAD and FMN centers.

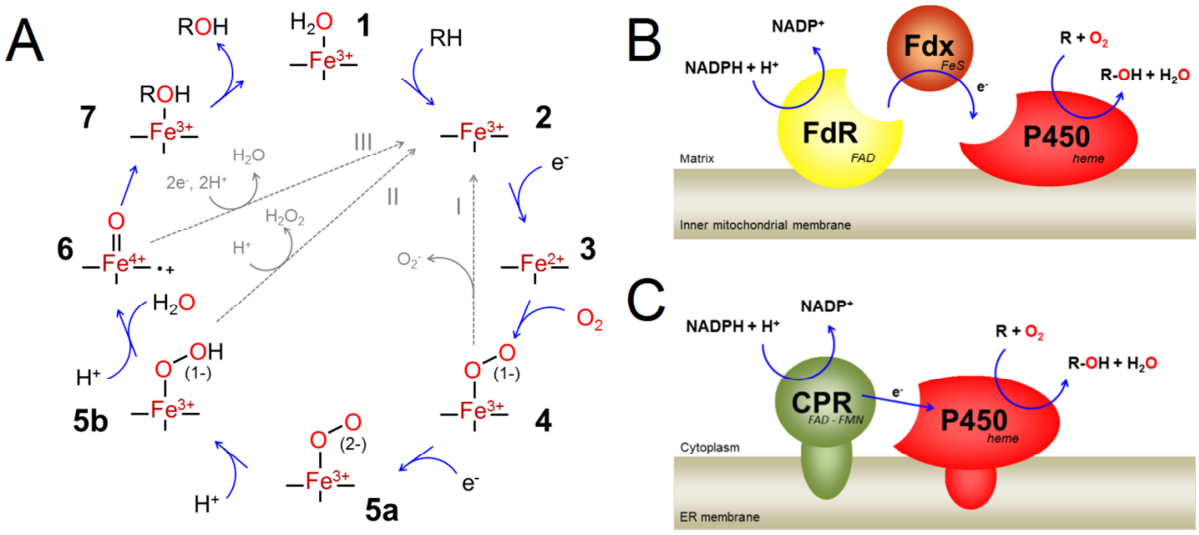

Fig. 1.2: Catalytic cycle of cytochromes P450 and electron transport systems. (**A**) – Catalytic cycle of cytochromes P450 according to (Denisov et al., 2005) with schematic representation of events leading to substrate hydroxylation at the heme iron. Numbers refer to the intermediate states described in the text. Grey arrows indicate the autoxidation shunt (I), peroxide shunt (II) and oxidase shunt (III). (**B**) Schematic organization of the mitochondrial *class I* P450 system. FdR = NADPH-dependent ferredoxin reductase, Fdx = ferredoxin, P450 = cytochrome P450. (**C**) Schematic organization of the microsomal *class II* P450 system, CPR = cytochrome P450 reductase, ER = endoplasmic reticulum. Prosthetic groups of each protein are indicated in italics. B and C were constructed according to (Hannemann et al., 2007) with modifications.

The absorbance spectrum of cytochromes P450 is sensitive to the oxidation state of the central heme iron as well as to the nature and concentration of the axial sixth ligand due to the effects of ligand binding on the spin state equilibrium of the heme iron. These spectroscopic characteristics make UV/vis spectroscopy an attractive tool for the quantification of P450s and the determination of ligand affinity. As already introduced, a characteristic absorbance maximum can be detected at ~450 nm for the reduced and CO-bound P450 complex due to the unique presence of a heme thiolate formed with a totally conserved cysteine. The respective heme iron coordination and spectrum are demonstrated in **Figure 1.3 A** and **B**. This spectrum is not just a qualitative verification for all P450s, but can also serve for the determination of P450 concentration by the specific molar extinction coefficient ε_{450nm}=91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964a; Omura and Sato, 1964b). The ferric P450 heme iron can exist with 2 different total spin angular momentums (*S*), which are either the *high spin* state of *S*=5/2 or the *low spin* state of *S*=1/2. Substrate-free P450 in its hexa-coordinated form with a water molecule as axial sixth ligand predominantly occurs in the *low spin* ferric state and therefore exhibits an absorbance maximum at ~417 nm (**Figure 1.3 A2**). Substrate binding and consecutive displacement of the axial water leads to a

(partial) transition of the spin state to the *high spin* form, which shifts the absorbance maximum to ~390 nm (**Figure 1.3 A3**). In contrast, the binding of a strong-field, in general nitrogen-containing, ligand stabilizes the *low spin* form and shifts the absorbance maximum to ~425 nm (**Figure 1.3 A4**) (Schenkman et al., 1967; Schenkman, 1970; Sligar, 1976; Schenkman et al., 1981; Gibson and Tamburini, 1984). Most of these ligands represent P450 inhibitors as they stabilize a low reduction potential and prevent oxygen binding, but also several *low spin* shift inducing, nitrogen-containing P450 substrates have been described (Locuson et al., 2007). The effect of ligand binding on spin state and absorbance spectrum can be used for the spectroscopic determination of dissociation constants (K_d). Therefore, difference spectra of the ligand-bound P450 deducting the spectrum of the ligand-free P450 are measured during the titration of the ligand. Binding of a putative substrate and transition to the *high spin* state lead to the formation of a type I-difference spectrum (**Figure 1.3 C**), while binding of a strong *low spin*-stabilizing ligand leads to the formation of a type II-difference spectrum (**Figure 1.3 D**) (Schenkman and Jansson, 2006). A K_d can be derived from the spectra by regression of the absorbance shift ($\Delta A_{max-min}$) as a function of the ligand concentration. Additionally, a rare reverse type I-difference spectrum with a minimum at ~390 nm and a maximum at ~420 nm can be observed, when a high amount of *high spin* P450 is converted into the *low spin* state by the particular ligand (Schenkman et al., 1972). However, it has to be noted that a ligand-induced *high spin* shift and P450 catalysis do not necessary depend on each other. Several substrates have been demonstrated to be converted without any observation of spectral changes (Girhard et al., 2010; Schmitz et al., 2014; Kiss et al., 2015b), while some type I-ligands are not converted (Schmitz et al., 2012; Khatri et al., 2013). Besides ligand-binding, the spin state equilibrium of the heme iron can be influenced by temperature (Sligar, 1976), redox partner interaction (Schiffler et al., 2001), and heme deformation (Groenhof, 2007).

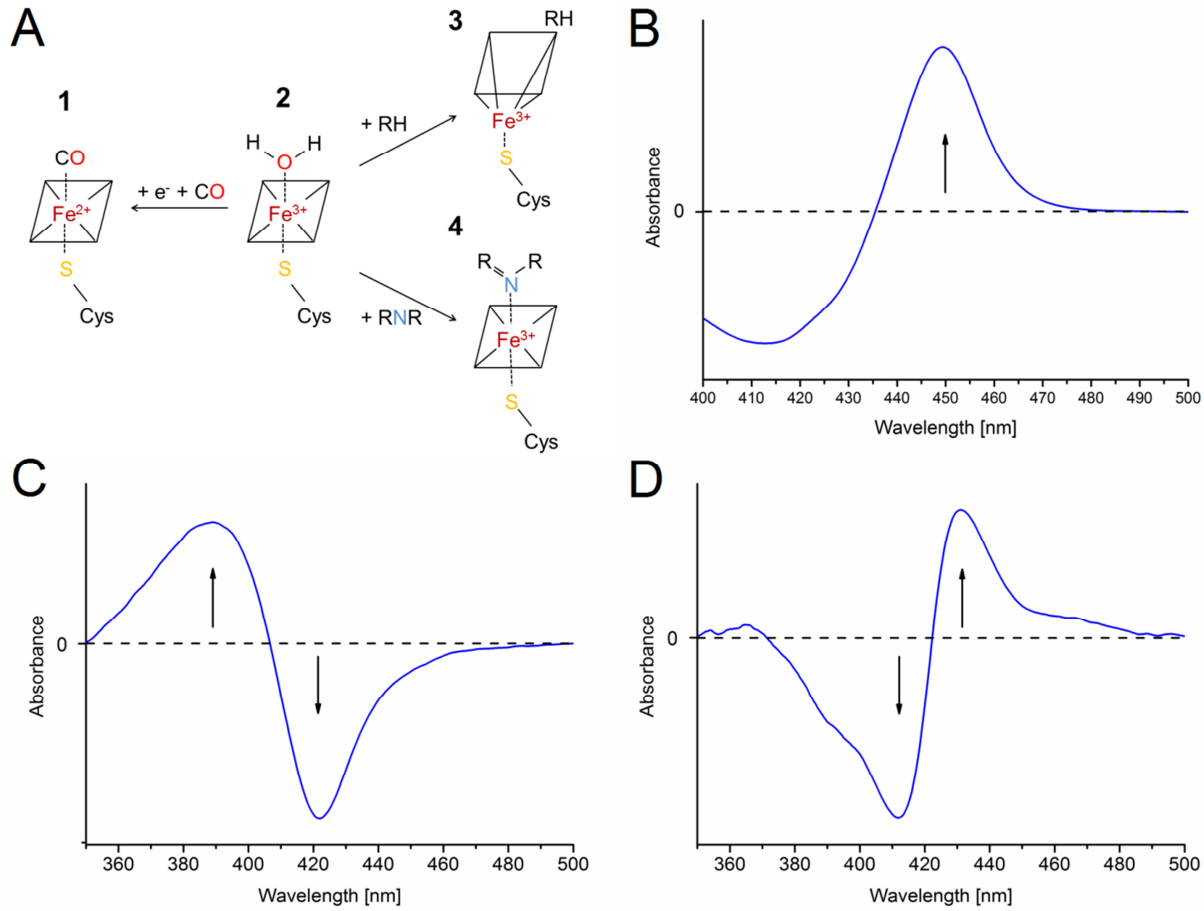

Fig 1.3: Heme iron coordination and resulting spectroscopic changes of the P450-ligand complex. (**A**) Schematic representation of the heme iron coordination by CO with preceding reduction (**1**), by water (**2**), upon substrate (RH) binding and displacement of the water (**3**) and by a nitrogen-containing ligand (RNR) (**4**). (**B**) Difference spectrum of the CO-bound reduced P450 complex (**1**) deducting the reduced P450 state with the eponymous maximum at 450 nm. (**C**) Type I-difference spectrum of the substrate-bound P450 complex (**3**) deducting the substrate-free water-bound form (**2**) with a maximum at ~385-390 nm and a minimum at ~420 nm resulting from a high spin shift of the heme iron. (**D**) Type II-difference spectrum of the P450 in complex with a nitrogen-containing ligand (**4**) deducting the ligand-free form (**2**) with a maximum at ~425-435 nm and a minimum at ~405-412 nm resulting from a *low spin* shift of the heme iron.

1.2 Functions of human cytochromes P450

In all biological kingdoms P450 functions can be divided into 2 major groups, which are the biodegradation of xenobiotics and the biosynthesis of essential compounds and secondary metabolites. Correspondingly, human P450s are traditionally categorized based on their major substrate classes and their critical role in the biotransformation of these substrates (Guengerich et al., 2005; Guengerich, 2008; Guengerich and Cheng, 2011). These include, on the one hand, xenobiotics and, on the other hand, endogenous compounds like sterols, fatty acids, eicosanoids and vitamins. The following sections will introduce the role and function of human P450s in, firstly, xenobiotic metabolism and, secondly, steroid hormone biosynthesis, which are central to the scientific contribution of this work.

1.2.1 Xenobiotic metabolism

The human body is permanently exposed to xenobiotic substances, which enter the body via its inner and outer surfaces, like the skin, gastrointestinal tract and lung, with the food, aerosols or cosmetic products among others. These agents include toxicants and carcinogens. Additionally, marketed pharmaceuticals are consumed intendedly for cure and prevention, as well as non-approved drugs for abuse. The application of a xenobiotic is followed by its resorption and distribution over the body with the blood flow. This allows the execution of its biological effects at its receptor sites, which might be desired or adverse. Furthermore, the substance can accumulate and be stored in particular tissues or compartments. All living organisms, however, own enzymatic systems for the biotransformation of xenobiotics to accelerate its clearance by increasing the polarity and solubility of the mainly hydrophobic xenobiotics. In humans, clearance takes place as renal and biliary excretion and in minor rates by intestinal secretion (**Figure 1.4**). Biotransformations of xenobiotics are divided into the phase-I and phase-II metabolism. During the phase-I metabolism functional groups are introduced or exposed. The most important reactions, thereby, are cytochrome P450-catalyzed oxidations. Other enzymes that contribute to the oxidative reactions of the phase-I metabolism are alcohol and aldehyde dehydrogenases, xanthine oxidase, amino oxidases and flavin-containing monooxygenases. Further reactions are carried out by reductases, esterases and epoxide hydrolases. Phase-II metabolism subsequently leads to the conjugation of functional groups with polar or negatively charged endogenous molecules. The most important conjugations are glucuronidation, sulfonation, methylation, acetylation and conjugation with amino acids and glutathione (Hofmann et al., 2013).

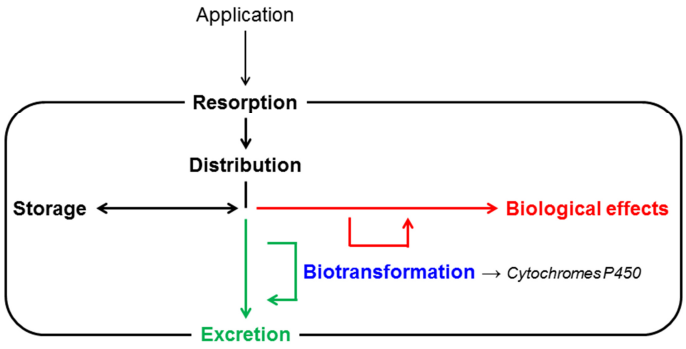

Fig 1.4: Overview of pharmacokinetics and pharmacodynamics with indication of P450 contribution. After its application a xenobiotic is resorbed and distributed, in general via the blood, to various tissues, where it can be stored and execute its biological functions, prior to its excretion. Chemical modifications of the xenobiotic (biotransformations) carried out by cytochromes P450s among other enzymes facilitate its excretion and modulate its bioactivity. Figure was adopted from (Hofmann et al., 2013) with modifications.

From the 18 human P450 families only the families 1, 2 and 3 contribute to the biotransformation of xenobiotics (Zanger and Schwab, 2013). They are expressed in the liver and represent microsomal P450s. Among them, only 4, which are CYP3A4, CYP2C9, CYP2C19 and CYP2D6, catalyze ~95 % of all known P450 reactions in the metabolism of marketed drugs (Guengerich, 2008). P450 oxygenation supports xenobiotic clearance by increasing the solubility and delivery of conjugation sites for the phase-II metabolism. Nevertheless, their activity can also greatly alter the biological activity of the parent compound by modifying toxic effects or bioactivation to reactive products (Nelson, 1982; Guengerich et al., 1985), which might lead to desired but also dangerous adverse effects. Therefore, the American Food and Drug Administration (FDA) released a guideline for the safety testing of drug metabolites (Food and Drug Administration, 2008). Metabolites, which are formed at greater than 10 % of the parent compound are recommended to be safety assessed by general toxicity, genotoxicity, embryo-fetal development toxicity studies and carcinogenicity studies. However, one can also profit from the concept of the bioactivation of a parent compound by the application of *prodrugs*, which are only activated after resorption during their distribution over the body when they are metabolized by the respective enzymes. Cytochromes P450 are greatly responsible for inter- and intra-individual variability in drug pharmacokinetics and response (Zanger and Schwab, 2013). P450 metabolism varies due to genetic polymorphisms, which alter enzymatic activity, the copy number of the gene or introduce promoter variants. This enables a classification of human populations into poor, intermediate, excessive and ultra-rapid metabolizers for particular drugs, which is reported to have clinical significance regarding adverse effects, drug efficacy and, thus, dose requirements. Moreover, P450 expression

changes in response to xenobiotics, hormone levels, disease state, sex and age of an individual. External factors like nutrition and the administration of other drugs can influence metabolism by P450 inhibition or induction. All these factors and their specific influence on the CYP1, 2 and 3 families were recently reviewed by (Zanger and Schwab, 2013). The characterization of drug-metabolizing P450s and the identification and biological characterization of emerging metabolites are, thus, challenging, but crucial tasks to ensure the design of safe and efficient pharmaceuticals.

1.2.2 Steroid hormone biosynthesis

Steroid hormones are synthesized from the membrane lipid cholesterol in the adrenal cortex and gonads by the P450s from the mitochondrial CYP11 family and the microsomal families 17, 19 and 21 together with 2 hydroxy steroid dehydrogenases (Bernhardt and Waterman, 2007; Miller and Auchus, 2011). **Figure 1.4** gives an overview of the reaction cascades leading to the formation of the three main classes of steroid hormones, which are mineralocorticoids, glucocorticoids and sex hormones. They exert their diverse functions via classic cytosolic steroid hormone receptors. These are hormone-activated transcription factors, which activate the transcription of specific target genes upon ligand binding. **Table 1.1** summarizes the central functions for all steroid hormone classes, the tissue/compartment of their biosynthesis and the major system responsible for the regulation of biosynthesis. The enzymatic properties of the different P450s contributing to their synthesis will be introduced in the following.

Table 1.1: Functions and biosynthesis of steroid hormones. For each class of steroid hormones the most important representative, the central function, localization and regulation of the biosynthesis are indicated (Norman and Litwack, 1997a; Norman and Litwack, 1997b; Norman and Litwack, 1997c; Norman and Litwack, 1997d; Norman and Litwack, 1997e).

			Biosynthesis	
Class	**Representative**	**Function**	**Localization**	**Regulation**
Mineralocorticoids	Aldosterone	Water/electrolyte homeostasis	Adrenal *zona glomerulosa*	Hypothalamic-pituitary-adrenal axis
Glucocorticoids	Cortisol	Energy mobilization Immune response	Adrenal *zona fasciculata*	Renin-angiotensin-system
Sex hormones Androgens Estrogens	 Testosterone Estrone	Sexual characteristics: Male Female Menstrual cycle	 Testis Ovaries	Hypothalamic-pituitary-gonadal axis
Gestagens	Progesterone	Gestation	Corpus luteum Placenta	Hypothalamic-pituitary-gonadal axis

CYP11A1 (cholesterol side-chain cleavage enzyme) catalyzes the initial and enzymatically rate-limiting step of steroid hormone biosynthesis in all steroidogenic tissues, which is the side-chain cleavage of cholesterol yielding pregnenolone, the common precursor of all steroid hormones. The side-chain cleavage requires six electrons and three molecules of O_2 and proceeds via the two intermediates 22(*R*)-OH-cholesterol and 20,22-diOH-cholesterol (Boyd et al., 1971; Katagiri et al., 1976; Chung et al., 1986). Cholesterol is delivered for this reaction by the steroidogenic acute regulatory (StAR) protein from the outer to the inner mitochondrial membrane. This transport step represents the overall rate-limiting step of steroidogenesis. CYP11A1 is also capable to catalyze the side-chain cleavage of other sterols, like 7-dehydrocholesterol, lumisterol, the fungal sterol ergosterol, the plant sterols campesterol and β-sitosterol and hydroxy-sterols like 24-OH-cholesterol, 25-OH-cholesterol and 27-OH-cholesterol. Additionally, CYP11A1 accepts vitamin D-type substrates for hydroxylation, but not for side-chain cleavage. Vitamin D_3 can be hydroxylated in positions 17, 20, 22, 23 by CYP11A1 and vitamin D_2 in positions 1, 17 and 20. The present knowledge about the CYP11A1 reaction repertoire was recently reviewed by (Slominski et al., 2015). The additional activities towards vitamin D and plant compounds seem physiologically important as CYP11A1 is also expressed in tissues different than the classical steroidogenic ones including the gastrointestinal tract (Guo et al., 2003), where it comes into contact with nourishment, and the skin, where vitamin D is generated from 7-dehydrocholsterol by UV energy and temperature-dependent isomerization. Moreover, the steroids androstenedione, dehydroepiandrosterone (DHEA), 11-deoxycorticosterone (DOC) and testosterone can undergo hydroxylation by CYP11A1 in the positions 2β, 6β and 16β (Mosa et al., 2015).

Human **CYP17A1** (steroid 17α-hydroxylase/17,20-lyase) possesses a steroid 17α-hydroxylase and 17,20 carbon-carbon lyase activity and is, in this way, an important branch point for the biosynthesis of glucocorticoids and sex hormones. It synthesizes the glucocorticoid precursor 17-OH-progesterone in the adrenal *zona fasciculata* and the testosterone and estrogen precursors DHEA and androstenedione from progesterone and pregnenolone in the adrenal *zona reticularis* and other steroidogenic tissues. CYP17A1 carbon-carbon lyase activity thereby depends on the presence of the allosteric effector cytochrome b_5, which is absent in the adrenal *zona fasciculata* (Katagiri et al., 1995; Auchus and Miller, 1999; Pandey and Miller, 2005; Bernhardt and Waterman, 2007). Furthermore, CYP17A1 has a preference regarding the C-C scission for the 3-OH-Δ_5 steroid 17-OH-pregnenolone over the 3-keto-Δ_4 steroid 17-OH-progesterone due to differential hydrogen bonding and specific allosteric effects of cytochrome b_5 (Gregory et al., 2013; Khatri et al., 2014). For progesterone an additional 16α-hydroxylase activity was demonstrated (Petrunak et al., 2014). Moreover, a role of CYP17A1 in the metabolism of sulfonated steroids was proposed, as bovine CYP17A1 was shown to convert pregnenolone sulfate to its 17-OH derivative, without a subsequent lyase reaction (Neunzig et al., 2014).

Following androgen biosynthesis by CYP17A1, **CYP19A1** (aromatase) synthesizes estrogens from the androgen precursors in both, men and women, but in significantly higher levels in women. Synthesis takes place in testis or ovaries and several extra gonadal tissues like skin, brain and adipose tissues (Simpson and Davis, 2001). Testosterone and androstenedione are converted to estradiol and estrone, respectively, by removal of the C_{19} carbon and aromatization of the A-ring. 16α-OH-testosterone represents an alternative substrate for this reaction. The aromatization requires three mols of O_2 and three mols of NADPH (Thompson and Siiteri, 1974a; Thompson and Siiteri, 1974b) and was shown to be a 3-step reaction which goes through 19-hydroxy and 19-aldehyde intermediates followed by a C_{10}-C_{19} lyase reaction leading to the A-ring aromatization (Akhtar and Skinner, 1968; Braselton et al., 1974; Akhtar et al., 1982; Ghosh et al., 2009). Aromatization is accompanied by stereospecific hydrogen eliminations in 1β and 2β positions (Townsley and Brodie, 1968; Fishman et al., 1969).

CYP21A2 (steroid 21-hydroxylase) produces the precursors for mineralo- and glucocorticoid biosynthesis in the adrenal by catalyzing the 21-hydroxylation of progesterone and 17-OH-progesterone yielding DOC and 11-deoxycortisol (Reichstein's substance S, RSS), respectively. This steroid 21-hydroxlyation was the first P450-mediated reaction discovered in adrenal microsomes in 1963 to be involved in steroid biosynthesis (Estabrook et al., 1963). For bovine CYP21 a further 20β-oxidase activity for 20β-hydroxy-C21-steroids has been demonstrated (Tsubaki et al., 1998).

The final steps of gluco- and mineralocorticoid biosynthesis are subsequently catalyzed by the 2 CYP11B subfamily members, **CYP11B1** (steroid 11β-hydroxylase) and **CYP11B2** (aldosterone synthase), which exhibit overlapping but distinct reaction repertoires despite a primary sequence identity of 93 % and the localization of all diverging residues outside the active site (Strushkevich et al., 2013). The current knowledge about the occurrence, evolution and the contribution of the enzymes from the CYP11B subfamily to the biosynthesis of steroid hormones in different species, as well as their regulation at different levels (gene expression, cellular regulation, regulation on the level of proteins) was reviewed in detail by the author of this thesis in a publication, which is printed in chapter 2.5 (Schiffer *et al*. 2015, Review article). CYP11B1 catalyzes the 11β-hydroxylation of RSS in the adrenal *zona fasciculata* and thereby produces cortisol, the major human glucocorticoid (Kawamoto et al., 1990b; Denner et al., 1995; Mulatero et al., 1998; Schiffer et al., 2015). It is also expressed in small quantities in the *zona reticularis*, but due to the low expression of the 3β-hydroxy steroid dehydrogenase this zone mainly produces DHEA (Gell et al., 1998). DOC also represents an efficient substrate for the 11β-hydroxylation by CYP11B1. Besides this, minor activities as 18- and 19-hydroxylase for DOC could be ascribed to CYP11B1 (Kawamoto et al., 1990b; Mulatero et al., 1998). CYP11B2 completes the biosynthesis of the major human mineralocorticoid aldosterone from DOC by a 3-step reaction sequence consisting of 11β- and 18-hydroxylation and an oxidation to the 18-aldehyde (Kawamoto et

al., 1990a; Mulatero et al., 1998; Hobler et al., 2012). The ability to perform an additional 18-oxidation distinguishes it from CYP11B1. The formation and secretion of aldosterone exclusively takes place in the adrenal *zona glomerulosa*, which is ensured by the restriction of *CYP11B2* expression to that zone in combination with the absence of CYP17A1 (Rainey, 1999). Additionally, CYP11B2 can catalyze an analogous 3-step reaction sequence on RSS yielding 18-oxo-cortisol (Mulatero et al., 1998; Freel et al., 2004) and shows a minor activity as 19-hydroxylase of DOC (Hobler et al., 2012). Both CYP11B isoforms have also been demonstrated to contribute to the metabolism of progesterone and the two androgens testosterone and androstenedione by forming the respective 11β-OH derivatives (Strushkevich et al., 2013; Swart et al., 2013).

In summary, steroidogenic P450s have been characterized to conduct highly stereo- and regio-selective hydroxylations and C-C cleavages with substrate specificities limited to particular steroid intermediates. This enables a straight-forward synthesis of different classes of steroid hormones, which is essential as P450-mediated functionalization is crucial for an efficient but specific activation of the respective target receptors. Only recently, the findings that CYP11B1, CYP11B2 and CYP21A2 may contribute to the metabolism of the synthetic anabolic androgenic steroid (AAS) metandienone (Zöllner et al., 2010b; Parr et al., 2012) and that CYP11B1 putatively catalyzes the bioactivation of an insecticide metabolite (Lund and Lund, 1995) began to change our perspective on the metabolic potential of steroid hormone synthesizing P450s, which will further be elucidated in one part of this work.

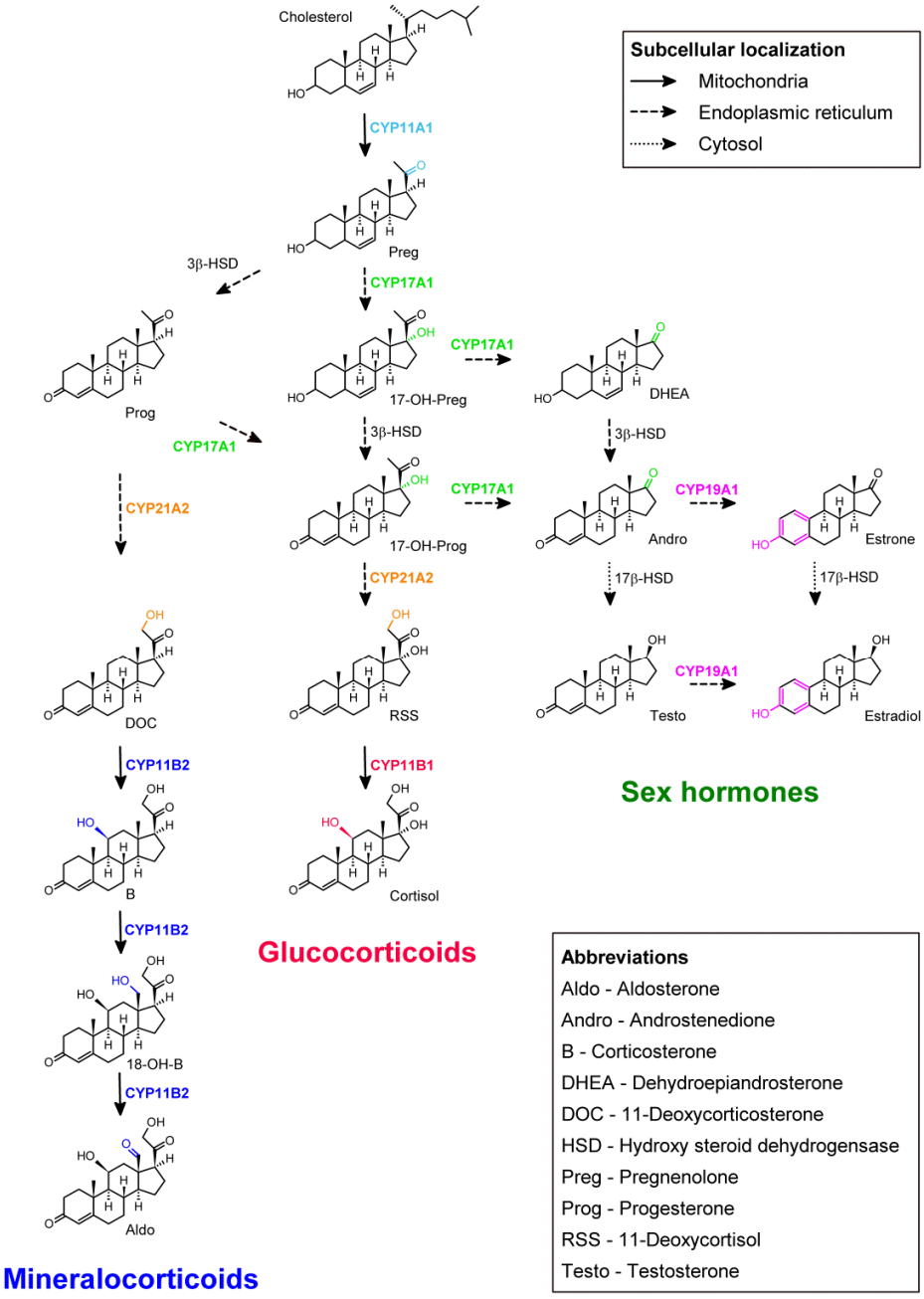

Fig. 1.4: Human steroid hormone biosynthesis. Reaction cascades catalyzed by cytochromes P450 and hydroxy steroid dehydrogenases lead to the formation of mineralocorticoids, glucocorticoids and sex hormones. Oxidative changes introduced to the steran scaffold are highlighted by color together with the respective P450 for each step. Subcellular localization of the reaction steps is indicated by the arrow style. Note that P450 expression and activity and, thus, formation of the different steroid hormones are tissue and compartment specific.

1.3 Biotechnological potential and limitations

Biocatalysis is defined as the application of enzymes and microbes in synthetic chemistry for purposes they have not evolved for (Bornscheuer et al., 2012). It respects in particular the principles of *Green Chemistry* (Anastas and Warner, 1998) by its environmental and economic advantages. Biocatalysts grow from renewable feedstocks, are non-toxic and biodegradable. They enable catalysis at ambient temperature, atmospheric pressure and neutral pH and their selectivity facilitates down-stream processing.

Cytochromes P450 own a unique capability to activate molecular oxygen for stereo- and regio-selective oxidations of inactive C-H bonds. This catalytic potential turns them into promising catalysts for biotechnological applications in the industrial synthesis of fine chemicals and chiral pharmaceuticals. They represent attractive alternatives to classical chemical synthesis, which often requires harsh reaction conditions and lacks selectivity in oxy functionalization (Loughlin, 2000; Labinger, 2004). Nevertheless, the presence and stereochemistry of functional groups, *i.e.* hydroxylations, are important determinants for the bioactivity of pharmaceuticals and the introduction of new stereo-centers with oxy functions opens the paths for further chemical modifications. Interesting reactions hereby are the formation of halogen, azide or amino derivatives (Zhang et al., 2012) as well as esterification, which can subsequently serve for the inversion of the stereo-center by hydrolysis (Tapolcsányi et al., 2004). This selectivity can be enabled by P450 application. Furthermore, the employment of human drug-metabolizing P450s in the production of metabolites for toxicity studies is of great interest (Zöllner et al., 2010a).

The minimum requirements for a putative industrial application, which are defined as a space-time-yield of 0.1 $g*L^{-1}*h^{-1}$ and a final product concentration of 1 $g*L^{-1}$ for fine chemicals and 0.001 $g*L^{-1}*h^{-1}$ and a final product concentration of 0.1 $g*L^{-1}$ for pharmaceuticals (Straathof et al., 2002; Julsing et al., 2008), are to date, however, only hardly met by P450 systems due to their complex multicomponent nature and the need for the costly co-factor NAD(P)H. Examples of industrial application of P450s are the biotransformation of RSS to cortisol with fungi of the species *Curvularia* (*i.e.*, *Curvularia lunata*), which possess an endogenous steroid 11β-hydroxylase ($P450_{lun}$) (Petzoldt et al., 1982) and the *de novo* synthesis of artemisinic acid, a precursor of the anti-malaria drug artemisinin, with recombinant *Saccharomyces cerevisiae* including CYP71AV1 from *Artemisia annua* (Ro et al., 2006; Paddon et al., 2013). For P450s, the usage of whole-cell biocatalysts expressing the complete functional redox system is thereby preferable over the use of purified enzymes (Schmid et al., 2001). The intracellular environment provides a stabilizing effect for the enzymes and NAD(P)H can be obtained and regenerated by the cellular metabolism. For the establishment of a whole-cell system, one can either

make use of an organism's endogenous P450 systems, which might require great screening efforts to select a suitable strain, or follow a recombinant approach by expressing the P450 system of interest in an appropriate microorganism. The second strategy provides the advantages that the host organism can be chosen with regard to the particular P450 and reaction and that the sequences of the P450 and its redox partners are directly available for enzyme engineering. Nevertheless, recombinant expression implies the knowledge of a P450 with the respective activity and the availability of its gene or cDNA sequence. This requires basic preliminary work in the fields of *substrate screening* and *genome mining* to establish characterized libraries of P450 biocatalysts.

Subsequent to the establishment of a P450 system, its improvement towards a stable, selective and productive catalyst in large scale processes faces a variety of challenges. Limitations can be approached on different levels by engineering of the enzyme, the reaction conditions and the host cell, which is summarized in **Table 1.2** including methodical approaches. Optimization can be completed by process engineering, which considers down-stream process issues (Julsing et al., 2008) and is not further relevant for this work. Enzyme engineering can optimize, on the one hand, the recombinant expression and stability by sequence modifications (Gillam, 2008), which is especially important for the application of membrane-associated mammalian and plant P450s, and, on the other hand, the activity and selectivity of the P450. Optimizing the latter properties is traditionally performed by either side-directed mutagenesis (SDM), a rational approach relying on established structure-function relations, or by molecular evolution (ME) consisting of several rounds of random mutagenesis and selection for the desired properties (Bloom et al., 2005). The first approach is, however, hampered by the limited availability and quality of 3D structures of P450s. This is especially valid for mammalian and plant P450s, which have a tremendous biotechnological potential due to their outstanding selectivity and diverse functions in secondary metabolism, but are demanding to express, to purify and to crystalize. Where limited structure-function information is available, molecular evolution represents the tool of choice. Its close-to-nature principal thereby opens up the discovery of synergistic mutational effects. The greatest challenge is the establishment of a sensitive screening system with a high throughput to select mutants with the desired properties in a time- and cost-efficient way (Kuchner and Arnold, 1997). Ideally, colorimetric and fluorimetric detection methods are established (Gillam, 2007; O'Reilly et al., 2011). The screening effort can further be reduced by the design of smart mutant libraries via the incorporation of reduced amino acid alphabets, the use of codon degeneracies or the stringent definition of randomization sites (Acevedo-Rocha et al., 2015).

Electron delivery from NAD(P)H by electron transfer proteins is essential for P450 catalysis and is therefore introduced as an extra level of engineering in **Table 1.2** for the optimization of P450 application. The nature of redox partners, which are co-expressed with a particular P450, as well as their

final ratio have to be considered for optimal activity. Due to the high conservation of the interaction domains (Sirim et al., 2010; Gricman et al., 2014), the employment of heterologous redox partners is feasible and eukaryotic redox systems can be replaced by bacterial systems (Ringle et al., 2013) as well as *class II* systems by *class I* systems (Pechurskaya et al., 2007). Another highly sophisticated, but to date not yet practicable approach, is the construction of artificial self-sufficient P450s by fusion of the P450 with a reductase domain (Munro et al., 2007; Gillam, 2008; Sadeghi and Gilardi, 2013).

The third level of engineering targets the host cell, whose physiology is crucial for protein synthesis and NAD(P)H supply and can be modulated by metabolic engineering or recombinant co-expression of proteins. Popular host organisms are the yeast species *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Pichia pastoris* and bacteria like *Pseudomonas putida* and *Bacillus subtilis* (Hanlon et al., 2007). The currently favored organism is, however, *Escherichia coli* (*E. coli*), which provides well-established molecular biology techniques and the advantage of the absence of endogenous P450s.

Engineering on the level of the reaction aims at stabilizing an efficient and continuous production. Cell growth, protein synthesis and the P450 reaction reduce nutrient and oxygen levels in the culture and alter the pH, which can be controlled by fermentation with feed optimization. Such approaches have been demonstrated to enhance P450 expression by up to 10-fold (Vail et al., 2005). Substrate solubility can represent another limiting factor, especially for the production of pharmaceutical steroids, which can be overcome by the addition of organic solvents or other solubilizing agents (Donova and Egorova, 2012).

Due to their catalytic potential and selectivity, P450s conclusively represent promising candidates for an economically and ecologically sustainable production of chiral compounds, but still require diverse optimization efforts to be established as stable and efficient biocatalysts, which can be achieved with consideration of all levels introduced above.

Table 1.2: Levels of engineering for the optimization of P450 whole-cell biocatalysts. Levels were divided as proposed previously (Julsing et al., 2008) with the addition of "Redox system" as independent level. Limitations that can be targeted on each level are summarized together with respective methodical approaches (Urlacher and Eiben, 2006; O'Reilly et al., 2011; Bernhardt and Urlacher, 2014). Limitations and strategies targeted in this work are highlighted in blue. SDM: side-directed mutagenesis; ME: molecular evolution.

Level of engineering	Targeted limitations	Methodical approaches
P450	**Expression and stability**	Sequence truncation or replacement, codon usage
	Activity Selectivity Substrate specificity Un-coupling[1]	SDM, **ME**, gene shuffling
Redox system	**Expression ratio**	Alteration of **gene copy number** or promoter
	Interaction/electron transfer	**Exchange or fusion of redox partners** SDM, ME
Reaction	**Substrate solubility**	**Co-solvents**, 2-phase-systems
	Substrate availability	Membrane permeabilization
	Product toxicity	*In-situ* product removal
	Medium-catalyst interaction NADPH availability	Modification of reaction medium Temperature, pH, O_2, feeding control by fermentation
Host cell	NADPH availability	Use of non-growing cells Metabolic engineering Co-expression: regenerating systems
	Protein synthesis	Modifications of the expression system (promoter, RNA polymerase activity) Enhancement of heme biosynthesis Co-expression of chaperones
	Substrate availability	Co-expression of uptake systems Modulation of efflux systems

[1] Uncoupling of NAD(P)H oxidation and product formation, see **Figure 1.2**.

1.4 Aim of the work

This work can be thematically divided into two parts. In the first part, it should be studied, if human steroidogenic P450s can contribute to the biotransformation of drugs in addition to their biosynthetic functions. Therefore, all 6 steroidogenic P450s should be expressed in *E. coli* and purified to homogeneity for substrate conversion assays in an *in-vitro* system reconstituted with their natural redox partners. Steroidal drugs from the groups of anabolic androgenic steroids (AAS) and mineralocorticoid receptor antagonists were selected as promising candidates for a transformation, because individual compounds from these groups have recently been shown to be metabolized by CYP11B1 and CYP11B2 (Zöllner et al., 2010b; Parr et al., 2012; Hobler, 2013). The turnover of positive hits from the substrate conversion assay should be kinetically characterized in detail with the reconstituted system to evaluate the putative physiological relevance of product formation. Structural characterization of the metabolites should by performed by nuclear magnetic resonance spectroscopy or mass spectrometry to elucidate the new enzymatic properties of the respective steroidogenic P450s. As P450 metabolism is known to significantly influence the bioactivity of drugs leading to severe adverse effects, selected metabolites will be functionally characterized regarding their effect on the mineralocorticoid receptor, whose activation is a crucial step in the regulation of blood pressure and hence the development of hypertension.

In the second part, the establishment of efficient, economic CYP11B1- or CYP21A2-dependent biocatalysts should be realized. CYP11B1 and CYP21A2 exhibit selective steroid hydroxylase activities for positions 11β and 21, respectively, and their employment for a sustainable production of pharmaceutical glucocorticoids is desirable, but currently available systems with recombinant yeast suffer from poor efficiencies (Szczebara et al., 2003; Hakki et al., 2008; Zehentgruber et al., 2010). Therefore, the respective natural P450 redox system will be reconstituted in *E. coli* by recombinant expression for substrate conversion with intact viable cells. In order to overcome the limitations of poor expression in bacteria, limited catalytic activity of the P450 itself and low electron transfer rates, the system should be engineered by side directed mutagenesis and molecular evolution of the P450, modulation of the expression level of the redox partners and replacement of the autologous redox partners by heterologous ones.

2. Publications

The results of this work were published in the articles listed below (2.1, 2.2 and 2.3).

A forth manuscript (2.4) is under review for publication after submission during the release of this thesis.

Additionally, a review article (2.5) was published by the author during the time of the doctoral research.

2.1 Schiffer *et al.* 2015a

A recombinant CYP11B1 dependent *Escherichia coli* biocatalyst for selective cortisol production and optimization towards a preparative scale

Lina Schiffer, Simone Anderko, Anna Hobler, Frank Hannemann, Norio Kagawa and Rita Bernhardt

Microbial Cell Factories (2015) 14:25

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MICROBIAL CELL FACTORIES

RESEARCH Open Access

A recombinant CYP11B1 dependent *Escherichia coli* biocatalyst for selective cortisol production and optimization towards a preparative scale

Lina Schiffer, Simone Anderko, Anna Hobler, Frank Hannemann, Norio Kagawa and Rita Bernhardt*

Abstract

Background: Human mitochondrial CYP11B1 catalyzes a one-step regio- and stereoselective 11β-hydroxylation of 11-deoxycortisol yielding cortisol which constitutes not only the major human stress hormone but also represents a commercially relevant therapeutic drug due to its anti-inflammatory and immunosuppressive properties. Moreover, it is an important intermediate in the industrial production of synthetic pharmaceutical glucocorticoids. CYP11B1 thus offers a great potential for biotechnological application in large-scale synthesis of cortisol. Because of its nature as external monooxygenase, CYP11B1-dependent steroid hydroxylation requires reducing equivalents which are provided from NADPH via a redox chain, consisting of adrenodoxin reductase (AdR) and adrenodoxin (Adx).

Results: We established an *Escherichia coli* based whole-cell system for selective cortisol production from 11-deoxycortisol by recombinant co-expression of the demanded 3 proteins. For the subsequent optimization of the whole-cell activity 3 different approaches were pursued: Firstly, *CYP11B1* expression was enhanced 3.3-fold to 257 nmol∗L^{-1} by site-directed mutagenesis of position 23 from glycine to arginine, which was accompanied by a 2.6-fold increase in cortisol yield. Secondly, the electron transfer chain was engineered in a quantitative manner by introducing additional copies of the *Adx* cDNA in order to enhance *Adx* expression on transcriptional level. In the presence of 2 and 3 copies the initial linear conversion rate was greatly accelerated and the final product concentration was improved 1.4-fold. Thirdly, we developed a screening system for directed evolution of CYP11B1 towards higher hydroxylation activity. A culture down-scale to microtiter plates was performed and a robot-assisted, fluorescence-based conversion assay was applied for the selection of more efficient mutants from a random library.

Conclusions: Under optimized conditions a maximum productivity of 0.84 g cortisol∗L^{-1}∗d^{-1} was achieved, which clearly shows the potential of the developed system for application in the pharmaceutical industry.

Keywords: Cortisol, Human CYP11B1, Steroid biotransformation, Whole-cell biocatalysis, *E. coli*

Background

Cortisol, the major human glucocorticoid, plays a crucial role in the physiological adaption to stress, the regulation of energy mobilization and immune response [1]. Its anti-inflammatory and immunosuppressive effects render it a powerful agent for the abatement of classical inflammatory symptoms like pain or swelling that occur in the course of acute and chronic inflammatory or autoimmune diseases. Moreover, cortisol serves as an intermediate in the production of synthetic glucocorticoids, which can exhibit even greater glucocorticoid effects but less mineralocorticoid side effects. Prednisolone, for example, is derived from cortisol by a microbial 1,2-dehydrogenation [2]. The hydroxyl group in position 11β of the cortisol molecule and its synthetic derivatives is the key functionalization that provides its glucocorticoid effects. It is the same functionalization that is the most difficult one to be introduced chemically or microbially in the preparative synthesis of cortisol. In current industrial production it is carried out as the final step of a hemi synthesis by microbial transformation of 11-deoxycortisol with fungal cultures of the genus *Curvularia* in a scale of about 100 tons per year [3] by taking advantage of the organism's endogenous

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steroid 11β-hydroxylase activity [4]. However, this process suffers from poor selectivity. Purification and characterization of the responsible enzyme revealed low regioselectivity as 11β-hydroxylation of the substrate is accompanied by 14α-hydroxylation [5,6]. Consecutively, it is of great interest to develop alternative biocatalysts for a more selective and efficient introduction of the 11β-hydroxyl group into synthetic glucocorticoids.

In the human adrenal cortex, which represents the principal tissue for the biosynthesis and secretion of gluco- and mineralocorticoids, cortisol is formed selectively from 11-deoxycortisol by the 11β-hydroxylase CYP11B1 (human steroid 11β-hydroxylase) (Figure 1) [7-10]. Hence, in the context of cortisol production CYP11B1 also constitutes an attractive candidate for a biotechnological application.

CYP11B1 belongs to the evolutionary highly conserved superfamily of cytochromes P450 (P450). P450s catalyze versatile biotransformations of a wide range of substrates in all domains of life. It is mainly their capability to activate molecular oxygen and to incorporate one oxygen atom into a substrate molecule leading to a regio- and stereoselective hydroxylation that vests them a tremendous biotechnological potential in the synthesis of pharmaceuticals and fine chemicals [11-14]. Due to their nature as external monooxygenases, P450s require an external electron donor, which is in general NAD(P)H, and an electron delivering system composed of one or more additional proteins [15]. In case of CYP11B1, which represents a mitochondrial P450, the respective electron transfer chain is constituted of AdR, an NADPH-dependent flavoprotein, and Adx, a [2Fe-2S]-cluster protein that interacts with the P450. Such complexity of P450 systems along with the necessity of a costly cofactor is so far one of the determining factors that restrict the employment of P450 catalysts in a larger scale. The most promising approach to overcome these limitations is the employment of whole-cell systems that offer cofactors from their metabolism and a cellular environment for the support of protein stability and do not require time-consuming purification steps [12,16]. The current state of molecular biology and recombinant protein expression enables the exploitation of different microbial hosts for application of biotechnologically interesting enzymes. CYP11B1 could already be applied in engineered yeast strains (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) that convert 11-deoxycortisol to cortisol [17-19] or even accept simple carbon sources as substrate when additional sterol providing and modifying genes are engineered and introduced [20]. However, optimization of these systems towards a relevant scale is a great challenge. Our laboratory previously reported the first expression of *CYP11B1* in a bacterial host (*Escherichia coli*, *E. coli*) for purification and enzymatic characterization [21]. Subsequently, we decided to use this fast-growing and genetically amenable microorganism, which does not possess any endogenous, by-product generating P450s, and established the first bacterial whole-cell system for application of CYP11B1 in cortisol preparative scale biosynthesis. The entire redox chain consisting of AdR, Adx and the P450 was introduced into *E.coli*. For optimization, CYP11B1 expression was enhanced by site-directed mutagenesis, the co-expression of Adx was quantitatively adjusted on transcriptional level and CYP11B1 was engineered by molecular evolution towards higher activity.

Results

Establishment of a CYP11B1 based whole-cell system for cortisol synthesis in *E. coli*

In order to employ human CYP11B1 for steroid hydroxylation in *E. coli*, we created the plasmid Twin_11B1 which is based on the pET-17b vector and carries the cDNAs of human CYP11B1 including the modifications described in the Material and methods section, bovine AdR and bovine Adx in a tricistronic transcription unit separated by ribosomal binding sites. This enables the reconstitution of a functional P450 system in the host organism. The *E. coli* strain C43(DE3), which has previously been reported as advantageous for the synthesis of membrane proteins [22], was co-transformed with the new plasmid and the chaperone encoding plasmid pGro12 [23]. Chaperone synthesis supports the proper

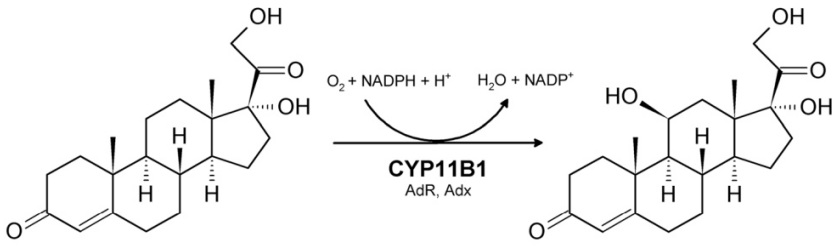

Figure 1 Scheme of the CYP11B1-catalyzed 11β-hydroxylation of 11-deoxycortisol yielding cortisol. Electrons are transferred from NADPH via AdR and Adx to CYP11B1, which activates molecular oxygen and incorporates one oxygen atom into the steroid substrate by means of a hydroxylation in a regio- and stereoselective manner, while the other oxygen is reduced to water.

folding of membrane proteins in the prokaryotic host [21,24-26]. Protein production was carried out in a complex medium and could be confirmed by Western Blot analysis with primary antibodies raised against CYP11B1, AdR and Adx, respectively.

Subsequent transformation of 11-deoxycortisol was conducted with non-growing cells in buffer supplemented with glycerol as carbon source to ensure a sufficient availability and regeneration of NADPH for the P450 reaction [27]. Thereby, a fixed cell density of 25 g_{wcw}/L was adjusted in all experiments. HPLC analysis of extracts from the resting cells demonstrates a selective CYP11B1 dependent 11β-hydroxylation of 11-deoxycortisol yielding cortisol (Figure 2). Steroids were identified via their retention times in comparison with standards from commercial sources.

As the solubilty of steroidal compounds can be a limiting factor for their bioconversion [12], we subsequently evaluated the effect of different dissolving agents for the addition of 11-deoxycortisol on the activity of the new whole-cell system. Each agent was added to a final concentration of 6% (vol/vol). While the employment of ethanol lead to the lowest cortisol yield and cyclodextrines and a 1:1 mixture of EtOH and PEG-400 slightly improved the final yield, the best results were obtained with DMSO, which was consecutively used for substrate supply in all subsequent experiments.

Optimization of CYP11B1 expression

In order to improve the *CYP11B1* expression in *E. coli* and thus the activity of the recombinant system, we performed site-directed mutagenesis in position 23 of the *E. coli* adapted sequence. Glycine, which corresponds to the published wildtype amino acid in that position [28,29], was replaced by the hydrophilic amino acid arginine. An analogous replacement from glycine to arginine, which was introduced into human *CYP19* when performing N-terminal replacements with related sequences from other P450s by Kagawa et al. [30], was reported to significantly enhance the expression in *E. coli.* The corresponding residue in human CYP11B1 was identified by primary sequence alignment. The expression level of the 2 CYP11B1 variants was estimated by CO-difference spectroscopy (Figure 3). The introduction of the arginine residue could succesfully enhance CYP11B1 level from 79 to 257 nmol∗L^{-1} and no significant reduction over the conversion period could be observed.

Cortisol formation by both enzyme variants in the whole-cell system was monitored in a time-dependent manner. In general, the system exhibited a linear volumetric productivity in an initial phase of at least 12 h. Afterwards the velocity of cortisol formation decreased and a final product concentration was reached after 30 h. The deployment of CYP11B1 G23R for the whole-cell conversion of 11-deoxycortisol increased the final cortisol yield after 30 h by a factor of 2.6 compared with the G23 variant from 239 to 631 mg∗L^{-1} (Figure 4). The initial linear productivity was enhanced in the same range from 11 to 27 mg∗L^{-1}∗h^{-1}. Therefore, CYP11B1 G23R is applied for all further experiments and will be

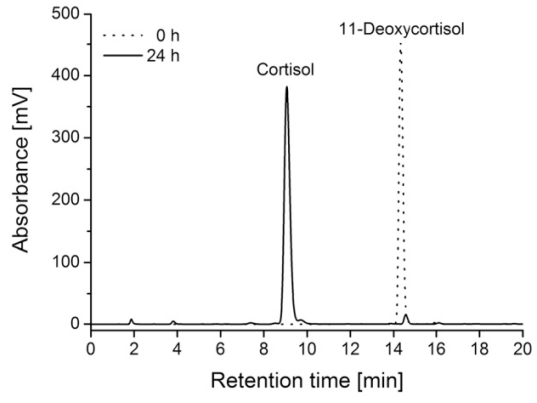

Figure 2 HPLC chromatogram of the CYP11B1 dependent conversion of 11-deoxycortisol to cortisol by recombinant *E. coli*. Chromatograms were obtained from extracted samples of *E. coli* cultures after 0 h (dotted line) and 24 h (solid line) of incubation after transfer into potassium phosphate buffer and addition of 1.2 mM substrate. Substance peaks obtained with an acetonitrile/water gradient represent cortisol with a retention time of 9.0 min and 11-deoxycortisol with a retention time of 14.3 min.

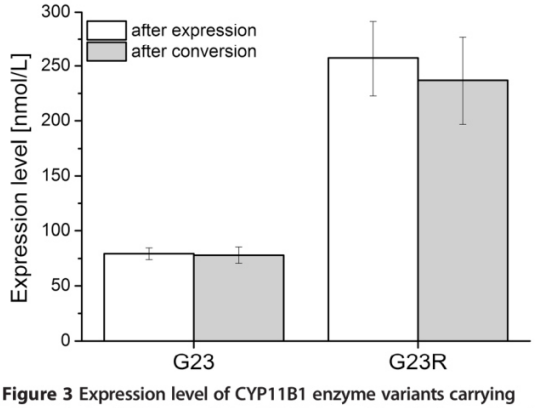

Figure 3 Expression level of CYP11B1 enzyme variants carrying either glycine (G23) or arginine (G23R) in position 23. Recombinant *E. coli* cultures expressing either wild type or mutant were harvested after the expression period (white bars) or at the end of substrate conversion (grey bars) under the conditions described in Material and methods. P450 concentration was determined by CO-difference spectroscopy with the supernatant of the cell lysate after ultracentrifugation. Values represent the mean of three experiments with the respective standard deviation.

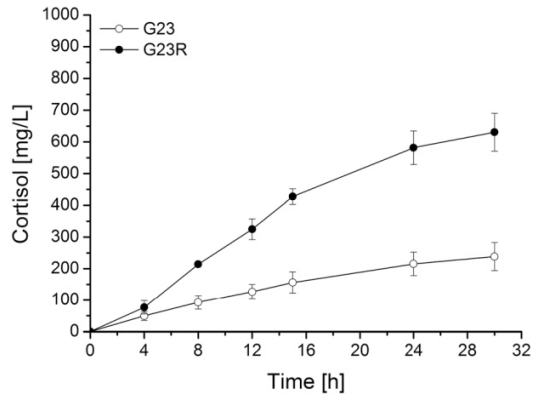

Figure 4 Comparison of the whole-cell cortisol formation by CYP11B1 enzyme variants carrying either glycine (G23) or arginine (G23R) in position 23 in recombinant *E. coli*. Reactions were performed after a 21-h expression period in TB medium with resting cells in the presence of 6% DMSO and 3 mM 11-deoxycortisol. Extracted steroids were analyzed by RP-HPLC. Values represent the mean of three conversion experiments conducted in parallel with respective standard deviation.

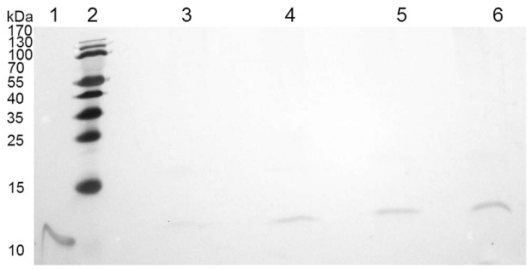

Figure 5 Western blot analysis of Adx expression by recombinant *E. coli* cells. *Lane 1* shows purified bovine Adx_{4-108} (~11,8 kDa). *lane 2* shows a pre-stained protein marker with molecular weights indicated on the left of the picture. *Lanes 3* to *6* represent the lysate of *E. coli* cells expressing the CYP11B1 redox system from variants of the Twin_11B1 plasmid carrying 1 to 4 copies of bovine Adx_{1-108} (~12,0 kDa). Adx detection was carried out with polyclonal antibodies and a peroxidase-based color reaction. The relative expression level of Adx was determined by measuring the band intensity with the Image Lab software.

termed Pa1 (Parent generation 1) in the following molecular evolution studies.

Influence of Adx copy number

As electron supply frequently constitutes the limiting step in the efficiency of P450 systems [31-33], our next approach for improving the whole-cell activity of CYP11B1 was an increase of the amount of Adx in the system, in order to enhance electron transfer to CYP11B1. For that, we constructed variants of the expression plasmid Twin_11B1 with up to 4 copies of the Adx cDNA by successively integrating additional copies including a 5′-ribosomal binding site at the end of the trancription unit according to Blachinsky et al. [34]. The relative increase of *Adx* expression was estimated by Western Blot (Figure 5) and evaluation of the Adx signal with an imaging software. With the introduction of a second cDNA copy *Adx* expression was increased approximately 2.4-fold and a maximum of *Adx* expression (3.3-fold increase) was reached with the insertion of a third copy which could not be further augmented by a fourth copy. No influence on *CYP11B1* expression was observed by CO-difference spectroscopy, when 2 or 3 copies of *Adx* were present on the expression vector. With the insertion of a fourth *Adx* copy, the CYP11B1 titer was, however, reduced by 50% to approximately 120 $nmol*L^{-1}$ and the construct was thus not further investigated.

Subsequent whole-cell conversions conducted with 2 and 3 *Adx* copies increased the final product concentration from 631 $mg*L^{-1}$ for the initial system with 1 Adx to 877 and 828 $mg*L^{-1}$, respectively (Figure 6). Although both plasmids enabled a comparable final yield, the presence of 2 Adx copies also greatly enhanced the initial productivity over the first 12 h from 27 to 52 $mg*L^{-1}*h^{-1}$, while a third copy diminished this rate again to 37 $mg*L^{-1}*h^{-1}$ but exhibited a longer phase of linearity in time-dependent product formation leading to a comparable final yield.

Development of a screening system for CYP11B1 activity

For further improvement of the CYP11B1 activity using molecular evolution, we adapted the biotransformation

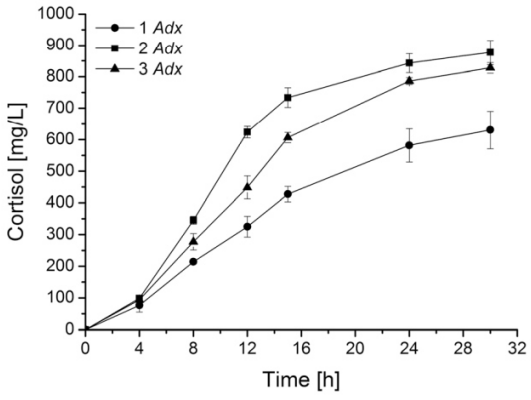

Figure 6 Comparison of the cortisol formation by recombinant *E. coli* transformed with different versions of the P450 system encoding plasmid Twin_11B1 with 1 (circles), 2 (squares) or 3 (triangles) copies of the Adx cDNA. Reactions were performed after a 21-h expression period in TB medium with resting cells in the presence of 6% DMSO and 3 mM 11-deoxycortisol. Extracted steroids were analyzed by RP-HPLC. Values represent the mean of three conversion experiments conducted in parallel with respective standard deviation.

with the *E. coli* whole-cell system and its subsequent evaluation to a microtiter plate format which enables a high and robot-assisted throughput. Culture size was scaled down to 1 mL and substrate conversion was carried out in TB medium with inoculation, induction of protein expression and addition of the substrate 11-deoxycortisol at the same time in order to reduce working steps to a minimum. For evaluation of enzyme activity, we employed a fluorescence assay, which makes use of the fluorescence developed by steroids with an intensity in dependence on the substitution of the steran scaffold [35-37]. As cortisol exposes a higher fluorescence than 11-deoxycortisol due to the additional hydroxyl group that is introduced in position 11β, enzyme variants with an increased hydroxylation activity can easily be selected. In order to ensure optimal conditions for the detection of mutants with improved activity, conversion with the parental enzyme variant Pa1 (CYP11B1 G23R) was tested with different substrate concentrations and the fluorescence was determined after 48 h of incubation in comparison with control cultures that were incubated with the respective 11-deoxycortisol concentration but without induction of protein expression. A clear difference between unspecific and steroid specific fluorescence could be observed as well as a significant increase of the relative fluorescence by a factor of more than 3 upon induction of protein expression, which proofs the presence of an active P450 system and thus the applicability of the system for monitoring 11-deoxycortisol conversion to cortisol (Figure 7).

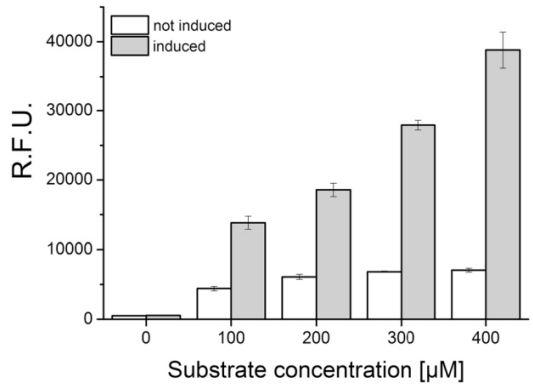

Figure 7 Relative fluorescence developed by recombinant *E. coli* cultures after incubation with different 11-deoxycortisol concentrations. Cells were transformed with Twin_11B1 and pGro12 and incubated with 11-deoxycortisol for 48 h in TB medium containing 1 mM δ-Ala (white bars) that has been additionally supplemented with 1 mM IPTG and 4 mg/mL arabinose for induction (grey bars). Steroid specific fluorescence was generated by the described assay and measured in relative units (R.F.U.) at λ_{ex} 485 nm and λ_{em} 535 nm. Values represent the mean of triplicates with standard deviations.

Higher substrate concentrations result in an increased activity, which is displayed by an increased fluorescence signal and which was confirmed by HPLC analysis. Steroids were identified by their retention time and relative quantification depicted 20.2 ± 2.9% conversion of 11-deoxycortisol to cortisol at a concentration of 400 μM, which was subsequently chosen for the selection of activity improved mutants.

Generation and screening of a CYP11B1 mutant library

A random CYP11B1 mutant library was created by epPCR applying the sequence of CYP11B1 Pa1 as a template. The emerged sequence variants were cloned into the Twin plasmid and C43(DE3) *E. coli* cells were co-transformed with the mutant Twin_11B1 plasmid library as well as pGro12 and were spread on agar plates. Sequencing of 10 randomly picked clones revealed a mutational frequency of 2.83 base exchanges per kilobase. Approximately 1000 clones were screened with the fluorescence assay for enhanced hydroxylation activity towards 11-deoxycortisol. From these, 53 clones, which exhibited an at least 1.5-fold higher fluorescence signal than the CYP11B1 Pa1 control incubated on the same plate, were re-screened in triplicates. For 3 mutants, which still showed an average increase of the fluorescence signal by more than the 1.5-fold, an activity increase between the 1.7- and 2.4-fold under screening conditions could be confirmed by HPLC. Sequencing identified the amino acid replacements H171L, Q166R/L271M and S168R/M286I/Q315E, respectively, for the selected clones. For the identification of the residues which cause the increased activity in case of the double and triple mutant all observed exchanges were introduced separately into CYP11B1 Pa1 by site-directed mutagenesis and analyzed for their whole-cell activity in microtiter plates using HPLC. Their activities regarding cortisol formation in comparison with the CYP11B1 Pa1 enzyme are shown in Figure 8.

The amino acid exchange Q166R, which occurred in the double mutant Q166R/L271M, reduced the CYP11B1 activity to about 50% when introduced individually. The removal of this unfavorable exchange leading to the single mutant L271M increased activity of CYP11B1 Pa1 approximately 3.4-fold. Two of the exchanges of the triple mutant, S168R and Q315E, did not show any or only a slightly beneficial effect on product formation, while the third exchange, M286I, alone enhanced product formation by a factor of 2.7 compared with CYP11B1 Pa1, which represents an additional slight increase compared with the parental triple mutant. The best mutant, L271M, was chosen for subsequent experiments. No significant differences in expression level in the microtiter plate were observed between CYP11B1 Pa1 and L271M, which were synthesized with 57 and 69 nmol∗L^{-1}, respectively. This

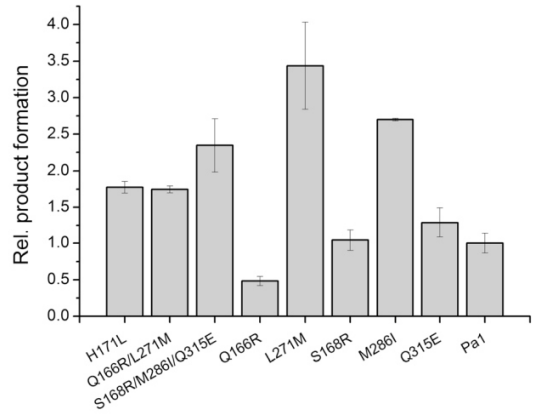

Figure 8 Relative cortisol formation of CYP11B1 enzyme variants in microtiter plates evaluated by HPLC. Mutants H171L, Q166R/L271M and S168R/M286I/Q315E were selected in the fluorescence based screening of a random mutant library. All other mutants were created by site-directed mutagenesis. Conversion took place for 48 h as described under Material and methods with 400 μM 11-deoxycortisol and cortisol formation was evaluated by HPLC and is presented in a relative manner as mean of triplicates with respective standard deviation. Product formation of CYP11B1 Pa1 was assigned as 1.

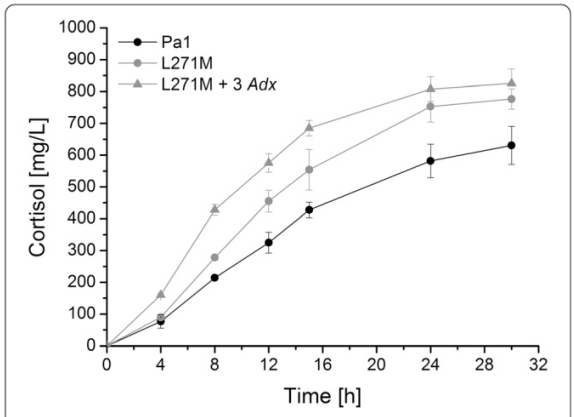

Figure 9 Time-dependent whole-cell cortisol formation by the parental CYP11B1 enzyme (Pa1) and the selected mutant L271M in shaking flasks. Pa1 (black circles) was expressed from the Twin_11B1 plasmid carrying 1 *Adx* copy, while L271M was expressed from plasmids with either 1 *Adx* (grey cirlces) or 3 *Adx* (grey triangles) copies. Reactions were performed after a 21-h expression period in TB medium with resting cells in presence of 6% DMSO and 3 mM 11-deoxycortisol. Extracted steroids were analyzed by RP-HPLC. Values represent the mean of three conversion experiments conducted in parallel with respective standard deviation.

excludes enzyme stability as underlying cause for the enhanced product yield.

Large-scale cortisol production by the selected CYP11B1 mutant

In order to verify the reliability of the microtiter plate screening for activities in larger scale, mutant L271M was analyzed for its time-dependent capacity of cortisol formation with resting cells in shaking flasks as described in Material and methods. As expected, the mutant turned out to be more productive than its parent CYP11B1 Pa1 and its application enabled an increase of the final product concentration from 631 to 777 $mg*L^{-1}$ (Figure 9). The expression level of L271M in shaking flasks was ascertained as approximately 240 $nmol*L^{-1}$ and is thus comparable to the expression level of CYP11B1 Pa1. Moreover, we combined the mutant in a plasmid with 3 *Adx* copies, as this number maximized Adx synthesis in the preceding experiments. This combination revealed an additive effect during the initial phase of the reaction and the productivity of L271M over the first 12 h was stimulated by the enhanced Adx availability in a dimension comparable to the effect of 3 *Adx* copies on CYP11B1 Pa1, leading from 38 to 48 $mg*L^{-1}*h^{-1}$ (Figure 9).

Discussion

CYP11B1, which synthesizes cortisol from 11-deoxycortisol in the human adrenal cortex, also exposes a great potential as biocatalyst in the industrial synthesis of cortisol, a pharmaceutically and thus commercially important steroid due to its anti-inflammatory and immunosuppressive effects. In this work, the successful reconstitution of a CYP11B1 system in a recombinant *E. coli* whole-cell biocatalyst, which is capable of forming cortisol from 11-deoxycortisol by 11β-hydroxylation, is presented. Because of the selectivity of 11-deoxycortisol hydroxylation by CYP11B1 in combination with a high volumetric productivity and the absence of side-product formation by *E. coli*, the developed system provides distinct advantages over other bioprocesses that have been established for cortisol production. Current industrial synthesis from 11-deoxycortisol by means of an 11β-hydroxylation via biotransformation by the fungus *C. lunata* [4] is accompanied by side-product formation [6], while alternative systems in recombinant yeast suffer from poor efficiencies [17-20]. Figure 10 compares the volumetric productivities [$mg*L^{-1}*d^{-1}$] of publically accessible systems for cortisol production by CYP11B1 dependent biotransformation. Our new *E. coli* based system, which enabled a maximum volumetric productivity of 843 $mg*L^{-1}*d^{-1}$, exhibits a productivity which is nearly one order of magnitude higher than the best value published. In order to realize this, strategies have been developed to successfully target factors which frequently limit the application of P450s in industrial biocatalysis.

Recent studies hint at a favorable effect of N-terminal replacements of hydrophobic amino acids by positively charged ones for the expression and stability of eukaryotic P450s in *E. coli* [30,38]. The introduction of a

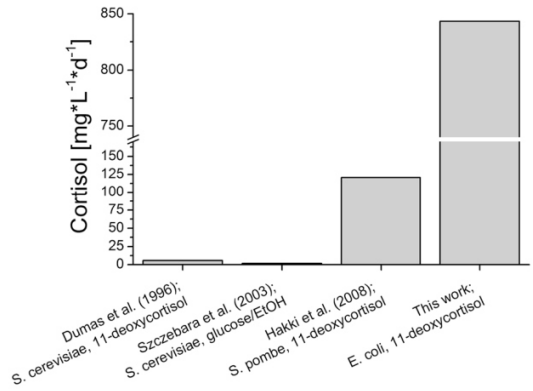

Figure 10 Comparison of the volumetric productivities of publically available CYP11B1 dependent whole-cell biocatalysts for cortisol production. The respective publications as well as the host organisms and the substrates are indicated.

hydrophilic amino acid near the N-terminus of CYP11B1 by site-directed mutagenesis of position 23 from glycine to arginine greatly enhanced the expression level, while maintaining catalytic activity, and thus improved initial productivity as well as the final product yield by a factor of 2.6.

After successfully increasing CYP11B1 expression, our second aim was an optimization of the electron flux from cellular NADPH towards CYP11B1 by engineering the redox chain which is reconstituted in *E. coli* by co-expressing AdR and Adx with the P450 from a tricistronic plasmid. Especially the electron transfer from Adx to the P450 is known to be an activity limiting step in P450 catalysis, which can be at least partially rescued by truncation and mutagenesis of Adx [31,32] or by increasing the Adx availability in the system which could already be demonstrated for CYP11B1 in in-vitro experiments with purified enzymes [21,39], as well as in recombinant cell cultures [40] and yeast [19]. In-vitro studies proof a dependency of CYP11B1 activity on Adx concentration following the Michealis-Menten equation at a stable AdR concentration [21] and describe that upon excess of Adx the maximum CYP11B1 hydroxylation activity is already achieved at molar ratios of AdR/CYP11B1 lower than 1 [41-43]. Additionally, investigations of a *class I* P450 system reconstituted in *E. coli* show a ratio for P450: ferredoxin:reductase of 1:6:1 under expression conditions optimized for substrate hydroxylation with CYP105A1 [44]. Therefore, we decided to engineer the ratio of *CYP11B1* to *Adx* expression and introduced additional copies of the Adx cDNA into the polycistronic expression unit in order to enhance *Adx* expression on transcriptional level and to reduce the rate-limiting factor of Adx availability in the *E. coli* biocatalyst. The approach succeeded to improve the productivity to a maximum possible within the polycistronic transcriptional strategy. Initial productivity was greatly accelerated and the final product concentration was increased by a factor of 1.4 at the maximum Adx level. This indicates that the limitations of the whole-cell activity caused by a reduced Adx availability were successfully overcome by the presented approach. Moreover, the high Adx concentration might have a general positive effect on the viability of the whole-cells as the [2Fe-2S]-cluster can function as a scavenger by trapping reactive oxygen species [45] which can be formed in the course of the P450 catalytic cycle [11].

In a parallel approach for the optimization of the whole-cell activity, new CYP11B1 variants with an increased activity of cortisol formation from 11-deoxycortisol were generated. Directed evolution, which consists of one or several cycles of enzyme mutagenesis, screening for the desired enzyme properties and selection of favorable mutants, represents a classical tool for such kind of enzyme engineering towards improved catalytic efficiencies, reduced uncoupling, altered selectivity or substrate specificity [46]. However, the crucial step in the establishment of a system for directed evolution is the development of a screening assay which enables a sensitive and accurate selection of the desired enzyme features with a high throughput. In order to meet these criteria, we performed a down-scale of the steroid-converting *E. coli* system to microtiter plates and employed a fluorescence based activity assay [35] in a robot-assisted manner. This assay has already been successfully applied for the improvement of the catalytic activity of CYP106A2 from *Bacillus megaterium* towards its steroidal substrates 11-deoxycortisol and progesterone [37,47]. It is premised on the fluorescence developed by steroids in an acidic, hygroscopic environment whose intensity can vary between substrate and product of a hydroxylation reaction. This is true for the transformation of 11-deoxycortisol to cortisol in the presented *E. coli* system with increasing fluorescence intensity upon formation of cortisol which proofs the applicability of the assay for the detection of activity enhanced mutants. Because of the little information about structure-activity relation of adrenocortical P450s in the literature, we conducted a random PCR mutagenesis of the entire CYP11B1 gene and examined the arising mutant library with the fluorescence screening test. We were able to select 3 mutants, H171L, Q166R/L271M and S168R/M286I/Q315E, that exhibited an approximately 2-fold increased activity in the microtiter scale and retained selectivity as shown by HPLC measurements. The activity improvements measured via fluorescence screening in the microtiter plates were supported by HPLC analysis underlining reliability of the screening procedure. The predicted localizations of mutated residues are summarized in Table 1.

The subsequent individual analysis of amino acid exchanges from the double and triple mutant revealed

Table 1 Localization of amino acid exchanges of CYP11B1 mutants with increased activity

Amino acid exchange	Localization
H171L	E'-helix, protein surface
Q166R	E'-helix, protein surface
L271M	H-helix, protein surface
S168R	E'-helix, protein surface
M286I	I-helix
Q315E	J-helix, protein surface

Localization of the respective residues discovered during the screening of a random CYP11B1 library for mutants with increased 11β-hydroxylation activity towards 11-deoxycortisol was deduced from the latest homology model of CYP11B1 [25].

activity impairing effects of mutations that introduce a positive charge into the E'-helix (S168R, Q166R), while the elimination of a potentially positively charged residue (H171L) increases the CYP11B1 activity. The removal of the unfavorable mutations further enhanced cortisol formation up to 3.4-fold compared with Pa1, when using L271M or M286I. L271M, a conservative exchange which is predicted to be localized in the H-helix on the protein surface (Figure 11), was identified as the exchange that contributes most efficiently to CYP11B1 activity. As the H-helix represents the link between the I-helix which traverses the active site and the mobile F/G-loop, which is involved in substrate access to the active site [48], the slight alteration in the residue's physicochemical properties might positively influence the flexibility of CYP11B1. M286I, the second activity increasing amino acid replacement, resides in the I-helix (Figure 11). It is not part of the active site pocket but can be assumed to strengthen structural integrity

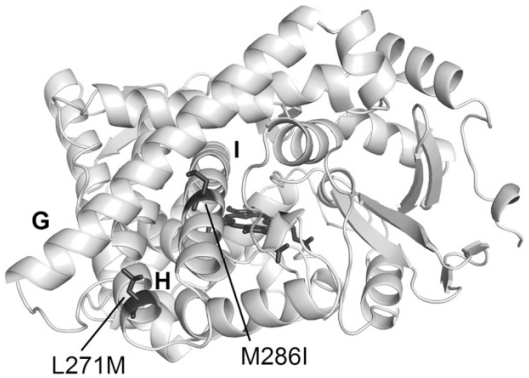

Figure 11 Localization of amino acid exchanges of CYP11B1 that increase hydroxylation activity towards 11-deoxycortisol. L271M is located on the protein surface in the H-helix, while M286I resides in the I-helix. Helices are labeled with their eponymous letter. The structure represents the latest homology model [25] and figures were created with PyMOL Builds. Mutated amino acids and the heme are highlighted in black.

of this core element. Q315E leads to the introduction of a charged group in the J-helix on the protein surface and does not have significant impact on the CYP11B1 activity which identifies M286I as determining mutation for the selection of the triple mutant. An upscale of the system from microtiter plates to shaking flasks using the most efficient mutant, L271M, could successfully reproduce the improvement of the whole-cell activity and enhanced the initial productivity as well as the final product concentration in comparison to CYP11B1 Pa1. The proposed screening procedure can thus be regarded as reliable for the optimization of large-scale processes by laboratory evolution. The mutagenesis approach can be combined with the strategy of engineering the redox partner co-expression leading to additive effects (Figure 9). However, the activity of all systems flattens after approximately 24 h and cannot be rescued with the approaches presented in this work. This points at the necessity of further optimization on the levels of process and strain engineering.

Conclusions

Taken together, we report the establishment of an *E. coli* based biocatalyst for cortisol production by a heterologous CYP11B1 system, which enables a maximum productivity of 0.84 $g*L^{-1}*d^{-1}$ under simple shaking flask conditions and thus clearly meets efficiency requirements for potential application in the pharmaceutical industry [49,50]. In total, our optimization approaches could increase the cortisol yield by a factor of 3.7. The presented strategy to overcome activity limits due to low protein ratios of Adx to CYP11B1 can be transferred to other biotechnologically interesting P450 redox chains in whole-cell application as Adx represents an efficient electron transfer partner not only for mitochondrial but also for microsomal and bacterial P450s [31,51]. The successful establishment of an accurate and fast screening system for CYP11B1 activity in combination with new structure-activity insights from the first mutant generation can be used for further directed evolution of the enzyme [52]. The system might additionally be applicable for the screening of CYP11B1 inhibitors, which can be important drugs for the treatment of for example Cushing's syndrome [53].

Material and methods

Chemicals and enzymes

All chemicals and reagents were purchased from standard sources in the highest purity available. Restriction enzymes were obtained from New England Biolabs (Ispwich, MA, USA), *Pfu* polymerase from Promega (Madison, WI, USA), and FastLink Ligase from Epicentre Biotechnologies (Chicago, IL, USA).

Bacterial strains and cultivation

Plasmid construction was performed with *E. coli* TOP10F' (*F-mcrA (mrrhsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*). All experiments involving protein expression and steroid conversion were conducted with *E. coli* C43(DE3) (*F– ompT gal hsdSB (rB- mB-) dcm Ion* λ). Transformation was carried out by electroporation and transformed cells were stored on agar plates supplemented with the appropriate antibiotics (100 μg/mL ampicillin and/or 50 μg/mL kanamycin) at 4°C.

Plasmid construction and mutagenesis

All methods of molecular biology were performed according to standard protocols described by [54]. The plasmid Twin_11B1 served as template for the preparation of all further plasmids. It is based on the pET-17b expression vector (MerckMillipore Novagen, Darmstadt, Germany), which enables a selection on ampicillin containing medium, and carries the cDNAs of human CYP11B1 cloned into the vector via NdeI/HindIII, bovine AdR via HindIII/KpnI and bovine Adx_{1-108} via KpnI/EcoRI in a polycistrionic transcription unit [55]. CYP11B1 was modified for expression in *E. coli* as described by [21]. Additionally, residue 29 in the *E. coli* adapted sequence (corresponding to residue 52 in the full length sequence) is mutated from leucine to methionine taking previous activity studies in recombinant fission yeast [19] into account. All utilized primers are shown in Additional file 1: Table S1.

Insertion of additional gene copies

In order to consecutively insert additional copies of the Adx cDNA into the Twin_11B1 plasmid behind the preexisting copy, the following strategy, which takes advantage of restriction site compatibility of EcoRI and MfeI, was pursued referring to [34]. In a first step, Twin_11B1 was used as a template to amplify the Adx cDNA by PCR including its ribosomal binding site and to introduce MfeI and XhoI restriction sites at the 5′ and 3′ end, respectively. The PCR product was then digested by MfeI and XhoI and ligated into the EcoRI/XhoI digested Twin_11B1 plasmid resulting in a plasmid carrying 2 Adx copies. In additional cycles of restriction and ligation further copies were inserted. In subsequent cloning attempts inserts of different Adx copy numbers could then be introduced into new plasmids by restriction and ligation via KpnI and EcoRI.

Site directed mutagenesis of CYP11B1

Targeted exchange of single amino acids was undertaken by QuikChange® mutagenesis with *Pfu* polymerase following manual instructions form Agilent Technologies (Santa Clara, USA).

Random mutagenesis of CYP11B1

Random mutagenesis of CYP11B1 was conducted by error prone PCR employing the GeneMorph II random mutagenesis kit (Stratagene, La Jolla, CA, USA). pET17b_hCYP11B1, which contains the modified cDNA of human CYP11B1 between the NdeI and HindII restriction sites of its multiple cloning site, was used as a template for the amplification of CYP11B1 with the standard primers T7 and T7term. Parameters for an average mutation frequency of 0–3 mutations per kb were chosen according to the manufacturer's protocol. The PCR product was digested by NdeI and HindIII and ligated into the likewise digested Twin_11B1 plasmid.

Whole-cell biocatalysis in shaking flasks

Protein expression

The synthesis of CYP11B1, AdR and Adx in *E. coli* took place as co-expression with the chaperone genes *GroEL* and *GroES* to ensure proper folding. Protein synthesis was carried out in 2 L Erlenmeyer flasks containing 150 mL TB medium (24 yeast extract technical, 12 g peptone, 4 mL glycerol, 4,62 g KH_2PO_4, 25 g K_2HPO_4 and distilled water ad. 1 L) supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin. The main culture was inoculated from an overnight culture of *E. coli* C43 (DE3), that had been freshly co-transformed with the respective variant of Twin_11B1 and the chaperone vector pGro12 (kanamycin resistance and arabinose inducible promoter) [23], and was grown at 37°C and 210 rpm (Excella 25 shaker incubator, New Brunswick Scientific, Eppendorf, Ensfield, CT, USA). When an $OD_{600\ nm}$ of 0.5 was reached expression was induced by addition of 1 mM IPTG, 4 mg/mL arabinose, 1 mM of the heme precursor δ-aminolevulinic acid and 50 μg/mL ampicillin. Cultures were further incubated at 27.5°C and 200 rpm for 21 h.

Steroid conversion with resting cells

Subsequent to the expression period cultures were harvested by centrifugation (3200 *g*, 10 min, 18°C) and cells were washed in 50 mM potassium phosphate buffer (pH 7.4). Steroid conversion took place at 27.5°C and 170 rpm in 300-mL baffled flasks using 25 mL of a cell suspension of 25 g wet cell weight (wcw) per L in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 1 mM IPTG, 4 mg/mL arabinose, 1 mM δ-aminolevulinic acid, 50 μg/mL ampicillin and 2% glycerol. The substrate 11-deoxycortisol (17,21-dihydroxypregn-4-ene-3,20-dione) was added from a stock solution in either EtOH, DMSO, 22.5% (m/vol) 2-hydroxypropyl-β-cyclodextrin or a 1:1 (vol:vol) mixture of EtOH and polyethyleneglycol-400. Each agent was added to the

culture in a final concentration of 6% (vol/vol). Samples were taken at defined time points.

Reversed phase HPLC analysis

For product quantification via HPLC, samples were extracted twice with one volume of chloroform. After evaporation of the organic solvent remaining steroids were suspended in acetonitrile and separated on a Jasco reversed phase HPLC system of the LC900 series (Jasco, Groß-Umstadt, Germany) using a 4.6 m × 125 mm NucleoDur C18 Isis Reversed Phase column (Macherey-Nagel, Düren, Germany) with an acetonitril/water gradient (Phase A: 10% acetonitrile, Phase B: 100% acetonitril; 0 min 20% B, 5 min 20% B, 13 min 40% B, 20 min 80% B, 21 min 80% B, 22 min 20% B, 30 min 20% B) at 40°C and a flow rate of 0.8 mL/min. Steroid pattern was monitored by an UV/Vis detector (UV-2 075 Plus, Jasco) at 240 nm.

Screening for improved CYP11B1 activity in microtiter plates

Protein expression and steroid conversion in microtiter plates as well as a fluorescence assay for the selection of CYP11B1 mutants with an improved hydroxylation activity towards 11-deoxycortisol was performed as previously reported [36], but TB medium was additionally supplemented with 50 μg/mL kanamycin and 4 mg/mL arabinose to ensure chaperone synthesis from pGro12 and did not contain a salt solution.

Analysis of protein expression

Cell lysis

For the analysis of CYP11B1 expression levels cells were harvested by centrifugation (4500 *g*, 20 min, 4°C), suspended in lysis buffer (50 mM potassium phosphate buffer (pH 7.4), 500 mM sodium-acetate, 0.1 mM EDTA, 1.5% sodium-cholate, 20% glycerol, 1.5% Tween 20, 0.1 mM phenylmethylsulfonylfluorid and 0.1 mM dithioerythritol) and disrupted with an ultrasonic homogenizer (Sonopuls HD 3200, Bandelin, Berlin, Germany). Cell debris were removed by ultracentrifugation (30000 g, 30 min, 4°C; hinac CP75, Hitachi, Tokyo, Japan) and the supernatant was surveyed for recombinant proteins.

Determination of cytochrome P450 concentration

P450 concentration was determined by CO-difference spectroscopy using a molar extinction coefficient of 91 mM^{-1} cm^{-1} as described by [56].

Western blot analysis of Adx synthesis

Cell pellets from 90 μL of the 25g_{wcw}/L cell suspension were suspended in 100 μL of SDS loading buffer (1 M Tris–HCl (pH 6.8), 40% glycerol, 20% SDS, 8% β-mercaptoethanol, 0.1% bromphenol blue) and boiled for 10 min in a water bath. Aliquots of 12 μL were separated by SDS-PAGE according to [57]. 8 μL of a 5 μM solution of purified bovine $Adx_{4–108}$ in SDS loading buffer were applied as positive control and Protein Marker IV from PEQLAB (Erlangen, Germany) served as molecular weight standard. Proteins were blotted onto a hybond™ ECL™ nitrocellulose membrane (Amersham, GE Healthcare, UK) with the help of a semi-dry transfer system (Trans-Blot SD, Bio-Rad, Munich, Germany) and the membrane was blocked by incubation with 3% milk powder in TBS (50 mM Tris–Cl pH 7.4, 200 mM NaCl, 0.1% Tween 20) overnight. The membrane was washed 3 times for 10 minutes in fresh TBS and was incubated for 2 h with the respective polyclonal antiserum from rabbit diluted in TBS. Subsequent to 3 further washing steps in TBS the membrane was incubated for 2 h with a dilution of the horseradish peroxidase-linked goat anti rabbit IgG secondary antibody (Dako, Glostrup, Denmark) in TBS. After washing the membrane 3 times for 5 minutes with PBS (10 mM potassium phosphate buffer pH 7.4, 150 mM NaCl) staining of the antigen-antibody-complexes took place by adding 5 mg 4-chloro-1-naphtol dissolved in 2 mL ethanol and 10 μL 30% H_2O_2 in 25 mL PBS. Relative intensity of the protein bands was measured with Image Lab 3.0 from BioRad (München, Germany).

Additional file

Additional file 1: Table S1. Primers used in this work with sequence and purpose of application. Restriction sites are marked with bold letters, introduced nucleotide exchanges are underlined.

Abbreviations

AdR: Adrenodoxin reductase; Adx: Adrenodoxin; CYP11B1: 11β-hydroxylase; *E. coli*: *Escherichia coli*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LS carried out the presented experiments and drafted the manuscript. SA established the whole-cell steriod conversion and screening conditions. AH constructed the tricistronic expression plasmids. FH participated in the design of the study, interpretation of the results and manuscript drafting. NK established the expression of CYP11B1 in *E. coli*. RB participated in the interpretation of the results and assisted in manuscript drafting. All authors read and approved the final manuscript.

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References

1. Clark JK, Schrader WT, O'Malley BW. Mechanism of steroid hormones. In: Wilson JD, Foster DW, editors. Williams textbook of endocrinolog. Philadelphia: WB Sanders Co; 1992. p. 35–90.

2. Sambamurthy K, Kar A. Pharmaceutical biotechnology. New Delhi: New Age International (P) Ltd. Publishers; 2006.
3. Van Beilen JB, Duetz WA, Schmid A, Withold B. Practical issues in the application of oxygenases. Trends Biotechnol. 2003;21:170–7.
4. Petzoldt K, Annen K, Laurent H, Wiechert R. Process for the preparation of 11-beta-hydroxy steroids. Berlin, Germany: US Patent 1982, Schering Aktiengesellschaft; 1982.
5. Jänig G-R, Pfeil G, Müller-Frohne M, Riemer H, Henning M, Schwarze W, et al. Steroid 11β-Hydroxylation by a fungal microsomal cytochrome P450. J Steroid Biochem Mol Biol. 1992;43:1117–23.
6. Suzuki K, Sanga K-I, Chikaoka Y, Itagaki E. Purification and properties of cytochrome P-450 (P-450_{Lun}) catalyzing steroid 11β-hydroxylation in *Curvularia lunata*. Biochim Biophys Acta. 1993;1203:215–23.
7. Bureik M, Lisurek M, Bernhardt R. The human steroid hydroxylases CYP11B1 and CYP11B2. Biol Chem. 2002;383:1537–51.
8. Curnow KM, Tusie-Luna MT, Pascoe L, Natarajan R, Gu JL, Nadler JL, et al. The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. Mol Endocrinol. 1991;5:1513–22.
9. Denner K, Doehmer J, Bernhardt R. Cloning of CYP11B1 and CYP11B2 from normal human adrenal and their functional expression in COS-7 and V79 Chinese hamster cells. Endocr Res. 1995;21:443–8.
10. Lisurek M, Bernhardt R. Modulation of aldosterone and cortisol synthesis on the molecular level. Mol Cell Endocrinol. 2004;215:149–59.
11. Bernhardt R. Cytochromes P450 as versatile biocatalysts. J Biotechnol. 2006;124:128–45.
12. Bernhardt R, Urlacher VB. Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. Appl Microbiol Biotechnol. 2014;98:6185–203.
13. Sakaki T. Practical application of cytochrome P450. Biol Pharm Bull. 2012;35:844–9.
14. Urlacher VB, Girhard M. Cytochrome P450 monooxygenases: an update on perspectives for synthetic application. Trends Biotechnol. 2012;30:26–3.
15. Hannemann F, Bichet A, Ewen KM, Bernhardt R. Cytochrome P450 systems - biological variations of electron transport chains. Biochim Biophys Acta. 2007;1770:330–44.
16. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B. Industrial biocatalysis today and tomorrow. Nature. 2001;409:258–68.
17. Dragan C-A, Zearo S, Hannemann F, Bernhardt R, Bureik M. Efficient conversion of 11-deoxycortisol to cortisol (hydrocortisone) by recombinant fission yeast *Schizosaccharomyces pombe*. FEMS Yeast Res. 2005;5:621–5.
18. Dumas B, Cauet B, Lacour T, Gegryse G, Laruelle L, Ledoux C, et al. 11β-Hydroxylase activity in recombinant yeast mitochondria. Eur J Biochem. 1996;238:495–504.
19. Hakki T, Zearo S, Dragan C-A, Bureik M, Bernhardt R. Coexpression of redox partners increases the hydrocortisone (cortisol) production efficiency in CYP11B1 expressing fission yeast *Schizosaccharomyces pombe*. J Biotechnol. 2008;133:351–9.
20. Szczebara FM, Chandelier C, Villeret C, Masurel S, Bourot S, Duport C, et al. Total biosynthesis of hydrocortisone from a simple carbon source in yeast. Nature Biotechnol. 2003;21:143–9.
21. Zöllner A, Kagawa N, Waterman MR, Nonaka Y, Takio K, Shiro Y, et al. Purification and functional characterization of human 11β-hydroxylase expressed in *Escherichia coli*. FEBS J. 2008;275:799–810.
22. Miroux B, Walker JE. Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high level. J Mol Biol. 1996;260:289–98.
23. Nishihara K, Kanemori M, Kitagawa M, Yanagi H, Yura T. Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroELGroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli*. Appl Environ Microbiol. 1998;64:1694–9.
24. Arase M, Waterman MR, Kagawa N. Purification and characterization of bovine steroid 21-hydroxylase (P450c21) efficiently expressed in *Escherichia coli*. Biochem Biophys Res Commun. 2006;344:400–5.
25. Hobler A, Kagawa N, Hutter MC, Hartmann MF, Wudy SA, Hannemann F, et al. Human aldosterone synthase: recombinant expression in *E. Coli* and purification enables a detailed biochemical analysis of the protein on the molecular level. J Steroid Biochem Mol Biol. 2012;132:57–65.
26. Kagawa N, Hiri H, Waterman MR, Yoshioka S. Characterization of stable human aromatase expressed in E. coli. Steroids. 2004;69:235–43.
27. Walton AZ, Stewart JD. Understanding and Improving NADPH-Dependent Reactions by Nongrowing *Escherichia coli* Cells. Biotechnol Prog. 2004;20:403–11.
28. Kawamoto T, Mitsuuchi Y, Toda K, Miyahara K, Yokoyama Y, Nakao K, et al. Cloning of cDNA and genomic DNA for human cytochrome P-$450_{11\beta}$. FEBS Lett. 1990;269:345–9.
29. Mornet E, Dupont J, Vitek A, White PC. Characterization of Two genes encoding human steroid 11β-hydroxylase (P-$450_{11\beta}$). J Biol Chem. 1989;35:20961–7.
30. Kagawa N, Cao Q, Kusano K. Expression of human aromatase (CYP19) in *Escherichia coli* by N-terminal replacement and induction of cold stress response. Steroids. 2003;68:205–9.
31. Ewen KM, Kleser M, Bernhardt R. Adrenodoxin: The archetype of vertebrate-type [2Fe-2S] cluster ferredoxins. Biochim Biophys Acta. 1814;2011:111–25.
32. Grinberg AV, Hannemann F, Schiffler B, Müller J, Heinemann U, Bernhardt R. Adrenodoxin: structure, stability, and electron transfer properties. Proteins. 2000;40:590–612.
33. Renault H, Bassard J-E, Hamberger B, Werck-Reichhardt D. CytochromeP450-mediated metabolic engineering: current progress and future challenges. Curr Opin Plant Biol. 2014;19:27–34.
34. Blachinsky E, Marbach I, Cohen R, Grably MR, Engelberg D. Procedure for controlling number of repeats, orientation, and order during cloning of oligonucleotides. Biotechniques. 2004;36:933–6.
35. Appel D, Schmid RD, Dragan C-A, Bureik M, Urlacher VB. A fluorimetric assay for cortisol. Anal Bioanal Chem. 2005;383:182–6.
36. Hannemann F, Virus C, Bernhardt R. Design of an *Escherichia coli* system for whole cell mediated steroid synthesis and molecular evolution of steroid hydroxylases. J Biotechnol. 2006;124:172–81.
37. Virus C, Bernhardt R. Molecular Evolution of a Steroid Hydroxylating Cytochrome P450 Using a Versatile Steroid Detection System for Screening. Lipids. 2008;43:1133–41.
38. Chen C-K, Leung SSF, Guilbert C, Jacobson MP, McKerrow JH, Podust LM. Structural Characterization of CYP51 from Trypanosoma cruzi and Trypanosoma brucei Bound to the Antifungal Drugs Posaconazole and Fluconazole. PLoS Negl Trop Dis. 2010;6:e651.
39. Strushkevich N, Gilep AA, Shen L, Arrowsmith CH, Edwards AM, Usanov SA, et al. Structural insights into aldosterone synthase substrate specificity and targeted inhibition. Mol Endocrinol. 2013;27:315–24.
40. Cao P-R, Bernhardt R. Interaction of CYP11B1 (cytochrome P-$450_{11\beta}$) with CYP11A1 (cytochrome P-450_{scc}) in COS-1 cells. Eur J Biochem. 1999;262:720–6.
41. Lambeth JD, Seybert DW, Lancaster JR, Salerno JC, Kamin H. Steroidogenic electrone transport in adrenal cortex mitochondria. Mol Cell Biochem. 1982;45:13–31.
42. Seybert DW, Lambeth JD, Kamin H. The participation of a second molecule of adrenodoxin in cytochrome P-450-catalyzed 11β-hydroxylation. J Biol Chem. 1978;253:8355–8.
43. Seybert DW, Lancaster JR, Lambeth JD, Kamin H. Participation of the membrane in the side chain cleavage of cholesterol. J Biol Chem. 1979;254:12088–98.
44. Janocha S, Bernhardt R. Design and characterization of an efficient CYP105A1-based whole-cell biocatalyst for the conversion of resin acid diterpenoids in permeabilized *Escherichia coli*. Appl Microbiol Biotechnol. 2013;97:7639–49.
45. Imlay JA. Iron-sulphur clusters and the problem with oxygen. Mol Microbiol. 2006;59:1073–82.
46. Gillam EMJ. Engineering Cytochrome P450 Enzymes. Chem Res Toxicol. 2008;21:220–31.
47. Nguyen KT, Virus C, Günnewich N, Hannemann F, Bernhardt R. Changing the Regioselectivity of a P450 from C15 to C11 Hydroxylation of Progesterone. ChemBioChem. 2012;13:1161–6.
48. Peterson JA, Graham SE. A close family resemblance: the importance of structure in understanding cytochromes P450. Structure. 1996;6:1079–85.
49. Julsing MK, Cornelissen S, Bühler B, Schmid A. Heme-iron oxygenases: powerful industrial biocatalysts? Curr Opin Chem Biol. 2008;12:177–86.
50. Straathof AJJ, Panke S, Schmid A. The production of fine chemicals by biotransformations. Curr Opin Biotechnol. 2002;13:548–56.
51. Pechurskaya TA, Harnastai IN, Grabovec IP, Gilep AA, Usanov SA. Adrenodoxin supports reactions catalyzed by microsomal steroidogenic cytochrome P450s. Biochem Biophys Res Commun. 2007;353:598–604.
52. Valetti F, Gilardi G. Improvement of biocatalysts for industrial and environmental purposes by saturation mutagenesis. Biomolecules. 2013;3:778–811.
53. Hakki T, Bernhardt R. CYP17- and CYP11B-dependent steroid hydroxylases as drug development targets. Pharmacol Ther. 2006;111:27–52.

54. Sambrook J, Russel DW. Molecular cloning. 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001.
55. Ringle M, Khatri Y, Zapp J, Hannemann F, Bernhardt R. Application of a new versatile electron transfer system. for cytochrome P450-based *Escherichia coli* whole-cell bioconversions. Appl Microbiol Biotechnol. 2013;97:7741–54.
56. Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. J Biol Chem. 1964;239:2379–85.
57. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–5.

2.2 Brixius-Anderko *et al.* 2015

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A CYP21A2 based whole-cell system in *Escherichia coli* for the biotechnological production of premedrol

Simone Brixius-Anderko[1], Lina Schiffer[1], Frank Hannemann[1], Bernd Janocha[2] and Rita Bernhardt[1*]

Abstract

Background: Synthetic glucocorticoids like methylprednisolone (medrol) are of high pharmaceutical interest and represent powerful drugs due to their anti-inflammatory and immunosuppressive effects. Since the chemical hydroxylation of carbon atom 21, a crucial step in the synthesis of the medrol precursor premedrol, exhibits a low overall yield because of a poor stereo- and regioselectivity, there is high interest in a more sustainable and efficient biocatalytic process. One promising candidate is the mammalian cytochrome P450 CYP21A2 which is involved in steroid hormone biosynthesis and performs a selective oxyfunctionalization of C21 to provide the precursors of aldosterone, the main mineralocorticoid, and cortisol, the most important glucocorticoid. In this work, we demonstrate the high potential of CYP21A2 for a biotechnological production of premedrol, an important precursor of medrol.

Results: We successfully developed a CYP21A2-based whole-cell system in *Escherichia coli* by coexpressing the cDNAs of bovine *CYP21A2* and its redox partner, the NADPH-dependent cytochrome P450 reductase (*CPR*), via a bicistronic vector. The synthetic substrate medrane was selectively 21-hydroxylated to premedrol with a max. yield of 90 mg L^{-1} d^{-1}. To further improve the biocatalytic activity of the system by a more effective electron supply, we exchanged the CPR with constructs containing five alternative redox systems. A comparison of the constructs revealed that the redox system with the highest endpoint yield converted 70 % of the substrate within the first 2 h showing a doubled initial reaction rate compared with the other constructs. Using the best system we could increase the overall yield of premedrol to a maximum of 320 mg L^{-1} d^{-1} in shaking flasks. Optimization of the biotransformation in a bioreactor could further improve the premedrol gain to a maximum of 0.65 g L^{-1} d^{-1}.

Conclusions: We successfully established a CYP21-based whole-cell system for the biotechnological production of premedrol, a pharmaceutically relevant glucocorticoid, in *E. coli* and could improve the system by optimizing the redox system concerning reaction velocity and endpoint yield. This is the first step for a sustainable replacement of a complicated chemical low-yield hydroxylation by a biocatalytic cytochrome P450-based whole-cell system.

Keywords: Methylprednisolone, Medrane, CYP21A2, Cytochrome P450, Whole-cell biocatalysis, *E. coli*, Etp1^{fd}, Arh1, CPR, Adx, Steroid

Background

Since the 1950's, the development of synthetic glucocorticoids is of growing interest with the aim to substitute the natural steroid hormone hydrocortisone as therapeutical compound. The superficial aim is to reduce severe hydrocortisone induced side effects, such as the disturbance of the electrolyte homeostasis, and to synthesize molecules with increased anti-inflammatory effects [1]. Based upon the artificial hydrocortisone derivative prednisolone the highly effective compound medrol (6-methylprednisolone) was developed by addition of a methyl group at carbon atom 6. Medrol turned out to have a far higher glucocorticoid activity than hydrocortisone without a comparative increase of electrolyte

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activity [2]. Medrol was synthesized from its precursor premedrol via a simple to perform biotechnological 1,2 dehydrogenation [3]. Today, medrol is a widespread drug in the treatment of autoimmune diseases, allergic reactions, multiple sclerosis and rheumatic arthritis [4]. Therefore, the demand for this pharmaceutically highly relevant glucocorticoid is still increasing. One bottleneck during contemporary premedrol and, therefore, medrol production is the hydroxylation of carbon atom 21. The chemical introduction of a hydroxyl group into a steran scaffold consists of a multistep-synthesis with the necessity to apply protecting groups and toxic compounds like iodine [5]. As by-products occur after each reaction step, a time consuming chromatographic purification is necessary, which leads to a reduced overall yield and a low efficiency factor [6, 7]. With regard to a more sustainable and less polluting production process and a regio- and stereoselective oxyfunctionalization at C21, the focus has shifted from the chemical process to an enzyme based biotechnological production of medrol and its precursor. A promising candidate for the enzymatic reaction is the mammalian cytochrome P450 21-hydroxylase (CYP21A2), which is a member of the cytochrome P450 (CYP, P450) superfamily. CYP21A2 is a protein of the endoplasmic reticulum and plays a crucial role in steroid hormone biosynthesis by providing the precursors of the most important mineralocorticoid, aldosterone, and the main glucocorticoid, cortisol, via a highly selective 21-hydroxylation, which is ensured by a unique amino acid arrangement within the active site [8–10]. A sufficient electron supply for the hydroxylation reaction is realized by its natural redox partner, the NADPH-dependent cytochrome P450 reductase (CPR), a membrane bound protein as well [11, 12]. Cytochromes P450 are external monooxygenases and exhibit, when reduced and in complex with CO, a unique absorption maximum at 450 nm due to the cysteinate coordinated heme group at the active site [13]. Their ability to functionalize molecular oxygen empowers them to catalyze a broad range of reactions, such as hydroxylations and even a chemically difficult to perform C–C bond cleavage. Apart from steroid hormone biosynthesis, they act as main detoxifying enzymes in the liver and are, therefore, involved in xenobiotics and drug metabolism. P450s are able to convert a great variety of substrates like steroids, terpenes as well as fatty acids, which shows their high potential as versatile biocatalysts [14, 15]. Since the 1960s, cytochromes P450 are crucially involved in the glucocorticoid synthesis in large scale by fermentation of species of the fungus *Curvularia,* whose later characterized P450 system was shown to be able to convert 11-deoxycortisol to cortisol [16–18]. In 2003, the application of a modified *Saccharomyces cerevisiae* strain was published, which performs cortisol production from a simple carbon source [19]. Aside from genetic manipulation of the yeast's ergosterol synthesis pathway, CYPs involved in steroid hormone synthesis, including CYP21A2, were expressed in this yeast strain, which shows the high importance of these enzymes for stereo- and regioselective steroid hydroxylation. To date, CYPs find their application in various industrial production processes and the number is still growing. Efforts are made to design whole-cell systems with single CYPs for the desired reaction in a suitable host to avoid by-products originating from homologous CYP systems like in case of *C. lunata*. Previously, a heterologous human CYP11B1 whole-cell system for a more selective cortisol production has been published [20]. Biocatalysis with whole cells ensures a better protein stability and a supply with costly cofactors, such as NADPH [21]. Concerning mammalian CYP21A2, a whole-cell system in the yeast *Schizosaccharomyces pombe* was already established with human CYP21A2, but with limited success due to a low recombinant protein yield and the host's long lasting generation time [22]. In other approaches to develop CYP based whole-cell systems, *Escherichia coli* emerged to be a suitable host attributed to its short generation time and the lack of intrinsic CYP systems [23]. Functional bovine CYP21A2 could already be successfully expressed with high yield in *E. coli* [24, 25]. These fundamentals were the starting point for our efforts to establish an efficient CYP21A2-based whole-cell system in *E. coli* for the production of premedrol, the precursor of medrol, via a simple one-step hydroxylation at C21. In the following, we demonstrate the successful expression and purification of bovine CYP21A2 and in vitro studies concerning the substrate-protein-interaction, the development of a biotransformation in whole cells and an improvement of the biocatalytic efficiency by using alternative redox systems for a more sufficient electron supply.

Results and discussion

Protein purification and in vitro characterization

Purification of bovine CYP21A2

Since bovine CYP21A2 could already be expressed in *E. coli*, we chose this mammalian CYP21A2 isoform for the initial examination of its suitability for a whole-cell system in *E. coli.* To ascertain whether bovine CYP21A2 is able to convert medrane to premedrol by a stereoselective 21-hydroxylation, the protein had to be expressed and purified according to Arase et al. [24]. The cDNA was subcloned into a pET17b vector, resulting in the vector pET17b_21b. The vector was co-transformed with the vector pGro12, which encodes for the *E. coli* chaperones GroEL/ES, into C43(DE3) cells. After protein

expression, cell lysis took place via sonification for the subsequent purification. The purification was performed via IMAC, anion and cation exchange chromatography and the protein was analyzed by SDS-PAGE, confirming the estimated molecular weight of 54.6 kDa, and by CO difference spectroscopy, which confirmed a correct insertion of the heme prosthetic group by showing a typical absorption maximum at 450 nm without a hint of inactive protein, indicated by a peak at 420 nm (Fig. 1). Taken together, the expression as well as the purification of bovine CYP21A2 was successful with an expression level of max. 398 nmol L^{-1} culture. The purified enzyme was used for further investigations.

In vitro conversion of medrane with purified CYP21A2

In order to prove a selective conversion of medrane to premedrol by a 21-hydroxylation, in vitro assays were carried out to perform a proof-of-principle, as medrane exhibits slight modifications within the steran skeleton compared with the natural CYP21A2 substrates progesterone (P4) and 17OH-progesterone (17OH-P4). Hence, substrate conversion with purified bovine CYP21A2 was performed with the synthetic substrate medrane characterized by its additional methyl group at carbon atom 6 and a hydroxyl group at carbon atom 11 compared with the natural substrate 17OH-P4. HPLC analysis revealed a 21-hydroxylation of medrane and demonstrated a stereoselective production of the wished product premedrol in an efficient biocatalytic one-step hydroxylation without by-product formation (Fig. 2b). It has been shown that bovine CYP21A2 is able to hydroxylate a synthetic substrate of high pharmaceutical interest.

Development of a whole-cell system for a biocatalytic premedrol production

After a conversion of medrane to premedrol by purified CYP21A2 was verified in vitro, the subsequent experiments focused on an establishment of a biotransformation using whole cells, showing advantages like an improved enzyme stability and the supply of costly co-factors by the cell itself [21]. For the development of a whole-cell system, the bicistronic vector p21b_bRED was constructed, carrying the cDNAs for bovine *CYP21A2* and *CPR* (Fig. 3). The natural redox partner CPR is responsible for electron supply in the endoplasmic reticulum through protein interaction. Cells were co-transformed with the respective vector and with the plasmid pGro12, encoding the chaperones GroEL/ES to ensure a proper folding of the membrane proteins CYP21A2 and CPR [26]. Although complex medium is suitable for bacterial cell growth and supports a high expression yield of recombinant proteins, it is inappropriate for whole-cell biotransformations with cytochromes P450 due to inhibitory effects of medium compounds and *E. coli* metabolites such as indole [27, 28]. For this reason, biotransformation with whole cells was performed with resting cells using potassium phosphate buffer as a conversion medium. As the metabolism, including protein biosynthesis, of resting cells is reduced to a minimum, more co-factors like NADPH can be recruited for the CYP dependent reaction [29]. By the addition of glycerol to the reaction mix, an NADPH-regeneration is ensured by the activity of metabolic enzymes like the isocitrate dehydrogenase. For initial examination, medrane was added to the whole-cell system and samples were taken after 24 h for HPLC analysis. Medrane was converted to premedrol by the constructed system without

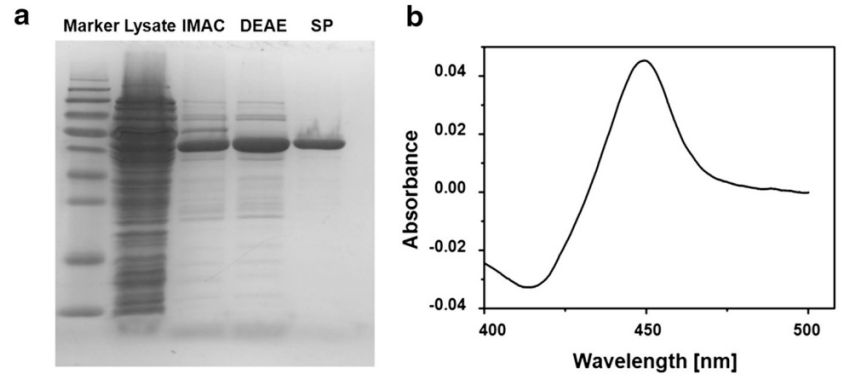

Fig. 1 Protein analysis of purified bovine CYP21A2 after three chromatographic steps. **a** SDS-PAGE analysis and protein staining shows the purification steps of CYP21A2 via IMAC, DEAE- and SP-Sepharose resulting in a single protein band with a size of approx. 55 kDa (estimated weight: 54.6 kDa); **b** Difference spectroscopy of purified bovine CYP21A2 was performed showing a typical absorption maximum at 450 nm in the reduced state in complex with CO

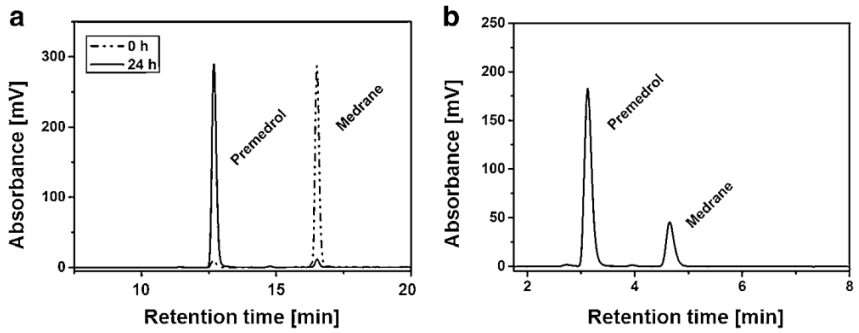

Fig. 2 HPLC chromatograms of the in vivo and in vitro conversion of medrane with bovine CYP21A2 and CPR. **a** Medrane was converted with resting cells of C43(DE3) containing the bovine CYP21A2 isoform and CPR encoding vector p21b_bRED. Samples were taken after 24 h and extracted for HPLC analysis. The steroids were separated by an acetonitril:water gradient. **b** Substrate conversions of medrane were performed with purified bovine CYP21A2 and its redox partner CPR for 30 min. Steroids were extracted and analyzed by HPLC to verify a selective conversion of medrane to premedrol. The steroids were separated by an acetonitril:water gradient, showing the 21-hydroxylated product premedrol

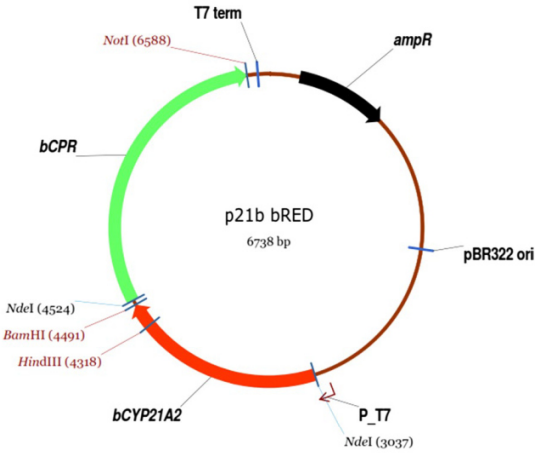

Fig. 3 Vector map of p21b_bRED containing the bicistronic transcription unit for bovine CYP21A2 and the CPR. For the whole-cell biotransformation, this bicistronic vector was constructed, based on the pET17b vector with a T7 promoter and an ampicillin resistance gene. The *CPR* cDNA encodes for the full length protein

by-product formation, verifying the highly specific 21-hydroxylation not only with purified enzymes but also by a biotransformation with whole cells (Fig. 2a). With a substrate concentration of 250 µM, a maximum premedrol yield of 93 mg L^{-1} d^{-1} could be achieved. Since the human CYP21A2 isoform shares a 79 % sequence homology to the bovine one, we additionally tested the human CYP21A2 isoform concerning its ability to produce premedrol. Just as the bovine enzyme, it performs a selective 21-hydroxylation of medrane, but exhibits a poor yield with 40 mg L^{-1} d^{-1} (Additional file 1: Fig. S1). Therefore, we concentrated on the bovine isoform in further experiments to improve the whole-cell system's efficiency.

Improvement of the whole-cell system by using different redox partners

CYP involved whole-cell systems require an efficient cytochrome P450/redox partner interaction to ensure a sufficient electron supply for the hydroxylation reaction [15]. Therefore, a stabilized redox partner synthesis as well as an optimal interaction with the CYP should be intended. In the following, we focused on these aspects by testing various redox systems of different origin.

Selection of alternative redox partners for CYP21

Bovine membrane-bound microsomal CPR represents the naturally occurring redox partner for CYP21 in the endoplasmic reticulum and was therefore the first choice for a co-expression in a whole-cell system. Though, this protein is difficult to produce recombinantly due to its property as a membrane protein. Therefore, efforts were undertaken to search for alternative redox partners of the bovine CYP21A2, which are easily expressed in *E. coli* in sufficient amounts, more stable and solvent resistant. On this account, we concentrated on the participating proteins of three naturally occurring redox systems, each consisting of two components, a ferredoxin or flavodoxin reductase and a ferredoxin as final electron donor for CYP21A2.

First, we focused on the soluble ferredoxin adrenodoxin (Adx), which reconstitutes the mitochondrial electron transfer system together with the membrane associated adrenodoxin reductase (AdR), which receives electrons from NADPH [12, 30–32]. This system is responsible for the electron supply of the mitochondrial

CYPs, CYP11A1, CYP11B1 and CYP11B2, which are also involved in steroid hormone biosynthesis. Pechurskaya et al. showed, that Adx is able to transfer electrons to purified CYP21A2, in the case of truncated CYP21A2 even more effectively than the CPR in in vitro assays [33]. In this work, we used the Adx version, Adx_{1-108}, which exhibits an increased electron transfer efficiency to some CYPs [34, 35].

Second, a redox system originating from the fission yeast *S. pombe* and consisting of the adrenodoxin reductase homologue 1 (arh1) and the ferredoxin domain of electron transfer protein 1 ($etp1^{fd}$) was considered, since Ewen et al. showed that this system is able to substitute Adx and AdR regarding an electron transfer to CYP11A1 [36]. In *S. pombe,* arh1 and $etp1^{fd}$ are involved in heme biosynthesis in the mitochondrium [37]. $Etp1^{fd}$ as iron-sulfur protein is highly homologous to adrenodoxin and is able to transfer electrons to mammalian steroidogenic CYPs [38, 39]. Here, we used the truncated version of $etp1^{fd}$ (516–618). Both proteins can be produced as cytosolic proteins in *E. coli*. Furthermore, it has been demonstrated, that arh1 can be reduced not only by NADPH like AdR, but also by NADH and that arh1 of *S. cerevisiae* is able to interact with bovine Adx [40]. Regarding a whole-cell system, a second electron pool could be of great advantage for a more efficient hydroxylation rate [36]. Janocha et al. already demonstrated a biotechnological application of arh1 and $etp1^{fd}$ from *S. pombe* with CYP105A1 from *Streptomyces griseolus* [41]. As in previous works of our laboratory, we used an arh1 variant with an improved FAD-binding behavior, ensuring co-factor stability [36].

Third, we applied the *E. coli* NADPH-flavodoxin reductase Fpr as an alternative reductase for a whole-cell system. It has been demonstrated previously that the soluble Fpr is able to transfer electrons to Adx and, therefore, represents an efficient substitution for AdR [27]. The Fpr together with flavodoxin A (FldA) is part of an *E. coli* redox system, which is involved in biosynthetic processes such as amino acid synthesis [42, 43].

To verify whether Adx and $etp1^{fd}$ as final electron transfer proteins are able to supply CYP21A2 with electrons, in vitro assays were carried out with different combinations of reductases and ferredoxins, listed in Table 1. HPLC analysis revealed, that both, Adx and $etp1^{fd}$, are able to transfer electrons to CYP21A2, no matter which reductase was chosen (Fig. 4). It was shown for the first time, that $etp1^{fd}$ is able to interact with CYP21A2 and, furthermore, to recruit the *E. coli* reductase Fpr as redox partner.

In the following, we show efforts to exchange the natural redox partner CPR by bovine Adx as well as $etp1^{fd}$ as final electron donors in combination with different reductases in a whole-cell system to achieve an enhanced premedrol yield.

Table 1 Vectors for the *E. coli* whole-cell system

Vector	Reductase	Origin	Ferredoxin	Origin
p21b_bRED	CPR	*Bos taurus*		
p21b_AdAx	AdR	*Bos taurus*	Adx	*Bos taurus*
p21b_ArAx	arh1	*S. pombe*	Adx	*Bos taurus*
p21b_FrAx	Fpr	*E. coli*	Adx	*Bos taurus*
p21b_ArEt	arh1	*S. pombe*	$etp1^{fd}$	*S. pombe*
p21b_FrEt	Fpr	*E. coli*	$etp1^{fd}$	*S. pombe*

Six pET17b based vectors were constructed, each carrying the *CYP21A2* cDNA, in either a bicistronic construct with the cDNA for *CPR* or in a tricistronic construct with the cDNAs for Adx_{1-108} or $etp1^{fd}$ as final electron donors. The origin of the respective protein is mentioned

Construction of vectors for a whole-cell system with various redox chains

We constructed three vectors with a tricistronic transcription unit, each containing Adx as final electron donor. The vector p21b_ArAx was constructed containing the ORF for bovine CYP21A2, followed by the ORF for bovine AdR and Adx_{1-108}, which represents the mitochondrial redox chain. Then, we replaced the AdR sequence with the one for arh1 from *S. pombe*. Finally, we used the *E. coli* reductase Fpr instead of AdR. The three resulting constructs are shown in Fig. S2 (Additional file 2).

Two more vectors were constructed, containing $etp1^{fd}$ as electron donor of CYP21A2, on the one hand in combination with its natural ferredoxin reductase from *S. pombe,* arh1 (p21b_ArEt), on the other hand with the *E. coli* reductase, Fpr (p21b_FrEt). The resulting constructs are shown in Fig. S3 (Additional file 3). All constructed vectors are listed in Table 1.

Evaluation of the CYP21A2 whole-cell systems with different redox partners

C43(DE3) cells were co-transformed with pGro12 and the constructed vectors, cultivated simultaneously to compare the initial productivity of each system as well as the endpoint yield of premedrol. Whole-cell biotransformation was carried out with resting cells in potassium phosphate buffer and samples for HPLC analysis were taken after 0, 2, 4, 6, 10 and 24 h to get a characteristic time course of the product formation depending on the respective redox system. A substrate concentration of 500 µM medrane was applied. HPLC analysis verified the biotransformation ability of each system. Regarding the endpoint yield, the systems containing the mitochondrial (AdR/Adx) and microsomal (CPR) redox partners produced the lowest amount of premedrol with 41 and

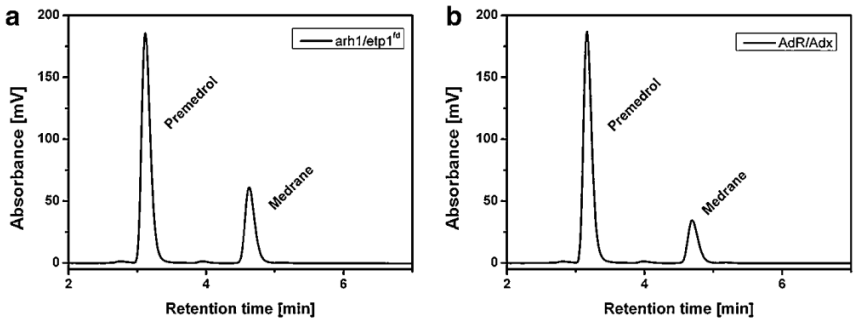

Fig. 4 HPLC chromatograms of the in vitro biotransformation of medrane with bovine CYP21A2 and Adx or etp1^{fd} as final redox partner. Substrate conversions of medrane were performed with purified bovine CYP21A2 and **a** Adx as final electron donor, here with its natural reductase AdR, and **b** etp1^{fd} as final electron donor, here with its natural reductase arh1 for 30 min. Steroids were extracted and analyzed by HPLC to verify a selective conversion to premedrol, respectively

87 mg L^{-1} d^{-1}, respectively. Taken the fact into account that AdR and CPR are membrane-associated and membrane-bound proteins, respectively, the recombinant synthesis and stability of these enzymes might be limited and, therefore, could represent a disadvantage for their application in a biotransformation process. Remarkably, the endpoint yield was dependent on the expressed reductases and not on the respective ferredoxin, suggesting that within this whole-cell system the functionality of the reductases is a limiting factor. The biotransformation overall yield with Fpr was higher than that with CPR with 127 and 115 mg L^{-1} d^{-1}, no matter which ferredoxin, Adx or etp1^{fd}, was the final electron donor for CYP21. The same was observed with arh1 as reductase with 156 mg L^{-1} d^{-1} together with Adx and 167 mg L^{-1} d^{-1} with its natural redox partner etp1^{fd}, emphasizing that the soluble proteins, Fpr and arh1, are more suitable for a whole-cell system (Fig. 5; Table 2).

Though the endpoint yields of the different systems are similar when using the same reductase, the time course revealed a crucial difference regarding reaction velocity. While the velocities of the redox partner combinations arh1/Adx, Fpr/Adx and Fpr/etp1^{fd} are similar within the first 4 h, the system containing the reductase as well as the ferredoxin from *S. pombe* exhibits a higher efficiency with about doubled product formation between 2 and 4 h of substrate conversion, obviously due to the fact that arh1 together with etp1^{fd} represents a natural redox chain with optimal protein–protein interaction properties. Table 2 lists the initial and final product formation rates.

Taken together, we clearly demonstrated, that a redox protein exchange for the CYP21 whole-cell system increased the overall yield from about 90 mg L^{-1} d^{-1} to about 167 mg L^{-1} d^{-1}, by use of arh1 and etp1^{fd} instead of CPR. It was also demonstrated that the reaction velocity

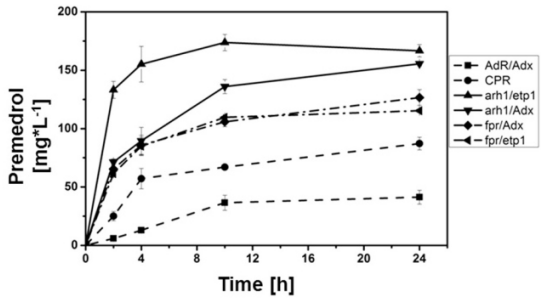

Fig. 5 Time-dependent premedrol formation by different CYP21A2 based whole-cell systems. Whole-cell biotransformation was performed with all constructed systems simultaneously by using resting cells. Samples were taken after 0, 2, 4, 10 and 24 h and analyzed via HPLC. The premedrol yield at several time points was determined for each system. All values represent the mean of triplicates with the respective standard deviation

is strongly dependent on the expressed redox proteins. With a substrate concentration of 1 mM medrane we could maximize the premedrol yield to 320 mg L^{-1} d^{-1} with the arh1 and etp1^{fd} redox chain in subsequent experiments. The fact that arh1 is able to receive electrons not only from NADPH but also from NADH (Additional file 3: Fig. S3) emphasizes the great potential of this reductase in a whole-cell application tapping an additional electron pool compared with the NADPH dependent Fpr and AdR, taking into account the fact that in *E. coli* NADH is the predominant co-factor under normal metabolic conditions of *E. coli* [44, 45].

To compare our established *E. coli* system with the human CYP21A2 based whole-cell system in *S. pombe*, we performed substrate conversions with the natural substrate 17OH-progesterone, since Zehentgruber et al. used it in an *S. pombe* whole-cell system [22]. With the *E.*

Table 2 Premedrol yield of different systems for whole-cell biotransformations performed with bCYP21A2 and various redox chains

Redox system	Overall yield premedrol (mg L^{-1} d^{-1})	Initial rate (mg L^{-1} h^{-1})
CPR	87 ± 5	13 ± 2
AdR/Adx	41 ± 6	3 ± 0.2
arh1/Adx	156 ± 2	36 ± 1
Fpr/Adx	127 ± 7	33 ± 2
arh1/etp1^{fd}	167 ± 5	67 ± 4
Fpr/etp1^{fd}	115 ± 1	31 ± 0.4

The premedrol overall yield was determined via HPLC analysis after 24 h conversion with all constructs simultaneously. The initial rate was calculated taking the first 2 h into account after starting the reaction by substrate addition. All values represent the mean of triplicates with the respective standard deviation

coli system we achieved 308 ± 16 mg L^{-1} d^{-1} of the product 11-deoxycortisol, which is an about fourfold higher product yield compared with the system in *S. pombe* producing 77 mg product per L and day. Taken into account that Zehentgruber et al. used a cell density of 360 g L^{-1}, which is tenfold higher than the applied *E. coli* density of 24 g L^{-1}, we achieved a productivity of 37 µmol g^{-1} cell wet weight, while only 0.625 µmol g^{-1} cell wet weight were produced with the *S. pombe* system, which is 60 times less. This data clearly demonstrates the high efficiency and productivity of the established *E. coli* whole-cell system.

To examine the stoichiometry of CYP21A2:arh1:etp1^{fd}, which are encoded by a tricistronic transcription unit, Western blot analysis was carried out for each enzyme after an expression time of 28 h according to Janocha et al. [41]. The highest expression level was determined for etp1^{fd} with approx. 880 nmol L^{-1}. The reductase arh1 expression level is estimated to be approx. 498 nmol L^{-1} and the lowest one is for CYP21A2 with approx. 119 nmol L^{-1}. Thus, the proteins are expressed with a ratio of 1:4:7 (CYP21A2:arh1:etp1^{fd}) demonstrating an excess of reductase and ferredoxin, which supports a sufficient electron supply to CYP21A2 and underlines the system's high efficiency (Fig. 6).

Determination of dissociation constants by difference spectroscopy

To compare the binding ability of bovine CYP21A2 to the synthetic substrate medrane to natural ones, progesterone and 17OH-progesterone, and to examine a possible limitation for medrane conversion due to a decreased protein binding, we determined the dissociation constant of the CYP21A2-medrane complex by difference spectroscopy. Complex formation between a potential substrate and CYP21A2 is spectroscopically detectable as type I shift due to the replacement of the heme coordinated H_2O molecule. Titration of CYP21A2 with increasing amounts of medrane shows a typical type I shift (Fig. 7a) and, therefore, underlines a medrane conversion by CYP21A2. The difference of the absorbance maximum and minimum plotted against the substrate concentration of each titrating step results in a hyperbolic regression curve revealing a K_D value of 11.27 ± 0.28 µM for medrane. To compare the K_D value

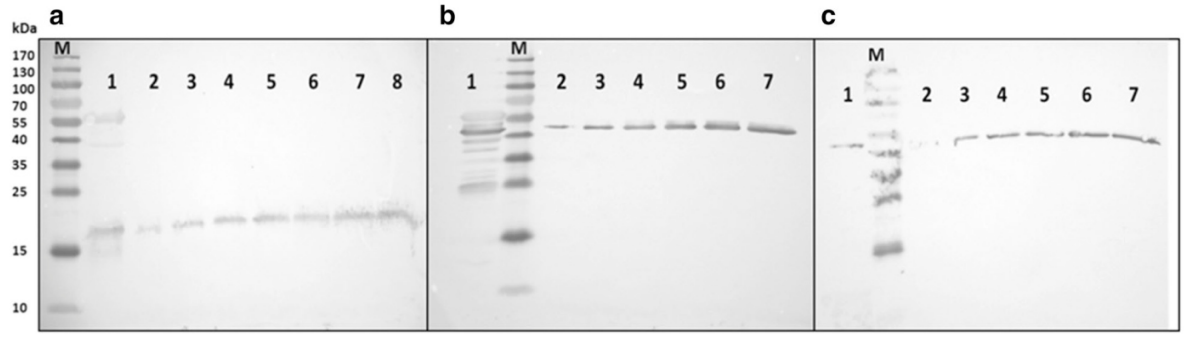

Fig. 6 Western blot analysis of CYP21A2 and redox proteins in the whole-cell system for determination of the stoichiometric ratio of the three proteins encoded by the tricistronic construct p21b_ArEt. Western blot analysis was performed with polyclonal antibodies against **a** etp1^{fd} (11.3 kDa), **b** arh1 (51 kDa), c bovine CYP21A2 (54.6 kDa). In each blot, lane 1 represents the *E. coli* cell extract expressing arh1, etp1^{fd} and CYP21A2 after 28 h. **a** *lane 2–8* represents purified etp1^{fd} in increasing amounts (10, 20, 30, 40, 50, 75 and 100 ng), **b**, **c** The *lanes 2–7* show purified arh1 and CYP21A2 in increasing amounts (arh1: 25, 50, 75, 125, 187.5 and 250 ng, CYP21A2: 24, 48, 95, 143, 191 and 239 ng). The with "M" marked *lanes* all represent a prestained protein marker. The relative lane intensities, which correlate with the respective protein masses, were determined and compared to the intensity of the whole-cell system sample. Mass values were converted into the amount of substances and extrapolated to the expression yield per liter culture. Note that etp1^{fd} is known to give a single band in the range of the double mass expected to see on the SDS-PAGE

of the non-natural substrate medrane with those of the natural substrates, 17OH-progesterone and progesterone, we additionally titrated CYP21A2 with increasing amounts of these steroids. Hyperbolic regression resulted in a K_D value for 17OH-progesterone of 0.14 ± 0.01 µM and for progesterone of 0.34 ± 0.01 µM (Fig. 7b, c), implicating a higher affinity of natural CYP21A2 substrates compared with the synthetic one, which is due to the amino acid arrangement in the active site of CYP21A2 to ensure a selective 21-hydroxylation of the natural substrates [10]. Considering the nearly 100-fold higher K_D value of medrane compared with the natural substrate 17OH-P4, an enzyme improvement could aspire a stronger binding of the synthetic substrate to promote a more efficient premedrol production. This hypothesis is underlined by a higher product formation when using 17OH-progesterone as a substrate displaying a yield of 889 ± 59 µM d^{-1} 11-deoxycortisol compared with a premedrol yield of 640 ± 13 µM d^{-1}. Hence, biotransformation with CYP21A2 using the natural substrate with the lowest dissociation constant shows a 40 % higher product formation than bioconversion with medrane.

Scale-up of the whole-cell system with CYP21A2 and the redox partners arh1 and etp1^{fd}

After the establishment of a CYP21A2 based whole-cell system and a further improvement of the system's efficiency by alternative redox chains in shaking flasks, we pursued a scale-up of the system by a fermentation approach with increased cell density and the possibility to supplement oxygen by the stir velocity, since a sufficient oxygen supply is indispensable for CYP

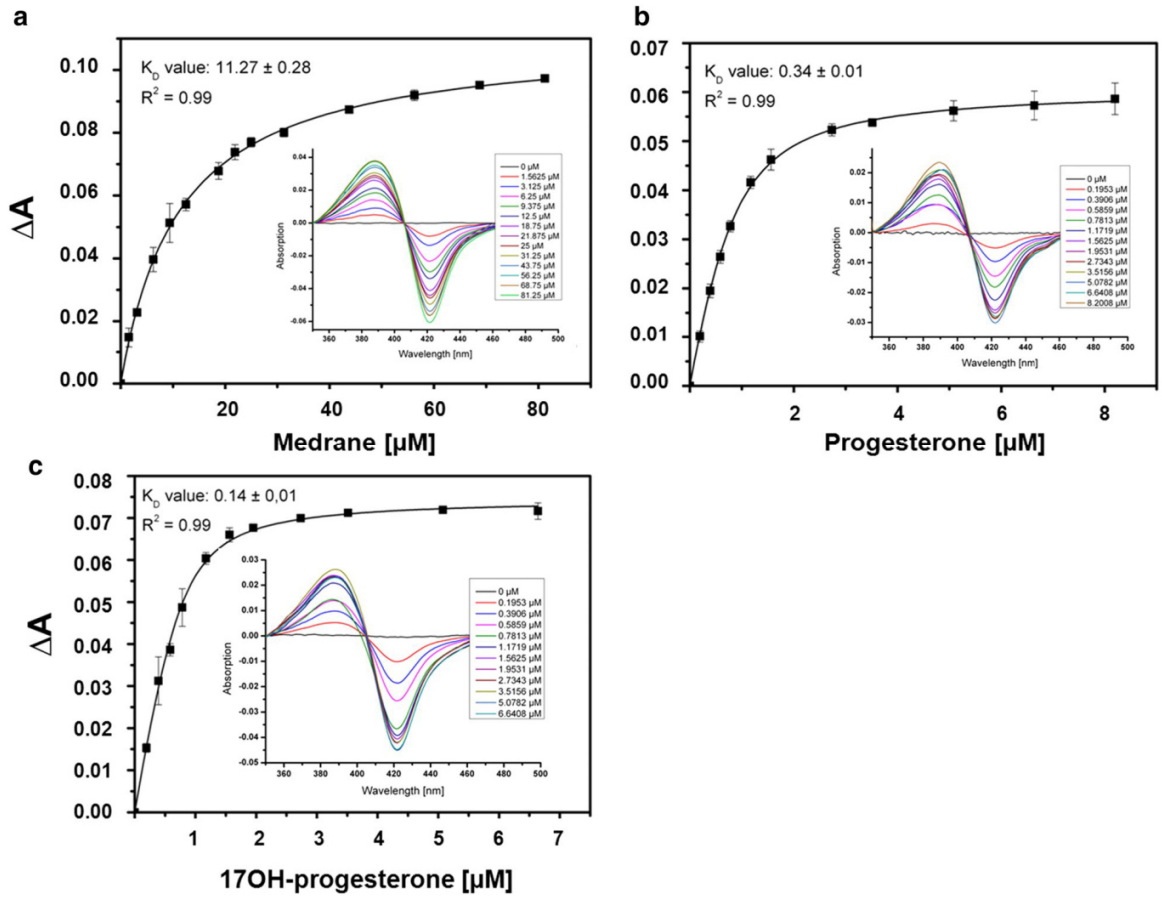

Fig. 7 Determination of dissociation constants (K_D values) with different substrates titrated to CYP21A2. Dissociation constants for **a** medrane, **b** progesterone and **c** 17OH-progesterone were determined by titration of bovine CYP21A2 with increasing concentrations of the particular substrate leading to a typical type I shift. Hyperbolic regression resulted in the respective K_D values, indicating the extent of the binding affinity towards the enzyme

dependent reactions. Therefore, we performed a scale-up of the most efficient whole-cell system consisting of bovine CYP21A2 and the heterologous redox partner proteins arh1 and etp1^{fd}. Protein expression was performed in Erlenmeyer flasks, and after a washing step the cell density for the biotransformation was adjusted to 72 g L^{-1}. The reaction took place in the bioreactor BiostatQ® with 500 mL resting cells in a defined buffer medium. 1000 mg medrane were added and the reaction was performed for 20 h with a stir velocity of 700 rpm. With this simple scale-up approach from a reaction volume of 25 mL in shaking flasks up to 500 mL in a bioreactor, a higher cell density was reached and a maximum product yield of 0.65 g premedrol per L d^{-1} could be achieved. Considering the reaction's time dependence it was shown that the initial rate of 88 mg L^{-1} h^{-1} within the first 3 h dropped to 15 mg L^{-1} h^{-1} within the last 15 h of the biotransformation (Fig. 8). The decrease of the reaction rate was already observed in shaking flasks and in other CYP dependent whole-cell systems indicating limiting factors for a continuous biotransformation [46]. In case of CYP21A2, protein stability as a limiting factor could be excluded by CO difference spectroscopy of samples taken before and after bioconversion, which showed a highly stable enzyme (Additional file 4: Fig. S4). Furthermore, we could confirm by Western blot analysis that there exists an optimal stoichiometry of CYP21A2 and the redox proteins arh1 and etp1^{fd}. Regarding a biotechnological application the next step would be the establishment of a controlled fermentation process ensuring a stable pH, carbon source as well as substrate feeding and, overall, a sufficient supply with oxygen needed for a CYP catalyzed reaction. Nevertheless, this scale-up approach implicates the potential to increase the whole-cell system's efficiency by a biotransformation in a bioreactor and already produced nearly gram amounts of product per liter and day (Additional file 5: Fig. S5, Additional file 6: Table S1).

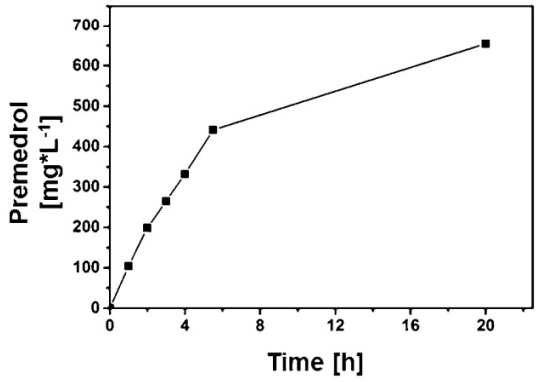

Fig. 8 Time-dependent premedrol formation by the CYP21A2 based whole-cell system with arh1 and etp1^{fd} in a bioreactor. Whole-cell biotransformation in a bioreactor was performed with cells of C43(DE3), transformed with the vector p21b_ArEt, which is encoding bovine CYP21A2 as well as arh1 and etp1^{fd}, by using resting cells in a volume of 500 mL and a cell density of 72 g L^{-1}. Samples were taken after certain time points and analyzed via HPLC to examine the temporal premedrol increase

Conclusions

In this work, we demonstrated that the mammalian CYP21A2, which is involved in mammalian steroid hormone biosynthesis and catalyzes a stereo- and regioselective 21-hydroxylation of progesterone and 17OH-progesterone, also exhibits a high potential as biocatalyst for medrol production. It could be demonstrated with purified enzyme as well as in a newly established *E. coli* whole-cell system that CYP21A2 is able to convert medrane to premedrol, an important precursor of medrol, via a selective oxofunctionalization at C21. Since a sufficient electron supply is an essential factor influencing CYP dependent reactions, the focus has shifted from the natural CYP21A2 redox partner, the membrane protein CPR, to alternative redox proteins. Therefore, five different redox systems were introduced and examined regarding velocity and endpoint yield. The systems containing the ferredoxin reductase arh1 from *S. pombe* were similar in their endpoint independent on the used ferredoxin. With regard of the initial reaction rate it is shown in Fig. 5, that the redox chain consisting of arh1 and its natural ferredoxin etp1^{fd} is two times faster than all other systems, possibly due to the fact that the electron transfer between a natural redox pair is much more effective and faster than in the system with Adx (Fig. 5; Table 2). Taken together, the use of soluble enzymes with a high expression yield seems to be the best choice for the system's stability and effectiveness, which is underlined by an ideal protein stoichiometry, confirmed by Western blot analysis of the three participating proteins (Fig. 6). Compared to the CPR based system the premedrol yield could be enhanced about the 3.6 fold by the use of other redox partners, which shows the high impact of a sufficient, stable and suitable electron supply during biotransformation. We could achieve 320 mg L^{-1} d^{-1} in shaking flasks experiments. A scale-up approach to 500 mL in a bioreactor, including an increase of the cell density, could further improve the overall yield up to a maximum of 0.65 g L^{-1} d^{-1} which shows the potential of the system for an industrial application and an important step towards the replacement of the chemical premedrol synthesis by a biocatalytic approach.

Methods

Chemicals, kits, enzymes and primary antibodies

All chemicals were from standard sources and of highest purity. Solvents used for chromatographic analysis were of gradient grade. Steroids for analysis and whole-cell biotransformation were from Sanofi, Frankfurt-Höchst (DE) and of highest purity. Restriction enzymes were obtained from New England Biolabs (Frankfurt, DE), kits for plasmid preparation and DNA purification from Machery-Nagel (Düren, DE) and the FastLink™ DNA Ligation Kit from Epicentre Biotechnologies (Madison, US). The primary antibody against arh1 was obtained from Charles River Laboratories (Sulzfeld, Germany), against etp1^{fd} from BioGenes (Berlin, Germany) and against bovine CYP21A2 from antikoerper-online.de (Aachen, Germany).

Bacterial strains and cultivation

Plasmid preparation and cloning experiments were carried out with *E. coli* TOP10 (Invitrogen, San Diego, CA, USA). Protein synthesis and whole-cell biotransformation were performed with *E. coli* strain C43(DE3) [47]. The cultivation took place in Luria–Bertani broth (BD, Heidelberg, DE) or in terrific broth (TB) complex medium. Transformed cells were stored as glycerol stock with a 1:1 mixture of an overnight culture and glycerol (50 %) at −80 °C.

Molecular cloning

Expression vector for bovine and human CYP21A2

The cDNAs for bovine and human *CYP21A2* was constructed according to Arase et al. with a replacement of the N-terminal hydrophobic anchor region with MAKK TSSKGK from CYP2C3 and a 6× Histidin tag for protein purification [24, 48]. It was digested with NdeI and BamHI and ligated into the pET17b expression vector (Novagen). The constructed vectors are subsequently designated as pET17b_21b and pET17b_21h.

Construction of vectors for a whole-cell biotransformation

All constructed vectors for a whole-cell biotransformation are based on the pET17b expression vector. All constructs consist of bi- or tricistronic transcription units with the *CYP21A2* cDNA sequence and one or two redox partner cDNAs downstream of it (Fig. 2, Additional file 1: Fig S1, Additional file 2: S2).

The vector pET17b_21b served as a backbone for the construction of the vector p21b_bRED, containing the cDNA for bovine *CYP21A2* and its natural redox partner, the bovine cytochrome P450 reductase (*CPR*), as a bicistronic transcription unit. A CPR containing vector was used for PCR amplification of *CPR* cDNA [49]. The forward primer contains a BamHI restriction side followed by a ribosomal binding side and the respective coding region. The appropriate reverse primer carries the C-terminal coding region including the stop codon and a NotI restriction side. The PCR product was digested and ligated between the BamHI and NotI sides of pET17b_21b, resulting in the bicistronic construct p21b_bRED. The vector p21h_bRED was cloned likewise.

The vector p21b_AdAx contains a tricistronic transcription unit, consisting of the cDNA for bovine *CYP21A2*, bovine adrenodoxin reductase (*AdR*) and truncated bovine adrenodoxin (Adx_{1-108}). Bovine AdR and Adx represent the mitochondrial redox system, which is proved to interact with CYP21A2. The vector Twin11B1 served as a backbone for the construction and carries the cDNA for human *CYP11B1*, bovine *AdR* and bovine *Adx* in a tricistronic arrangement [20]. Firstly, an undesired HindIII site had to be removed within the *CYP21A2* sequence by QuikChange® Site-Directed Mutagenesis. The resulting cDNA for bovine *CYP21A2* was amplified by PCR with pET17b_21b as a template. The forward primer is equal to the existing DNA sequence and contains an NdeI restriction site. The reverse primer carries the end of the coding region and a HindIII site. Both PCR product and the vector Twin11B1 were digested and the *CYP21A2* cDNA was ligated between the NdeI and HindIII sites of Twin 11B1 by a replacement of the *CYP11B1* sequence against the *CYP21A2* cDNA which results in the tricistronic vector p21b_AdAx.

The vector p21b_FrAx was constructed according to p21b_AdAx and contains the *E. coli* reductase Fpr instead of AdR, which is cloned through the HindIII and KpnI sites. Origin of the Fpr sequence was the vector pET_MR6 [27].

For cloning of the vectors harboring components of the redox system from *S. pombe* the vector pBar_Twin_pombe served as a template, carrying the cDNA for adrenodoxin reductase homologue 1 (*arh1*) and the ferredoxin domain of the electron transfer protein 1 (*etp1^{fd}*) [36, 38, 41]. In a first step, the AdR sequence of the vector p21b_AdAx was replaced by the *arh1* cDNA which was amplified via PCR using pBar_Twin_pombe as a template. The forward primer carried a HindIII restriction site as well as a following ribosomal binding site while the reverse primer was identical to the C-terminal sequence including a KpnI restriction site. The amplified PCR product was digested and cloned into the likewise digested p21b_AdAx. The resulting vector p21b_ArAx contains a tricistronic construct composed of the cDNAs for *CYP21A2*, *arh1* and *Adx*.

In the next step, the vector p21b_ArEt was constructed based on the backbone of the vector p21b_ArAx, which contains both components of the *S. pombe* redox system. Again, pBar_Twin_pombe served as a template for a PCR amplification of *etp1^{fd}*. The forward as well as the reverse primer were identical to the etp1^{fd} cDNA sequence, carrying a KpnI

and an EcoRI restriction site. The PCR product was digested and ligated between the KpnI and EcoRI restriction sites of the likewise digested p21b_ArAx resulting in an exchange of Adx by etp1^{fd}. The vector p21b_FrEt was cloned likewise. All used primers are listed in Additional file 6: Table S6.

Protein expression and purification

Expression and purification of electron transfer proteins

Bovine AdR and Adx as well as arh1, Fpr and etp1^{fd} were expressed in *E. coli* and purified as described before [27, 34, 36, 38, 50].

Bovine CPR was synthesized in *E. coli* and purified via Immobilized Metal Ion Affinity Chromatography (IMAC) as described elsewhere [49].

Expression and purification of bovine and human CYP21A2

C43(DE3) were co-transformed with the expression vector pET17b_21b and the vector pGro12 which carries the genes for the molecular *E. coli* chaperones GroEL/ES to ensure a proper protein folding and a correct integration of the heme cofactor. For the seed culture, 10 mL LB medium, supplemented with 100 µg mL^{-1} ampicillin for pET17b_21b selection and 50 µg mL^{-1} kanamycin for pGro12 selection, were inoculated with transformed cells from a glycerol stock and grown overnight at 37 °C with 160 rpm. For the main culture, 250 mL TB medium, supplemented with 100 µg mL^{-1} ampicillin and 50 µg mL^{-1} kanamycin, were inoculated with 1/100 (v/v) of the seed culture and grown at 37 °C with 190 rpm to an OD_{600} of 0.5. At this time point, gene expression was induced by adding 1 mM isopropylthiogalactopyranosid (IPTG), 1 mM δ-aminolevulinic acid as heme precursor and 4 mg mL^{-1} L-arabinose for the induction of the chaperones GroEL/ES. Protein synthesis was carried out at 27 °C with 150 rpm for 38 h. Cells were harvested at 4,000*g* for 20 min at 4 °C.

Cell pellets were diluted in lysis buffer, consisting of 50 mM potassium phosphate buffer (pH 7.4), 500 mM sodium acetate, 0.1 mM EDTA, 20 % glycerin, 1.5 % sodium cholate, 1.5 % Tween20, 0.1 mM PMSF and 0.1 mM DTE. Cells were disrupted by sonification and centrifuged with 30,000*g* at 4 °C for 30 min. The supernatant was taken for the subsequent purification. The 3 step protein purification by Immobilized Metal Ion Affinity Chromatography (IMAC) and DEAE Sepharose as well as SP Sepharose for ion exchange chromatography was done as previously described by Arase et al. [24].

UV/vis spectroscopy

CO difference spectroscopy of reduced CYP in complex with CO was carried out for a qualitative and quantitative enzyme characterization following the typical absorption maximum at 450 nm with an extinction coefficient of 91 mM^{-1} cm^{-1} [13].

Difference spectroscopy was performed to examine the binding behavior of CYP21A2 natural and unnatural CYP21A2 substrates as previously described by using tandem cuvettes. CYP21A2 was dissolved in buffer (50 mM potassium phosphate (pH 7.4), 20 % glycerol, 0.5 % sodium cholate and 0.05 % Tween 20) and titrated with increasing amounts of substrate in DMSO. Difference spectra were recorded from 350 to 500 nm. The values from three titrations were averaged and the K_D values were determined by fitting the plots with hyperbolic regression or tight binding quadratic equation with OriginPro 9.1G [51].

Reconstituted in vitro assays

The in vitro reconstitution assay was performed in a final volume of 250 µL with 50 mM HEPES buffer (pH 7.4) containing either 100 µM DLPC and 20 % glycerol for the CPR or 0.5 % Tween20 for all other redox proteins. The final concentration of CYP21A2 was 0.5 µM, the concentration of arh1 and AdR 0.5 µM, of Adx and etp1^{fd} 10 µM, of the Fpr 25 µM and of the CPR 1 µM, respectively. Additionally, the mixture contained a NADPH regeneration system consisting of 5 mM glucose-6-phosphate, 1 mM $MgCl_2$ as well as glucose-6-phosphate dehydrogenase. The particular steroid substrate was added in a concentration range of 100–400 µM. The reaction was started with 5 mM NADPH or NADH and incubated shaking for 30–40 min at 37 °C. The assay was stopped by addition of 250 µL chloroform, steroids were extracted twice with chloroform, dried and stored at −20 °C for HPLC analysis.

Whole-cell biotransformation with different redox systems in shaking flasks

Protein synthesis of bovine and human CYP21A2 and the respective redox partners for a whole-cell biotransformation was performed as described above by co-transformation of C43(DE3) cells with the particular bi-or tricistronic vector and the pGro12. After 28 h expression time, cells were harvested at 4,000*g* for 15 min at room temperature. The cell pellets were washed with 50 mM potassium phosphate buffer and cell wet weight was adjusted to 24 g L^{-1}. The whole-cell biotransformation was carried out with resting cells in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 2 % glycerol, 1 mM IPTG, 1 mM δ-Ala, 4 mg mL^{-1} arabinose and 25 µg mL^{-1} polymyxin B. The reaction volume was 25 mL in 300 mL baffled Erlenmeyer flasks. The steroid substrate was solved in DMSO and added in concentrations ranging from 200 to 1.2 mM. The whole-cell reaction mixture was incubated at 27 °C with 145 rpm for 24 h. Samples for HPLC analysis were taken at different time points, extracted twice with chloroform, dried and stored at −20 °C.

Whole-cell biotransformation in a bioreactor

Protein synthesis of bovine CYP21A2 and the redox partners arh1 and etp1^{fd} for a whole-cell biotransformation was performed as described above, by co-transformation of C43(DE3) cells with the p21b_ArEt vector and the pGro12. After 28 h expression time, cells were harvested at 4,000*g* for 15 min at room temperature. The cell pellets were washed with 50 mM potassium phosphate buffer and cell wet weight was adjusted to 72 g L^{-1}. The whole-cell biotransformation was carried out with resting cells in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 2 % glycerol, 1 mM IPTG, 1 mM δ-Ala, 4 mg mL^{-1} arabinose and 25 µg mL^{-1} polymyxin B. The reaction volume was 500 mL and the biotransformation was carried out in the bioreactor BiostatQ® with a stir velocity of 700 rpm at 27 °C. The steroid substrate was dissolved in DMSO and added in concentrations up to 1000 mg L^{-1}.

Steroid analysis via RP-HPLC

Steroid analysis was carried out by reversed-phase high performance liquid chromatography using a Jasco reversed phase HPLC system of the LC900 series and a 4.6 mm × 125 mm NucleoDur C18 Isis Reversed Phase column (Macherey–Nagel).

The reconstituted in vitro assays were analyzed with an acetonitril/water gradient at 240 nm within 15 min at 40 °C and a flow rate of 0.8 mL min^{-1}.

The whole-cell conversion was measured with an acetonitril/water gradient at 240 nm within 30 min at 40 °C and a flow rate of 0.8 mL min^{-1}.

Western blot analysis

Samples from the culture, co-expressing bovine CYP21A2, arh1 and etp1^{fd}, were taken, adjusted to OD 1 and centrifuged. The pellet was suspended in 100 µl SDS-PAGE loading buffer and boiled for 10 min. 6 µL of the sample in case of CYP21A2 and arh1 and 3 µL in case of etp1^{fd} were separated on a 12 % acrylamide gel according to Laemmli et al. [52]. For Western blot analysis, proteins were transferred to hybond-ECL nitrocellulose membranes (Amersham, GE Healthcare, England) [53]. The membranes were blocked overnight in 3 % milk powder in 30 mL TBS (50 mM Tris–Cl pH 7.5, 400 mM NaCl, 0.15 % Tween 20). After blocking, the membranes were washed three times for 15 min with TBS and afterwards incubated for 1.5 h with the respective primary antibody, dissolved 1:1000 in TBS. After three following washing steps with TBS, incubation with the secondary horseradish-linked goat antirabbit IgG antibody (Dako, Glostrup, Denmark), diluted 1:3000 in TBS, took place for 1.5 h. In the following step, the membranes were washed three times for 15 min with PBS (10 mM potassium phosphate buffer pH 7.4, 150 mM NaCl) and afterwards, the protein-antibody conjugates were visualized by addition of 4-chloro-1-naphthol (2 mL; 3 mg/mL in ethanol) in 25 mL PBS supplemented with 10 µL H_2O_2. Relative intensity of the protein bands was measured with Image Lab 3.0 from BioRad (München, Germany). The determination of the protein yield was performed by comparing the sample amount (Fig. 6a–c, lane 1) to increasing concentrations of purified protein, for etp1^{fd} 10, 20, 30, 40, 50, 75 and 100 ng (Fig. 6a, lanes 2–8), for arh1 25, 50, 75, 125, 187.5 and 250 ng (Fig. 6b, lanes 2–7) and for bovine CYP21A2 24, 48, 95, 143, 191 and 239 ng (Fig. 6c, lanes 2–7). The relative lane intensities, which correlate with the respective protein masses, were determined and compared to the intensity of the whole-cell system sample. Mass values were converted into the amount of substances and extrapolated to the expression yield per liter culture.

Additional files

Additional file 1: Fig. S1. HPLC chromatograms of the *in vivo* and *in vitro* conversion of medrane with human CYP21A2 and CPR. **a** Medrane was converted with resting cells of C43(DE3) containing the human CYP21A2 isoform and CPR encoding vector p21h_bRED. Samples were taken after 24 h and extracted for HPLC analysis. The steroids were separated by an acetonitril:water gradient. **b** Substrate conversions of medrane were performed with purified human CYP21A2 and its redox partner CPR for 30 min. Steroids were extracted and analyzed by HPLC to verify a selective conversion of medrane to premedrol, respectively. The steroids were separated by an acetonitril:water gradient, showing the 21-hydroxylated product premedrol.

Additional file 2: Fig. S2. Vector maps of constructed vectors for a CYP21A2 based whole-cell system in *E. coli* with Adx as final electron donor. Three vectors with tricistronic transcription units were constructed, based on the pET17b vector with an inducible T7 promoter and an ampicillin resistance gene, with Adx as final electron donor in combination with one of the reductases, AdR (p21b_AdAx), arh1 (p21b_ArAx) or Fpr (p21b_FrAx).

Additional file 3: Fig. S3. Vector maps of constructed vectors for a CYP21A2 based whole-cell system in *E. coli* with etp1^{fd} as final electron donor. Two vectors with tricistronic transcription units were constructed, based on the pET17b vector with an inducible T7 promotor and an ampicillin resistance gene, with etp1^{fd} as final electron donor in combination with the reductases arh1 (p21b_ArEt) or Fpr (p21b_FrEt).

Additional file 4: Fig. S4. *In vitro* conversion of medrane with the redox systems AdR/Adx/CYP21A2 or arh1/Adx/CYP21A2 with either NADH or NADPH. 400 µM Medrane was converted in a reconstituted *in vitro* assay with Adx based redox systems containing AdR or arh1 as reductase. To each system either NADH or NADPH was added to verify the ability of arh1 to receive electrons from NADH. AdR served as a negative control. All values represent the mean of triplicates with the respective standard deviation.

Additional file 5: Fig. S5. CO difference spectra of bovine CYP21A2 before and after 24 h biotransformation. Cells were harvested before and after 24 hours biotransformation. COD was performed with the respective lysate, each showing a typical absorption maximum at 450 nm in a reduced state in complex with CO which indicates a correct heme insertion. The solid line shows the COD before biotransformation, the dashed one after 24 h bioconversion in buffer.

Additional file 6: Table S1. Sequences of used primers with indication of their purpose. Restriction sites are in bold letters and base exchanges are signed in bold and cursive.

Abbreviations
CYP21A2: 21-hydroxylase; *E. coli*: *Escherichia coli*.

Authors' contributions
SA carried out the presented experiments and drafted the manuscript. LS participated in the establishment of the expression and purification of human CYP21A2. FH participated in the design of the study, interpretation of the results and manuscript drafting. BJ performed the biotransformation experiments in the bioreactor. RB participated in the design of experiments, the interpretation of the results and in manuscript drafting.

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Compliance with ethical guidelines

Competing interests
The authors declare that they have no competing interests.

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References

1. Flammer JR, Rogatsky I. Minireview: Glucocorticoids in autoimmunity: unexpected targets and mechanisms. Mol Endocrinol. 2011;25:1075–86.
2. Boland EW, Liddle GW. Metabolic and antirheumatic activities of 6-methyl-prednisolone (Medrol). Ann Rheum Dis. 1957;16:297–306.
3. Sambamurthy K, Ashotosh K. Pharmaceutical Biotechnology. New Delhi: New Age International (P) Ltd, Publishers; 2006.
4. Zingler VC, Strupp M, Jahn K, Gross A, Hohlfeld R, Brandt T. The effect of combined mitoxantrone and methylprednisolone therapy in primary and secondary progressive multiple sclerosis. An applied study in 65 patients. Nervenarzt. 2005;76:740–7.
5. Woodward RB, Brutcher FV. cis-Hydroxylation of a synthetic steroid intermediate with iodine, silver acetate and wet acetic acid. J Am Chem Soc. 1958;80:209–11.
6. Schmidlin J, Wettstein A. Synthese Aldosteron-ähnlicher Corticosteroide. d, l-18-Hydroxycorticosteron und d, l-18-Desoxyaldosteron. Helv Chim Acta. 1961;44:1596–607.
7. Boudi A, Lemoine P, Viossat B, Tomas A, Fiet J, Galons H. A convenient synthesis of 18-hydroxycorticosterone and 18-hydroxy-11-desoxycorticosterone via stereospecific hypoiodination of 20-hydroxysteroids. Tetrahedron. 1999;55:5171–6.
8. Ryan KJ, Engel LL. Hydroxylation of steroids at carbon 21. J Biol Chem. 1956;225:103–14.
9. Kominami S, Ochi H, Kobayashi Y, Takemori S. Studies on the steroid hydroxylation system in adrenal cortex microsomes. Purification and characterization of cytochrome P-450 specific for steroid C-21 hydroxylation. J Biol Chem. 1980;255:3386–94.
10. Mizrachi D, Wang Z, Sharma KK, Gupta MK, Xu K, Dwyer CR, Auchus RJ. Why human cytochrome P450c21 is a progesterone 21-hydroxylase. Biochemistry. 2011;50:3968–74.
11. Hubbard PA, Shen AL, Paschke R, Kasper CB, Kim JJ. NADPH-cytochrome P450 oxidoreductase. Structural basis for hydride and electron transfer. J Biol Chem. 2001;276:29163–70.
12. Hannemann F, Bichet A, Ewen KM, Bernhardt R. Cytochrome P450 systems—biological variations of electron transport chains. Biochim Biophys (BBA) Acta Gen Subj. 2007;1770:330–44.
13. Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. J Biol Chem. 1964;239:2370–8.
14. Bernhardt R. Cytochromes P450 as versatile biocatalysts. J Biotechnol. 2006;124:128–45.
15. Bernhardt R, Urlacher V. Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. Appl Microbiol Biotechnol. 2014;98:6185–203.
16. Petzoldt KD, Annen KD, Laurent HD. Process for the preparation of 11-beta-hydroxy steroids. Berlin: Schering Aktengesellschaft; 1983.
17. Jänig G-R, Pfeil D, Müller-Frohne M, Riemer H, Henning M, Schwarze W, Ruckpaul K. Steroid 11ß-hydroxylation by a fungal microsomal cytochrome P450. J Steroid Biochem Mol Biol. 1992;43:1117–23.
18. Suzuki K, Sanga K, Chikaoka Y, Itagaki E. Purification and properties of cytochrome P-450 (P-450lun) catalyzing steroid 11 beta-hydroxylation in *Curvularia lunata*. Biochim Biophys Acta. 1993;8:215–23.
19. Szczebara FM, Chandelier C, Villeret C, Masurel A, Bourot S, Duport C, Blanchard S, Groisillier A, Testet E, Costaglioli P, et al. Total biosynthesis of hydrocortisone from a simple carbon source in yeast. Nat Biotech. 2003;21:143–9.
20. Schiffer L, Anderko S, Hobler A, Hannemann F, Kagawa N, Bernhardt R. A recombinant CYP11B1 dependent *Escherichia coli* biocatalyst for selective cortisol production and optimization towards a preparative scale. Microb Cell Fact. 2015;14:25.
21. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B. Industrial biocatalysis today and tomorrow. Nature. 2001;409:258–68.
22. Zehentgruber D, Dragan CA, Bureik M, Lutz S. Challenges of steroid biotransformation with human cytochrome P450 monooxygenase CYP21 using resting cells of recombinant *Schizosaccharomyces pombe*. J Biotechnol. 2010;146:179–85.
23. Hannemann F, Virus C, Bernhardt R. Design of an *Escherichia coli* system for whole cell mediated steroid synthesis and molecular evolution of steroid hydroxylases. J Biotechnol. 2006;124:172–81.
24. Arase M, Waterman MR, Kagawa N. Purification and characterization of bovine steroid 21-hydroxylase (P450c21) efficiently expressed in *Escherichia coli*. Biochem Biophys Res Commun. 2006;344:400–5.
25. Zhao B, Lei L, Kagawa N, Sundaramoorthy M, Banerjee S, Nagy LD, Guengerich FP, Waterman MR. Three-dimensional structure of steroid 21-hydroxylase (cytochrome P450 21A2) with two substrates reveals locations of disease-associated variants. J Biol Chem. 2012;287:10613–22.
26. Nishihara K, Kanemori M, Kitagawa M, Yanagi H, Yura T. Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli*. Appl Environ Microbiol. 1998;64:1694–9.
27. Ringle M, Khatri Y, Zapp J, Hannemann F, Bernhardt R. Application of a new versatile electron transfer system for cytochrome P450-based *Escherichia coli* whole-cell bioconversions. Appl Microbiol Biotechnol. 2013;97:7741–54.
28. Girhard M, Klaus T, Khatri Y, Bernhardt R, Urlacher VB. Characterization of the versatile monooxygenase CYP109B1 from *Bacillus subtilis*. Appl Microbiol Biotechnol. 2010;87:595–607.
29. Walton AZ, Stewart JD. Understanding and Improving NADPH-dependent reactions by nongrowing *Escherichia coli* Cells. Biotechnol Prog. 2004;20:403–11.
30. Lambeth JD, Seybert DW, Lancaster JR, Salerno JC, Kamin H. Steroidogenic electron transport in adrenal cortex mitochondria. Mol Cell Biochem. 1982;45:13–31.
31. Ewen KM, Kleser M, Bernhardt R. Adrenodoxin: The archetype of vertebrate-type [2Fe–2S] cluster ferredoxins. Biochim Biophys Acta (BBA) Gen Subj. 2011;1814:111–25.
32. Ewen KM, Ringle M, Bernhardt R. Adrenodoxin—A versatile ferredoxin. IUBMB Life. 2012;64:506–12.
33. Pechurskaya TA, Harnastai IN, Grabovec IP, Gilep AA, Usanov SA. Adrenodoxin supports reactions catalyzed by microsomal steroidogenic cytochrome P450s. Biochem Biophys Res Commun. 2007;353:598–604.
34. Uhlmann H, Kraft R, Bernhardt R. C-terminal region of adrenodoxin affects its structural integrity and determines differences in its electron transfer function to cytochrome P-450. J Biol Chem. 1994;269:22557–64.

35. P-r Cao, Bernhardt R. Modulation of aldosterone biosynthesis by adrenodoxin mutants with different electron transport efficiencies. Eur J Biochem. 1999;265:152–9.
36. Ewen KM, Schiffler B, Uhlmann-Schiffler H, Bernhardt R, Hannemann F. The endogenous adrenodoxin reductase-like flavoprotein arh1 supports heterologous cytochrome P450-dependent substrate conversions in *Schizosaccharomyces pombe*. FEMS Yeast Res. 2008;8:432–41.
37. Barros MH, Nobrega FG, Tzagoloff A. Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*. J Biol Chem. 2002;277:9997–10002.
38. Bureik M, Schiffler B, Hiraoka Y, Vogel F, Bernhardt R. Functional expression of human mitochondrial CYP11B2 in fission yeast and identification of a new internal electron transfer protein, etp1†. Biochemistry. 2002;41:2311–21.
39. Schiffler B, Bureik M, Reinle W, Muller EC, Hannemann F, Bernhardt R. The adrenodoxin-like ferredoxin of *Schizosaccharomyces pombe* mitochondria. J Inorg Biochem. 2004;98:1229–37.
40. Lacour T, Achstetter T, Dumas B. Characterization of recombinant adrenodoxin reductase homologue (Arh1p) from yeast: implication in in vitro cytochrome P45011β monooxygenase system. J Biol Chem. 1998;273:23984–92.
41. Janocha S, Bernhardt R. Design and characterization of an efficient CYP105A1-based whole-cell biocatalyst for the conversion of resin acid diterpenoids in permeabilized *Escherichia coli*. Appl Microbiol Biotechnol. 2013;97:7639–49.
42. McIver L, Leadbeater C, Campopiano DJ, Baxter RL, Daff SN, Chapman SK, Munro AW. Characterisation of flavodoxin NADP+ oxidoreductase and flavodoxin; key components of electron transfer in *Escherichia coli*. Eur J Biochem. 1998;257:577–85.
43. Jenkins CM, Waterman MR. NADPH− flavodoxin reductase and flavodoxin from *Escherichia coli*: characteristics as a soluble microsomal P450 reductase. Biochemistry. 1998;37:6106–13.
44. Tseng H-C, Martin CH, Nielsen DR, Prather KLJ. Metabolic engineering of *Escherichia coli* for enhanced production of (R)- and (S)-3-hydroxybutyrate. Appl Environ Microbiol. 2009;75:3137–45.
45. Brumaghim JL, Li Y, Henle E, Linn S. Effects of hydrogen peroxide upon nicotinamide nucleotide metabolism in *Escherichia coli*: changes in enzyme levels and nicotinamide nucleotide pools and studies of the oxidation of NAD(P)H by Fe(III). J Biol Chem. 2003;278:42495–504.
46. Hernández-Martín A, von Bühler CJ, Tieves F, Fernández S, Ferrero M, Urlacher VB. Whole-cell biotransformation with recombinant cytochrome P450 for the selective oxidation of Grundmann's ketone. Bioorganic Med Chem. 2014;22:5586–92.
47. Miroux B, Walker JE. Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol. 1996;260:289–98.
48. von Wachenfeldt C, Richardson TH, Cosme J, Johnson EF. Microsomal P450 2C3 is expressed as a soluble dimer in *Escherichia coli* following modifications of its N-terminus. Arch Biochem Biophys. 1997;339:107–14.
49. Neunzig J, Sánchez-Guijo A, Mosa A, Hartmann MF, Geyer J, Wudy SA, Bernhardt R. A steroidogenic pathway for sulfonated steroids: the metabolism of pregnenolone sulfate. J Steroid Biochem Mol Biol. 2014;144(Part B):324–33.
50. Sagara Y, Wada A, Takata Y, Waterman MR, Sekimizu K, Horiuchi T. Direct expression of adrenodoxin reductase in *Escherichia coli* and the functional characterization. Biol Pharm Bull. 1993;16:627–30.
51. Khatri Y, Girhard M, Romankiewicz A, Ringle M, Hannemann F, Urlacher V, Hutter M, Bernhardt R. Regioselective hydroxylation of norisoprenoids by CYP109D1 from *Sorangium cellulosum* So ce56. Appl Microbiol Biotechnol. 2010;88:485–95.
52. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–5.
53. Renart J, Reiser J, Stark GR. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc Natl Acad Sci USA. 1979;76:3116–20.

2.3 Schiffer *et al.* 2015b

Metabolism of oral-turinabol by human steroid hormone-synthesizing cytochromes P450

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Metabolism of oral-turinabol by human steroid hormone-synthesizing cytochromes P450

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Abstract

The human mitochondrial cytochromes P450 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), which is a common doping agent. By contrast, microsomal steroid hydroxylases did not convert OT. Spectroscopic binding assays revealed dissociation constants of 17.7 µM and 5.4 µM for CYP11B1 and CYP11B2, respectively, while 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 mg-scale with a recombinant Escherichia coli-based whole-cell system. They could be identified by NMR to be 11β-OH-

OT for both CYP11B isoforms, whereby CYP11B2 additionally formed 11β,18-diOH-OT and 11β-OH-OT-18-al, which rearranges to its tautomeric form 11β,18-expoxy-18-OH-OT. CYP11A1 produces six metabolites, which are proposed to include 2-OH-OT, 16-OH-OT and 2,16-diOH-OT based on LC-MS/MS 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 impact was demonstrated for CYP11B2. These findings suggest that steroidogenic cytochromes P450 can contribute to drug metabolism and should be considered in drug design and toxicity studies.

Abbreviations: AAS, anabolic androgenic steroids; AdR, adrenodoxin reductase; Adx, adrenodoxin; CPR, cytochrome P450 reductase; CYP, cytochrome P450, DOC, 11-deoxycorticosterone; IMAC, immobilized metal ion affinity chromatography; OT, oral-turinabol

Introduction

In humans most steps of steroid biosynthesis are catalyzed by monooxygenases from the cytochrome P450 superfamily (CYP), 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. Additionally, CYP11A1 can convert a variety of other sterol derivatives as well as vitamins D_2 and D_3 (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. CYP19 can, finally, aromatize androgens to estrogens (Bernhardt and Waterman, 2007). CYP 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 CYPs, 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 CYPs CYP17A1, CYP19 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). Very recently, several studies hint at an involvement of steroidogenic CYPs in the biotransformation of xenobiotic compounds, which is, according to traditional classifications of human CYPs, believed to be solely conducted by microsomal isoforms 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 (Zöllner et al., 2010; Parr et al., 2012) and there are even indications for the contribution of CYP11B1 to the bioactivation of the non-steroidal environmental pollutant 3-methylsulfonyl-2,2-bis(4-chlorophenyl)-1,1-dichloroethene (Lund and Lund, 1995).

In this study, we aimed to characterize the putative metabolism of the AAS oral-turinabol (OT) by steroid hormone-synthesizing CYPs to further expand our understanding of their substrate specificity and possible participation in biotransformation. Anabolic agents and, in particular, AAS are widely misused for doping purposes in all sports. Among them, especially 17α-alkylated AAS like OT are popular for their

oral availability, which is attributed to a reduction of the first pass-effect in the liver due to the 17α-alkylation (Fragkaki et al., 2009). However, AAS also frequently appear in adulterated nutritional supplements leading to an unintentional intake of them (Geyer et al., 2008). In anti-doping controls, they represent the most frequently detected class of substances prohibited by the World Anti-Doping Agency (WADA, 2014). Numerous side effects that include physical phenomena, such as cardiovascular risks (Angell et al., 2012; Deligiannis and Kouid, 2012) and increased risks of breast and Leydig cell cancer (Chimento et al., 2012; Sirianni et al., 2012) as well as psychiatric disorders (Palmié et al., 2013) are attributed to AAS. 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 fr agmentary. 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, the recruitment of specific co-activators, 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 CYPs on the molecular level in order to explore their drug-metabolizing capabilities. Therefore, we took advantage of the recombinant, high-yield expression of these enzymes in the bacterial host *Escherichia coli* (*E. coli*), which only recently became feasible for all of these CYPs. Dissociation constants were determined by UV/Vis-spectroscopy to enable a comparison with affinities towards endogenous substrates and kinetic studies on OT metabolism were carried out using a reconstituted *in-vitro* system with purified enzymes combined with HPLC analysis. Metabolites were characterized by tandem mass spectrometry or produced with an *E. coli* whole-cell system and purified for NMR characterization. Additionally, potential physiological consequences of OT metabolism were investigated by demonstrating the influence of OT on the natural CYP function in a reconstituted system and by studying the effect of the metabolites on MR transactivation in a reporter gene assay.

Materials and Methods

Chemicals

All reagents were obtained from standard sources with the highest purity available. OT (4-Chlor-17β-hydroxy-17α-methylandrosta-1,4-dien-3-on)

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), isopropyl β-D-1-thiogalactopyranoside and 5-aminolevulinic acid from Carbolution Chemicals (Saarbruecken, Germany).

Protein expression and purification

Expression and purification of CYPs

All CYPs were expressed with a C-terminal poly-histidine tag from a pET-17b or pET-22b (Invitrogen, Life Technologies, Carlsbad, CA) vector 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 IMAC and ion-exchange as presented for bovine CYP11A1 (Neunzig and Bernhardt, 2014) with slight modifications demanded for ion-exchange due to differences in pI. After IMAC the eluate was dialyzed over night against buffer A (20 mM potassium phosphate, pH 6.8, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTE, 1% Na-Cholate, 0.1% Tween 20) and applied to the 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% Na-Cholate, 0.1% Tween 20), which was the replaced by buffer C (50 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTE, 1% Na-Cholate, 0.05% Tween 20) by dialysis. Human CYP11B1, CYP11B2 and CYP21A2 were expressed and purified by IMAC and ion exchange as previously described by (Zöllner 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 (Khatri et al., 2014a; Petrunak et al., 2014) as well as human CYP19 according to (Khatri et al., 2014b). Final CYP concentrations were determined by CO-difference spectroscopy (Omura and Sato, 1964) with a molar extinction coefficient ε=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 $\varepsilon_{450\ nm}$=11.3 mM^{-1} cm^{-1} mM for AdR and ε_{414nm}=9.8 mM^{-1} cm^{-1} for Adx. CPR was expressed in C43(DE) as N-terminally truncated version with a 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 by 1 % of sodium cholate in all buffers and imidazole was used for washing and elution at 30 and 200 mM concentrations, respectively. For the removal of imidazole the protein was finally dialyzed against buffer C described above. CPR concentration was determined using ε_{585nm}=2.4 mM^{-1} cm^{-1} (Vermilion and Coon, 1978) for the semi-quinone form. Bovine cytochrome b_5 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 (DLPC). Prior to use, the buffer was sonicated in a sonication water bath for 5 minutes for the reconstitution of DLPC vesicles. The system contained 0.5 µM of the CYP, 1 mM $MgCl_2$ and 1 mM NADPH as well as a NADPH-regenerating system composed of 5 mM glucose-6-phosphate, 4U/ml glucose-6-phosphate dehydrogenase. For mitochondrial CYPs 0.5 µM AdR and 10 µM Adx were added, in case of microsomal CYPs 1 µM of CPR. For CYP17A1 reactions including 2 µM of bovine cytochrome b_5 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 % in case of cholesterol. 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 CYP11A1due 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 impact 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. In order to monitor CYP11A1-dependent conversion of cholesterol to pregnenolone at 240 nm, the samples were boiled for 5 min in a water bath after the respective reaction time and a subsequent cholesterol oxidase reaction was performed for 1 h at 37 °C, which enables detection of the steroids as cholestenone 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 CYP, 0.25 µM AdR and 4 µM Adx in case of CYP11B1 and to 0.1 µM CYP, 0.1 µM AdR and 2 µM Adx in case of

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 20 to 25 minutes for CYP11A1.

High Performance Liquid Chromatography (HPLC)-UV/vis detection

Steroids were separated on a Jasco reversed phase HPLC system (Jasco, Gross-Umstadt, Germany) using a 4.6 ×125 mm NucleoDur C18 Isis Reversed Phase column (Macherey-Nagel, Dueren, Germany) with an acetonitrile/water gradient at 40 °C and a flow rate of 0.8 mL/min. Steroid pattern was monitored by an UV/Vis detector (UV-2 075 Plus, Jasco, Gross-Umstadt, Germany) at 240 nm.

Spectroscopic binding assay

The determination of dissociation constants was performed by difference spectroscopy using tandem cuvettes as described (Schenkmann, 1970) with a Jasco V-630 Spectrophotometer (Jasco, Gross-Umstadt, Germany). 1 µM of CYP11B1 or CYP11B2 was diluted in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 20 % glycerol, 0.5 % sodium cholate and 0.05 % Tween20 were titrated with increasing concentrations of the steroid from stock solutions in DMSO and difference spectra were recorded from 350-500 nm. Titrations were performed 3 times. For the determination of the binding dissociation constant (K_d) the averaged ΔA (peak-to-trough absorbance difference) was plotted against the ligand concentration and plots were fitted with Origin 8.6 software by either hyperbolic regression ($\Delta A=(A_{max}[S]/K_d+[S])$) or tight binding quadratic equation ($\Delta A = (A_{max}/2[E])\{(K_d+[E]+[S]) - \{(K_d+[E]+[S])2 – 4[E][S]\}½ \}$, whereby ΔA represents the peak-to-trough absorbance difference at every ligand concentration, Amax the maximum absorbance difference at saturation, [E] the enzyme concentration (1 µM) and [S] the substrate concentration. For the measurement of binding spectra of CYP11A1 a 5 µM concentration of the enzyme was used and 5 µM Adx were 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 (rB- mB-) dcm Ion λ) were transformed by electroporation with the pET-17b based plasmid Twin_11B2 encoding human CYP11B2, bovine AdR and bovine Adx_{1-108} and the plasmid pGro12 for the co-synthesis of the molecular chaperones GroEL/ES (Nishihara et al., 1998). 150 mL TB media (24 g yeast extract technical, 12 g peptone, 4 mL glycerol, 4.62 g KH_2PO_4, 25 g K_2HPO_4 and distilled water ad. 1L) were supplemented with 100 µg/mL of ampicillin and 50 µg/mL of kanamycin in a 2 L-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-1-thiogalactopyranoside, 1 mM δ-aminolevulinic acid, 4 mg/mL arabinose and 50 µg/mL ampicillin and took place at 27.5 °C and 200 rpm for 24 h. For subsequent substrate conversion cells were harvested by centrifugation (3200*g*, 10 min, 18 °C), washed in 50 mM potassium phosphate buffer (pH 7.4) and suspended in 150 mL buffer supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside, 4 mg/mL arabinose, 1 mM δ-aminolevulinic acid, 50 µg/mL ampicillin and 2 % glycerol in 2 L-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 24h. 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 (Jasco, Gross-Umstadt, Germany) with a NucleoDur 100-5 5 x 250 mm C18 ec column (Macherey-Nagel, Dueren, Germany) and an acetronitrile/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 LC/MS.

LC-Q/TOF setup

Liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry (LC-MS/MS) measurements were conducted using an Agilent (Waldbronn, Germany) 6550 iFunnel Q-TOF LC-MS/MS instrument equipped with a Dual AJS electrospray ion 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 manufacturers' protocol allowing for mass errors < 5 ppm for the period of analysis. The mass analyzer acquired data from *m/z* 50 to 600 with an acquisition time of 200 ms/spectrum, and collision energies of MS/MS experiments were adjusted between 15 and 25 eV. Liquid chromatography was accomplished by means of an Agilent 1290 Infinity LC system equipped with an Agilent Eclipse XDB-C18 column (5 µm, 4.6 x 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 min, followed by a 4 min re-equilibration period at starting conditions.

NMR characterization of the major metabolites

The NMR spectra were recorded in $CDCl_3$ with a Bruker DRX 500 or a Bruker Avance 500 NMR spectrometer at 298 K. The chemical shifts were relative to $CHCl_3$ at δ 7.26 (^{1}H NMR) and $CDCl_3$ at δ 77.00 (^{13}C NMR), respectively, using the

standard δ notation in parts per million. The 1D NMR (^{1}H and ^{13}C NMR, DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments were based on extensive NMR spectral evidence.

Mineralocorticoid receptor transactivation assay

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

Results

In-vitro metabolism assay of OT by human steroidogenic CYPs

Conversion of OT by the human steroid hormone-synthesizing CYPs was assayed with recombinant proteins purified from *E. coli* as previously described (for references, see Materials and Methods). The natural electron transfer chain from NADPH to the CYP was reconstituted with human Adx and AdR for mitochondrial CYPs and CPR for the microsomal ones 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 isoforms of the CYP11B subfamily. In contrast, the microsomal CYPs, CYP17A1, CYP19 and CYP21A2, did not show any conversion of this AAS. CYP11B1- and CYP11B2-dependent conversion of OT showed distinct, but partially overlapping product patterns (**Figure 1**). Metabolites with the same retention times in the HPLC measurements are assumed to be the same OT derivatives. While 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 3 intermediate (**2**) or side products (**3** and **6**). For the CYP11B2-catalyzed reaction time-dependency of the product pattern could be observed. Peak area portions of metabolites **1** and **2** are reduced over the time while those of **4** and **5** are increasing (**Figure 1 C** and **D**), which suggests that **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 (**7**, **8** and **10**-**12**; HPLC chromatogram not shown). As product formation was weak compared with CYP11B1 and CYP11B2 and a preparative set up did not seem to be feasible, we subsequently followed with an LC-MS/MS approach to earn the maximal information about the metabolites of the CYP11A1-catalyzed conversion of OT.

Table 1: Metabolism of OT by human steroidogenic CYPs.

OT conversion was assayed in a reconstituted *in-vitro* system with NADPH and the natural redoxsystem (mitochondrial: AdR and Adx; microsomal: CPR) and was analyzed by HPLC. Product formation is indicated by +, no product forming activity by -. A positive control was performed with a natural substrate. cyt b_5= cytochrome b_5, Prog=progesterone, Andro=androstenedione.

Cytochrome P450	Redox system	OT conversion	Control reaction
CYP11A1	mitochondrial	+	Cholesterol → Pregenenolone
CYP11B1	mitochondrial	+	DOC → Corticosterone
CYP11B2	mitochondrial	+	DOC → Corticosterone
CYP17A1	microsomal	-	Prog → 17-OH-Prog
	microsomal		17-OH-Prog → Andro
	+ cyt b_5		
CYP19A1	microsomal	-	Andro → Esterone
CYP21A2	microsomal	-	Prog → DOC

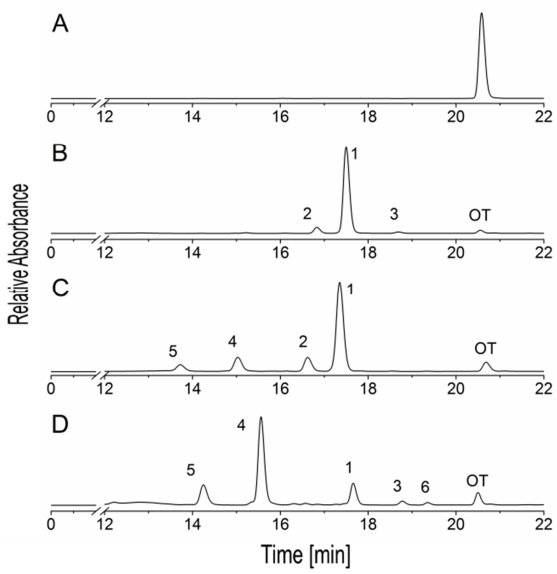

Fig. 1: RP-HPLC chromatograms of the *in-vitro* conversion assays of OT by human CYP11B1 and CYP11B2. 100 µM OT were incubated at 37 °C with a reconstituted CYP-system consisting of 0.5 µM CYP, 10 µM Adx and 0.5 µM AdR, 1 mM NADPH and an NADPH regenerating system. **A** - Incubation without NADPH for 30 min as negative control. **B** - CYP11B1-dependent conversion of OT for 30 min. **C** and **D** - CYP11B2-dependent conversion of OT for 10 min and 30 min, respectively.

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. In **Figure 2**, the extracted ion chromatograms of mono- and dihydroxylated OT are illustrated, suggesting the formation of at least six metabolites (metabolites **7-12**). The product ion mass spectrum of metabolite **7** is depicted in **Figure 3 A**, 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 **7** but at substantially different abundances (**Figure 3 B**). Moreover, intense product ions at *m/z* 205 and 207 complemented this spectrum, which were suggested to originate also from the steroidal A/B-ring based on the observed accurate masses and corresponding elemental compositions ($C_{12}H_{10}ClO$ and $C_{12}H_{12}ClO$, 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 (**Figure 3 C**), 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 while **9** predominantly generated a product ion at *m/z* 315.195 as a result of a HCl elimination (**Figure 3 C**, **inset**), which further corroborated an A/B-ring modification of this analyte.

In addition to the aforementioned monohydroxylated metabolites of OT, also dihydroxylated species were observed by means of the accurate mass of the protonated molecules (**Figure 2**, compounds **10**-**12**). Due to 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** (**Figure 3 D**). Here, the same product ion as observed in case of 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 π-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.

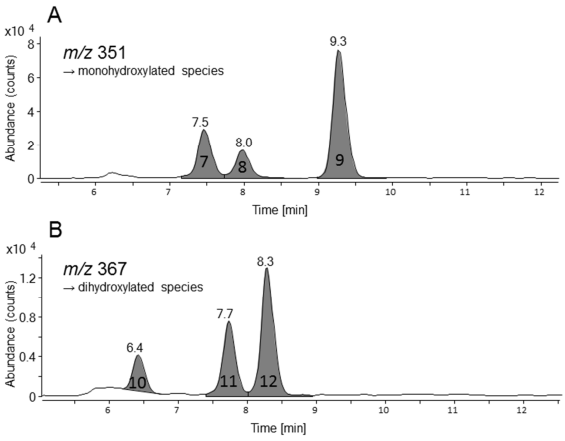

Fig. 2: Extracted ion chromatograms of CYP11A1-derived mono- and dihydroxylated OT. 100 µM OT were incubated at 37 °C with a reconstituted CYP-system consisting of 0.5 µM CY11A1, 10 µM Adx and 0.5 µM AdR, 1 mM NADPH and an NADPH regenerating system.Protonated molecules of steroids $[M+H]^+$ as measured by LC-ESI-MS are shown with **A** - metabolites **7**, **8** and **9** representing monohydroxylated OT (*m/z* 351), and **B** - metabolites **10**, **11** and **12** representing dihydroxylated OT (*m/z* 367).

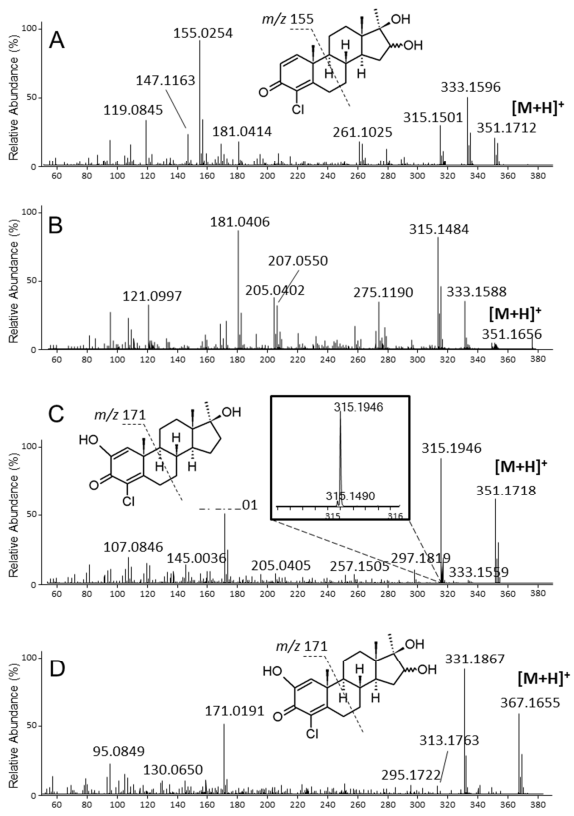

Fig. 3: Product ion mass spectra of protonated CYP11A1-derived OT products at *m/z* 351 and *m/z* 367. A - Product **7**: Product ion mass spectrum of *m/z* 351, collision energy: 15 eV; RT= 7.5 min. **B** – Product **8**: Product ion mass spectrum of *m/z* 351, collision energy: 15 eV; RT =8.0 min. **C** – Product **9**: Product ion mass spectrum of *m/z* 351, collision energy: 25 eV; RT = 9.3 min. **D** – Product **11**: Product ion mass spectrum of *m/z* 367, collision energy: 25 eV; RT = 7.7 min. Proposed structures of the respective OT metabolites are shown. Fragmentation and the resulting fragment ion masses are indicated.

Determination of dissociation constants and kinetic parameters

In order 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 the CYP with increasing concentrations of OT (**Figure 4**). CYP11B2 showed a K_d value of 5.4 ± 0.4 µM, while 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 CYP concentrations and in the presence of Adx (**Figure 4 C**). The observation of a type-II difference spectrum with OT arises unexpectedly 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. The spin state equilibrium can, however, 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 towards the low-spin state.

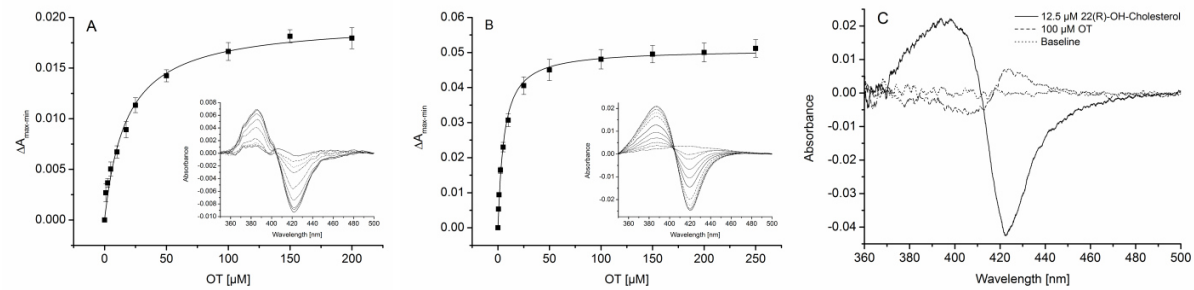

Fig. 4: Spectroscopic characterization of OT binding by the CYP11 family. A, **B** - Determination of OT affinity to CYP11B1 (**A**) and CYP11B2 (**B**) by difference spectroscopy and regression of the absorbance shift. 1 µM of the respective CYP11B isoform was titrated with increasing concentrations of OT in tandem cuvettes and difference spectra were recorded from 350-500 nm (**insets**). Peak to trough differences of the resulting type-I spectra were plotted against the OT concentration and dissociation constants were determined by hyperbolic regression for CYP11B1 (R^2 0.98) and tight binding quadratic equation for CYP11B2 (R^2 0.99). Measurements were performed in triplicates. **C** – Difference spectra of CYP11A1 upon addition of OT and 22(*R*)-OH-cholesterol. 5 µM of CYP11A1 were supplemented with its substrates in saturating concentrations in the presence of Adx at equimolar concentration.

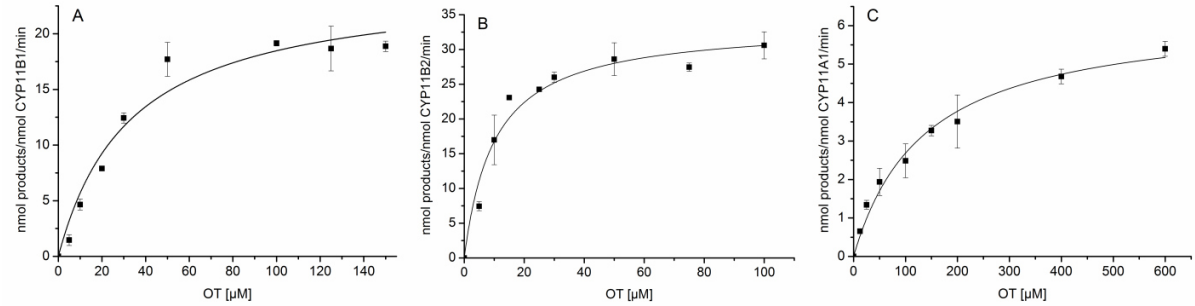

Fig. 5: Determination of kinetic parameters for OT conversion by human CYP11B1 (A), CYP11B2 (B) and CYP11A1 (C) in a reconstituted *in-vitro* system. The system was reconstituted with the respective CYP, Adx and AdR at a ratio of 1:20:1. Reactions took place at 37 °C in presence of an NADPH-regenerating system and were stopped under steady-state conditions. OT consumption was determined by HPLC using progesterone or cortisol as an internal standard. All values represent the mean of triplicates with standard deviation. Kinetic parameters were ascertained by hyperbolic regression (R^2 0.96 for CYP11B1 and CYP11B2, R^2 0.98).

Moreover, we determined kinetic parameters of OT conversion by performing *in-vitro* reactions under steady-state conditions and quantification of OT consumption (**Figure 5**). Molar ratios of the CYP and its redox partners were 1:20:1 (CYP:Adx:AdR) as the excess of Adx excludes the electron transfer to the CYP as limiting step and maximum activities of CYP11 systems are observed at CYP:AdR ratios ≥1 upon Adx excess (Seybert et al., 1978; Seybert et al., 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 efficieny k_{cat}/K_M are summarized in **Table 2**.

Table 2: Kinetic parameters of OT conversion by CYP11B1, CYP11B2 and CYP11A1 determined as shown in Figure 5.

	K_M [µM]	k_{cat} [min^{-1}]	$kcat/K_M$ [min-1mM-1]
CYP11B1	33 ± 9	25 ± 2	741
CYP11B2	10 ± 2	34 ± 2	3338
CYP11A1	136 ± 21	6 ± 0.4	46

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 OT to inhibit steroid biosynthesis on enzymatic level (**Figure 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 five-fold excess of OT over the respective natural substrate, CYP11A1- and CYP11B1-dependent product formation was reduced to 42 and 49 %, respectively, while 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.

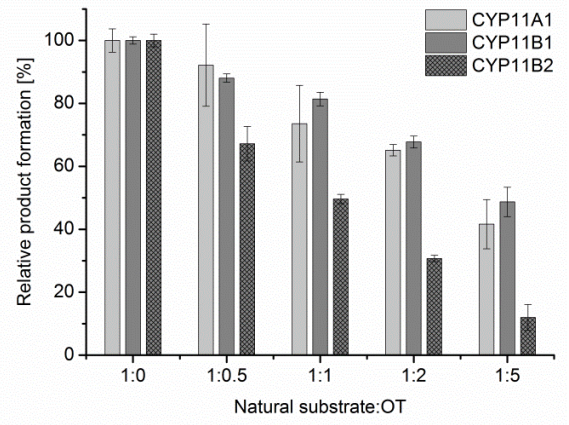

Fig. 6: Relative product formation from the natural substrates in the presence of OT in a reconstituted *in-vitro* system. The system consisted of 0.5 µM CYP, 10 µM Adx and 0.5 µM AdR, 1 mM NADPH and an NADPH regenerating system and the reaction took place at 37 °C for 1 min for the CYP11B isoforms and 10 min for CYP11A1. DOC was used as a substrate for CYP11B1 and CYP11B2, cholesterol for CYP11A1. The substrate concentration was 20 µM to ensure conditions below the saturation but in an excess over the CYP and OT was added in the indicated ratios. DOC consumption or pregnenolone formation was determined by HPLC using progesterone or cortisol, respectively, as an internal standard. Each bar represents the mean of three reactions with respective standard deviation.

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). As CYP11B1 products are also formed by CYP11B2, application of CYP11B2 was chosen. The complete redox chain was transferred into *E. coli* by co-expression of *CYP11B2*, bovine *AdR* and truncated bovine

Adx_{1-108} 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 non-growing conditions to maximize the availability of NADPH for the CYP11B2 reaction. After 24 h 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 mg amounts by preparative HPLC.

NMR characterization of the OT metabolites

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

Metabolite **1** - *11β-OH-OT* (4-Chlor-11β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one).
In comparison to oral-turinabol the ^{1}H and ^{13}C NMR spectra of its conversion metabolite **1** 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 2D NMR. For example its proton showed correlations to H-9 (δ1.07, dd) and H-12a (δ1.57, dd) and H-12b (δ1.64, dd) in the HHCOSY. H-11 must be in equatorial position due to its small coupling constants to H-9 (J=3.7 Hz) and to both H-12(J=2.5 and 3.7 Hz) and is therefore in α-orientation. In consequence of this fact the hydroxyl at C-11 is β-orientated. ^{1}H NMR ($CDCl_3$,500 MHz): 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, 3x H-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), 7.32 (d, 10.0 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz): 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), 38.67 (CH2, C-16), 41.00 (CH2, C-12), 44.78 (C, C-13), 46.79 (C, C-10), 50.94 (CH, C-14), 56.22 (CH, C-9), 70.36 (CH, C-11), 81.76 (C, C-17), 126.62 (CH, C-2), 127.23 (CH, C-4), 155.84 (CH, C-1), 163.37 (C, C-5), 178.57 (C, C-3).

Metabolite **4** - *11β,18-Di-OH-OT* (4-Chlor-11β,17β,18-trihydroxy-17α-methylandrosta-1,4-dien-3-one). The NMR spectra of metabolite **4** were similar to those of **1**, especially for the resonances of C-11 (δC 69.12) and H-11 (δH 4.39 q, 3.3 Hz) as well as the coupling pattern of H-11, but P2 lacked of resonances for methyl C-18. Therefore, signals for a primary hydroxyl group at δH 4.00 (d, 11.3 Hz), 4.04 (d, 11.3 Hz) and δC 63.49 appeared in the spectra. Data thus obtained led to the 11β, 18 dihydroxy derivative of oral-turinabol. 2D NMR measurements supported the structure and led to the full

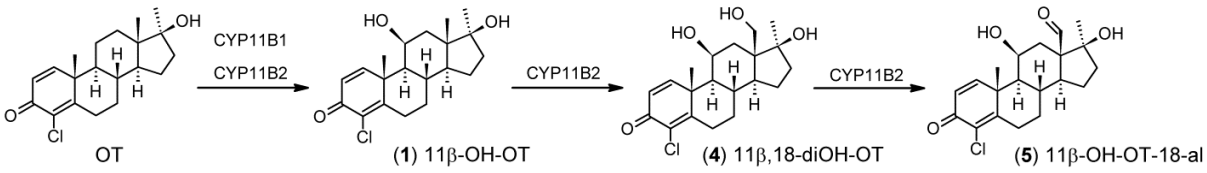

Fig. 7: Hydroxylation and oxidation of OT catalyzed by the human CYP11B subfamily. Structures were revealed by NMR spectroscopy after CYP-dependent whole-cell catalysis in *E. coli*.

assignments. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.03 (dd, 11.3 and 3.3 Hz, H-9), 1.05 (m, H-7a), 1.20 (s, 3xH-20), 1.21 (m, H-14), 1.29 (m, H-12a), 1.44 (m, H-15a), 1.54 (s, 3xH-19), 1.69 (m, H-15b), 1.82 (ddd, 14.0, 9.5 and 7.0 Hz, H-16a), 2.08 (m, H-16b), 2.09 (m, H-7b), 2.20 (m, H-8), 2.33 (m, H-12b), 2.39 (td, 14.0 and 5.4 Hz, H-6a), 3.25 (ddd, 14.0, 4.8 and 2.7 Hz, H-6b), 4.00 (d, 11.3 Hz, H-18a), 4.04 (d, 11.3 Hz, H-18b), 4.39 (q, 3.3 Hz, H-11), 6.39 (d, 10.0 Hz, H-2), 7.38 (d, 10.0 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 21.16 (CH3, C-19), 23.97 (CH2, C-15), 27.04 (CH3, C-20), 28.21 (CH2, C-6), 32.07 (CH, C-8), 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 (CH2, C-18), 69.12 (CH, C-11), 83.49 (C, C-17), 126.44 (CH, C-2), 127.24 (CH, C-4), 156.10 (CH, C-1), 163.08 (C, C-5), 178.57 (C, C-3).

Metabolite **5** - *11β,18-Epoxy-18-OH-OT* (4-Chlor-11β,18-epoxy-17β,18-dihydroxy-17α-methylandrosta-1,4-dien-3-one). The NMR spectra of metabolite **5** revealed a (11-18)-hemi-acetal function. This was obvious by the characterictic 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 derivate of oral-turinabol. ^{1}H NMR ($CDCl_3$, 500 MHz): 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 (td, 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), 7.14 (d, 10.0 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 20.40 (CH3, C-19), 23.34 (CH2, C-15), 27.40 (CH3, C-20), 28.83 (CH2, C-6), 31.07 (CH2, 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 (C, 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), 178.13 (C, C-3).

Mineralocorticoid receptor activation assay with the CYP11B-derived OT metabolites CYP11B-derived OT metabolites carry the same oxy-functionalizations as are introduced into the steran scaffold during the biosynthesis of natural

mineralocorticoids. Therefore, we evaluated their potential to activate the human mineralocorticoid receptor in order 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 2-20,000 pM, which resulted in full dose-response for the natural MR ligand aldosterone. The respective EC_{50} 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 30 % of the maximum aldosterone response (**Figure 8**). The modifications at C_{11} and C_{18} introduced by CYP11B2 did not alter the agonist potential of OT.

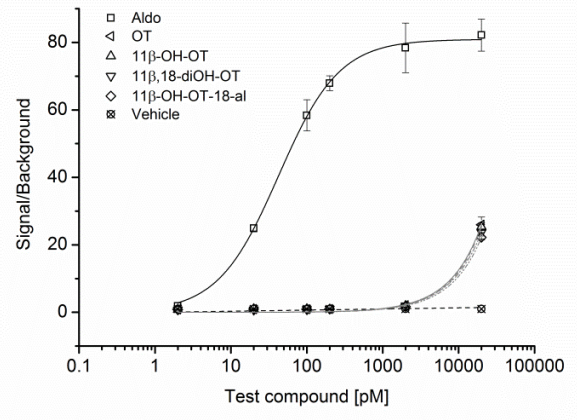

Fig. 8: Dose-response analysis of human MR activation by OT and its CYP11B-derived metabolites. Dilutions of each compound were analyzed with INDIGO Bioscience Human Mineralocorticoid Receptor Reported Assay System following the manufacturer's protocol. The luminescence signal of MR-responsive luciferase reporter gene expression was normalized to the signal of the vehicle control and is plotted against the test compound concentration in logarithmic scale. The assay was performed in triplicates for each concentration. The black line indicates the Hill-regression of the aldosterone induced response (R^2=0.998), the dashed line shows the vehicle background signal.

Discussion

Human CYPs are traditionally classified into a group of drug-metabolizing CYPs 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 CYPs were tested for their activity towards OT. While no conversion of OT was found using CYP17A1, CYP19 and CYP21A2, the three mitochondrial CYPs, 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 K_d only approximately four-fold higher than that for the natural substrate DOC reported to be 1.34 µM (Hobler et al., 2012) and the catalytic efficiency (k_{cat}/K_M) of OT conversion by CYP11B1 and CYP11B2 is even slightly higher than previously

determined for the natural substrates (Zöllner 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 towards 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 so far. We can hypothesize the structure of metabolites based on studies on natural substrates (Kawamoto 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, which 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 the second group in OT does conclusively not negatively influence the selectivity or advancement of the catalytic reaction. However, the 3keto-Δ^4 motif, which was already supposed to be conserved among all CYP11B substrates using endogenous steroids (Strushkevich et al.), 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 position 11β and 18 (Parr et al., 2012). When compared with the metabolites formed from OT by CYP11B2, the differences between OT and metandienone metabolism lead to the suggestion that the C_4 substitution of OT determines the processivity of the reaction.

It is worth mentioning that the conducted bioconversion of OT by CYP11B2 at preparative scale represents the first application of human CYP11B2 for substrate conversion in *E. coli*, which has so far only been used for biotechnological purposes in recombinant yeast strains (Bureik et al., 2002b). Despite working under non-optimized shake flask conditions, the volumetric productivity of OT consumption of approximately 30 $mg*L^{-1}*d^{-1}$ 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 (Ardhanari et al., 2015), whose medicinal treatment by selective CYP11B2 inhibitors currently regains interest (Andersen, 2013; Hargovan and Ferro, 2014; Namsolleck and Unger, 2014). The presented system might alternatively serve as simple, economic pre-screening for the effectiveness of potential inhibitors upon down-scaling to a multi-well format.

Compared with the two CYP11B enzymes, CYP11A1 exhibits a lower efficiency of OT conversion (46 min^{-1} mM^{-1} compared with 741 and 3338), which is also lower than for the natural substrate cholesterol. Bovine CYP11A1 shows an efficiency of approximately 85 min^{-1} mM^{-1} for cholesterol (Neunzig and Bernhardt, 2014; Mosa et al., 2015). The low catalytic efficiency for OT is, however, consistent with the observed activities towards endogenous steroids (Mosa et al., 2015) and the preference of CYP11A1 for longer side-chains (Masuo et al., 1980). Due to 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 a 2β-, 6β- and 16β-hydroxylase activity of CYP11A1 for steroids (Mosa et al., 2015) (**Figure 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 putative structure. Metabolite **11** seems to carry a combination of these two hydroxylations 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-hydroxymethyl derivatives (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.

Following the studies about the biotransformation of OT by the human mitochondrial CYPs, we were also interested to examine the effect of OT on the activity of these CYPs towards their endogenous substrates. An inhibition of the natural function of the OT metabolizing CYPs seemed likely, as both, OT and the natural substrate, 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 the CYPs for OT resulting in the highest inhibitory effect on CYP11B2. Potential physiological relevance is, however, the result of concentrations in the microenvironment of steroid-synthesizing enzymes and can hardly be assessed at this time. Cholesterol level in the inner mitochrondrial 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-norandrostenediol and its active metabolite nandrolone were reported to be in the range of several hundreds of nanogramms per 100 mL after a single dose of 25-100 mg (Schrader et al., 2006), which is in excess over DOC and 11-deoxycortisol plasma levels (Mason and Fraser, 1975). Exact 19-norandrostenediol 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 CYPs. 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 rat, whose MR-agonist properties induce increases of blood pressure (Colby et al., 1970). However, this phenomenon has so far only been explained on 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 hydrogen bond network between the steroid ligand C_{11} and C_{18} 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 do also not show a relevant activation of the MR, although they carry oxy-functionalization at C_{11} and C_{18}. 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, as they lack the C_{20}-carbonyl and C_{21}-hydroxyl functions, which interact with the receptor in case of the native ligands (Bledsoe et al., 2005). Additionally, 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 tetrahydrogestrinone, which has a C_{17}-ethyl group (Friedel et al., 2006b).

Summarizing the results, it was clearly demonstrated that OT is, in addition to metandienone (Parr et al., 2012), the second xenobiotic steroid whose metabolism by steroidogenic CYPs has been observed. Our

detailed *in-vitro* studies hint at a potentially systematic contribution of human steroidogenic CYPs 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 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 (post-operative) steroid replacement purposes. Product properties might differ from those of the parental compound and the interference with the endogenous steroid biosynthesis can cause severe adverse effects.

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Authorship Contributions

Participated in research design: Schiffer, Thevis, Bernhardt

Conducted experiments: Schiffer, Brixius-Anderko, Zapp, Neunzig

Performed data analysis: Schiffer, Zapp, Thevis

Wrote or contributed to the writing of the manuscript: Schiffer, Hannemann, Thevis, Bernhardt

References

Andersen K (2013) Aldosterone Synthase Inhibition in Hypertension. Curr Hypertens Rep 15:484-488.

Angell PJ, Chester N, Sculthorpe N, Whyte G, George K, and Somauroo J (2012) Performance enhancing drug abuse and cardiovascular risk in athletes: implications for the clinician. British Journal of Sports Medicine 46:i78-i84.

Arase M, Waterman MR, and Kagawa N (2006) Purification and characterization of bovine steroid 21-hydroxylase (P450c21) efficiently expressed in Escherichia coli. Biochemical and Biophysical Research Communications 344:400-405.

Ardhanari S, Kannuswamy R, Chaudhary K, Lockette W, and Whaley-Connell A (2015) Mineralocorticoid and Apparent Mineralocorticoid Syndromes of Secondary Hypertension. Advances in Chronic Kidney Disease 22:185-195.

Bernhardt R and Waterman MR (2007) Cytochrome P450 and Steroid Hormone Biosynthesis, in: The Ubiquitous Roles of Cytochrome P450 Proteins, pp 361-396, John Wiley & Sons, Ltd.

Bledsoe RK, Madauss KP, Holt JA, Apolito CJ, Lambert MH, Pearce KH, Stanley TB, Stewart EL, Trump RP, Willson TM, and Williams SP (2005) A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor. The Journal of biological chemistry 280:31283-31293.

Brownie AC, Bhasker CR, and Waterman MR (1988) Levels of adrenodoxin, NADPH-cytochrome P-450 reductase and cytochromes P-45011β, P-45021, P-450scc, in adrenal zona fasciculata-reticularis tissue from androgen-treated rats. Molecular and Cellular Endocrinology 55:15-20.

Brownie AC, Simpson ER, Skelton FR, Elliott wB, and Estabrook RW (1970) Interaction of androgens with the adrenal mitochondrial cytochrome system: The influence of androgen treatment on the levels of cytochrome in rat adrenal cortical mitochondria and

on substrate-induced spectral changes. Archives of Biochemistry and Biophysics 141:18-25.

Bureik M, Lisurek M, and Bernhardt R (2002a) The Human Steroid Hydroxylases CYP11B1 and CYP11B2. Biological Chemistry 383:1537-1551.

Bureik M, Schiffler B, Hiraoka Y, Vogel F, and Bernhardt R (2002b) Functional Expression of Human Mitochondrial CYP11B2 in Fission Yeast and Identification of a New Internal Electron Transfer Protein, etp1. Biochemistry 41:2311-2321.

Chimento A, Sirianni R, Zolea F, De Luca A, Lanzino M, Catalano S, Andò S, and Pezzi V (2012) Nandrolone and stanozolol induce leydig cell tumor proliferation through an estrogen-dependent mechanism involving IGF-I system. Journal of Cellular Physiology 227:2079-2088.

Chung BC, Matteson KJ, Voutilainen R, Mohandas TK, and Miller WL (1986) Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta. Proceedings of the National Academy of Sciences of the United States of America 83:8962-8966.

Colby HD, Skelton FR, and Brownie AC (1970) Testosterone-Induced Hypertension in the Rat. Endocrinology 86:1093-1101.

Deligiannis A, Björnstad H, Carre F, Heidbüchel H, Kouidi E, Panhuyzen-Goedkoop NM, Pigozzi F, Schänzer W, Vanhees L, and Cardiology obotESGoS (2006) ESC Study Group of Sports Cardiology Position Paper on adverse cardiovascular effects of doping in athletes. European Journal of Cardiovascular Prevention & Rehabilitation 13:687-694.

Deligiannis AP and Kouid EI (2012) Cardiovascular Adverse Effects of Doping in Sports. Hellenic Journal of Cardiology:447-457.

Fragkaki AG, Angelis YS, Koupparis M, Tsantili-Kakoulidou A, Kokotos G, and Georgakopoulos C (2009) Structural characteristics of anabolic androgenic steroids contributing to binding to the androgen receptor and to their anabolic and androgenic activities: Applied modifications in the steroidal structure. Steroids 74:172-197.

Friedel A, Geyer H, Kamber M, Laudenbach-Leschowsky U, Schänzer W, Thevis M, Vollmer G, Zierau O, and Diel P (2006a) 17β-Hydroxy-5alpha-androst-1-en-3-one (1-testosterone) is a potent androgen with anabolic properties. Toxicology Letters 165:149-155.

Friedel A, Geyer H, Kamber M, Laudenbach-Leschowsky U, Schänzer W, Thevis M, Vollmer G, Zierau O, and Diel P (2006b) Tetrahydrogestrinone is a potent but unselective binding steroid and affects glucocorticoid signalling in the liver. Toxicology Letters 164:16-23.

Funder M, John W. (1997) Glucocorticoid and Mineralocorticoid Receptors: Biology and Clinical Relevance. Annual Review of Medicine 48:231-240.

Galigniana MD, Piwien Pilipuk G, Kanelakis KC, Burton G, and Lantos CP (2004) Molecular mechanism of activation and nuclear translocation of the mineralocorticoid receptor upon binding of pregnanesteroids. Molecular and Cellular Endocrinology 217:167-179.

Gallant S, Alfano J, Charpin M, and Brownie AC (1991) Expression of adrenal cytochromes P-450 in testosterone-induced hypertension. Hypertension 18:523-528.

Geyer H, Parr MK, Koehler K, Mareck U, Schänzer W, and Thevis M (2008) Nutritional supplements cross-contaminated and faked with doping substances. Journal of Mass Spectrometry 43:892-902.

Groenhof AR (2007) Theoretical Investigation of the Hydroxylation Mechanism by Cytochromes P450, University of Amsterdam, Amsterdam.

Guengerich FP (2001) Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity. Chemical Research in Toxicology 14:611-650.

Hannemann F, Bichet A, Ewen KM, and Bernhardt R (2007) Cytochrome P450 systems—biological variations of electron transport chains. Biochimica et

Biophysica Acta (BBA) - General Subjects 1770:330-344.

Hargovan M and Ferro A (2014) Aldosterone synthase inhibitors in hypertension: current status and future possibilities. JRSM Cardiovascular Disease 3.

Hobler A, Kagawa N, Hutter MC, Hartmann MF, Wudy SA, Hannemann F, and Bernhardt R (2012) Human aldosterone synthase: Recombinant expression in E. coli and purification enables a detailed biochemical analysis of the protein on the molecular level. The Journal of Steroid Biochemistry and Molecular Biology 132:57-65.

Julsing MK, Cornelissen S, Bühler B, and Schmid A (2008) Heme-iron oxygenases: powerful industrial biocatalysts? Current Opinion in Chemical Biology 12:177-186.

Kawamoto T, Mitsuuchi Y, Ohnishi T, Ichikawa Y, Yokoyama Y, Sumimoto H, Toda K, Miyahara K, Kuribayashi I, Nakao K, Hosoda K, Yamamoto Y, Imura H, and Shizuta Y (1990) Cloning and expression of a cDNA for human cytochrome P-450aldo as related to primary aldosteronism. Biochemical and Biophysical Research Communications 173:309-316.

Khatri Y, Gregory MC, Grinkova YV, Denisov IG, and Sligar SG (2014a) Active site proton delivery and the lyase activity of human CYP17A1. Biochemical and Biophysical Research Communications 443:179-184.

Khatri Y, Luthra A, Duggal R, and Sligar SG (2014b) Kinetic solvent isotope effect in steady-state turnover by CYP19A1 suggests involvement of Compound 1 for both hydroxylation and aromatization steps. FEBS Letters 588:3117-3122.

Lambeth JD, Seybert D, Lancaster J, Jr., Salerno J, and Kamin H (1982) Steroidogenic electron transport in adrenal cortex mitochondria. Mol Cell Biochem 45:13-31.

Li Y, Suino K, Daugherty J, and Xu HE (2005) Structural and Biochemical Mechanisms for the Specificity of Hormone Binding and Coactivator Assembly by Mineralocorticoid Receptor. Molecular Cell 19:367-380.

Lund B-O and Lund J (1995) Novel Involvement of a Mitochondrial Steroid Hydroxylase (P450c11) in Xenobiotic Metabolism. Journal of Biological Chemistry 270:20895-20897.

Mason PA and Fraser R (1975) Estimation of Aldosterone, 11-Deoxycorticosterone, 18-Hydroxy-11-deoxycorticosterone, Corticosterone, Cortisol And 11-Deoxycortisol in human plasma by gas-liquid chromatography with electron capture detection. Journal of Endocrinology 64:277-288.

Masuo M, Carmenza D, Nobuo I, and Mikio S (1980) Substrate specificity of adrenocortical cytochrome P-450scc - I. Effect of structural modification of cholesterol side-chain on pregnenolone production. Journal of Steroid Biochemistry 13:545-550.

Melchert RB and Welder AA (1995) Cardiovascular effects of androgenic-anabolic steroids. Medicine & Science in Sports & Exercise 27:1252-1262.

Mosa A, Neunzig J, Gerber A, Zapp J, Hannemann F, Pilak P, and Bernhardt R (2015) 2β- and 16β-hydroxylase activity of CYP11A1 and direct stimulatory effect of estrogens on pregnenolone formation. The Journal of Steroid Biochemistry and Molecular Biology 150:1-10.

Namsolleck P and Unger T (2014) Aldosterone synthase inhibitors in cardiovascular and renal diseases. Nephrology Dialysis Transplantation 29:i62-i68.

Neunzig J and Bernhardt R (2014) Dehydroepiandrosterone Sulfate (DHEAS) Stimulates the First Step in the Biosynthesis of Steroid Hormones. PLoS ONE 9:e89727.

Neunzig J, Sánchez-Guijo A, Mosa A, Hartmann MF, Geyer J, Wudy SA, and Bernhardt R (2014) A steroidogenic pathway for sulfonated steroids: The metabolism of pregnenolone sulfate. The Journal of Steroid Biochemistry and Molecular Biology 144, Part B:324-333.

Nishihara K, Kanemori M, Kitagawa M, Yanagi H, and Yura T (1998) Chaperone Coexpression Plasmids: Differential and Synergistic Roles of DnaK-DnaJ-GrpE and GroEL-GroES in Assisting Folding of an Allergen of Japanese Cedar Pollen, Cryj2, in

Escherichia coli. Applied and Environmental Microbiology 64:1694-1699.

Omura T and Sato R (1964) The Carbon Monoxide-binding Pigment of Liver Microsomes: II. Solubilization, Purification and Properties. Journal of Biological Chemistry 239:2379-2385.

Palmié N, Peyrière H, Eiden C, and Condemine-Piron C (2013) Misuse of Anabolic Products in Sport: a Retrospective Study of Phone Calls at the Écoute Dopage Anti-doping Hot-line. Therapie 68:27-29.

Parr MK, Zöllner A, Fußhöller G, Opfermann G, Schlörer N, Zorio M, Bureik M, and Schänzer W (2012) Unexpected contribution of cytochrome P450 enzymes CYP11B2 and CYP21, as well as CYP3A4 in xenobiotic androgen elimination – Insights from metandienone metabolism. Toxicology Letters 213:381-391.

Petrunak EM, DeVore NM, Porubsky PR, and Scott EE (2014) Structures of Human Steroidogenic Cytochrome P450 17A1 with Substrates. Journal of Biological Chemistry 289:32952-32964.

Pozo OJ, Van Eenoo P, Deventer K, Grimalt S, Sancho JV, Hernández F, and Delbeke FT (2008) Collision-induced dissociation of 3-keto anabolic steroids and related compounds after electrospray ionization. Considerations for structural elucidation. Rapid Communications in Mass Spectrometry 22:4009-4024.

Rendic S, Nolteernsting E, and Schänzer W (1999) Metabolism of anabolic steroids by recombinant human cytochrome P450 enzymes: Gas chromatographic–mass spectrometric determination of metabolites. Journal of Chromatography B: Biomedical Sciences and Applications 735:73-83.

Rockhold RW (1993) Cardiovascular Toxicity of Anabolic Steroids. Annual Review of Pharmacology and Toxicology 33:497-520.

Sagara Y, Wada A, Takata Y, Waterman MR, Sekimizu K, and Horiuchi T (1993) Direct expression of adrenodoxin reductase in Escherichia coli and the functional characterization. Biol Pharm Bull 16:627-630.

Sandee D and Miller WL (2011) High-Yield Expression of a Catalytically Active Membrane-Bound Protein: Human P450 Oxidoreductase. Endocrinology 152:2904-2908.

Schänzer W (1996) Metabolism of anabolic androgenic steroids. Clinical Chemistry 42:1001-1020.

Schänzer W, Horning S, Opfermann G, and Donike M (1996) Gas chromatography/mass spectrometry identification of long-term excreted metabolites of the anabolic steroid 4-chloro-1,2-dehydro-17α-methyltestosterone in humans. The Journal of Steroid Biochemistry and Molecular Biology 57:363-376.

Schenkmann JB (1970) Studies on the nature of the type I and type II spectral changes in liver microsomes. Biochemistry 9:2081-2091.

Schiffer L, Anderko S, Hannemann F, Eiden-Plach A, and Bernhardt R (2015a) The CYP11B subfamily. The Journal of Steroid Biochemistry and Molecular Biology 151:38-51.

Schiffer L, Anderko S, Hobler A, Hannemann F, Kagawa N, and Bernhardt R (2015b) A recombinant CYP11B1 dependent Escherichia coli biocatalyst for selective cortisol production and optimization towards a preparative scale. Microbial Cell Factories 14:25.

Schiffler B, Zöllner A, and Bernhardt R (2004) Stripping Down the Mitochondrial Cholesterol Hydroxylase System, a Kinetics Study. Journal of Biological Chemistry 279:34269-34276.

Schrader Y, Thevis M, and Schänzer W (2006) Quantitative determination of metabolic products of 19-Norandrostenediol in human plasme using gas chromatography/mass spectrometry. Drug Metabolism and Disposition 34:1328-1335.

Seybert DW, Lambeth JD, and Kamin H (1978) The participation of a second molecule of adrenodoxin in cytochrome P-450-catalyzed 11beta hydroxylation. Journal of Biological Chemistry 253:8355-8358.

Seybert DW, Lancaster JR, Lambeth JD, and Kamin H (1979) Participation of the membrane in the side chain cleavage of cholesterol. Reconstitution of

cytochrome P-450scc into phospholipid vesicles. Journal of Biological Chemistry 254:12088-12098.

Sirianni R, Capparelli C, Chimento A, Panza S, Catalano S, Lanzino M, Pezzi V, and Andò S (2012) Nandrolone and stanozolol upregulate aromatase expression and further increase IGF-I-dependent effects on MCF-7 breast cancer cell proliferation. Molecular and Cellular Endocrinology 363:100-110.

Slominski AT, Li W, Kim T-K, Semak I, Wang J, Zjawiony JK, and Tuckey RC (2015) Novel activities of CYP11A1 and their potential physiological significance. The Journal of Steroid Biochemistry and Molecular Biology 151:25-37.

Sobolevsky T and Rodchenkov G (2012) Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine. The Journal of Steroid Biochemistry and Molecular Biology 128:121-127.

Strushkevich N, Gilep AA, Shen L, Arrowsmith CH, Edwards AM, Usanov SA, and Park H-W (2013) Structural Insights into Aldosterone Synthase Substrate Specificity and Targeted Inhibition. Molecular Endocrinology 27:315-324.

Sugiyama T and Yamano T (1975) Purification and crystallization of NADPH-adrenodoxin reductase from bovine adrenocortical mitochondria. FEBS Letters 52:145-148.

Thevis M and Schänzer W (2005) Mass Spectrometric Analysis of Androstan-17β-ol-3-one and Androstadiene-17β-ol-3-one Isomers. Journal of the American Society for Mass Spectrometry 16:1660-1669.

Uhlmann H, Beckert V, Schwarz D, and Bernhardt R (1992) Expression of bovine adrenodoxin in E. coli and site-directed mutagenesis of /2 FE・2S/ cluster ligands. Biochemical and Biophysical Research Communications 188:1131-1138.

Vermilion JL and Coon MJ (1978) Purified liver microsomal NADPH-cytochrome P-450 reductase. Spectral characterization of oxidation-reduction states. Journal of Biological Chemistry 253:2694-2704.

WADA (2014) 2013 Anti-Doping Testing Figures Report. (Agency WA-D ed.

Woods ST, Sadleir J, Downs T, Triantopoulos T, Headlam MJ, and Tuckey RC (1998) Expression of Catalytically Active Human Cytochrome P450scc inEscherichia coliand Mutagenesis of Isoleucine-462. Archives of Biochemistry and Biophysics 353:109-115.

Zöllner A, Kagawa N, Waterman MR, Nonaka Y, Takio K, Shiro Y, Hannemann F, and Bernhardt R (2008) Purification and functional characterization of human 11β hydroxylase expressed in Escherichia coli. FEBS Journal 275:799-810.

Zöllner A, Parr Maria K, Drăgan C-A, Dräs S, Schlörer N, Peters Frank T, Maurer Hans H, Schänzer W, and Bureik M (2010) CYP21-catalyzed production of the long-term urinary metandienone metabolite 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one: a contribution to the fight against doping. Biological Chemistry 391:119-127.

2.4 Müller and Schiffer *et al.* 2015, under review

Metabolism of spironolactone and canrenone by human CYP11B1 and CYP11B2 alters the mineralocorticoid receptor antagonistic properties

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Metabolism of spironolactone and canrenone by human CYP11B1 and CYP11B2 alters the mineralocorticoid receptor antagonistic properties

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Abstract

Spironolactone and its major metabolite canrenone are potent mineralocorticoid receptor antagonists and are, therefore, applied as drugs for the treatment of primary aldosteronism and essential hypertension. We report that both compounds can be converted by the purified adrenocortical cytochromes P450 CYP11B1 and CYP11B2, while no conversion of the selective mineralocorticoid receptor antagonist eplerenone was observed. As their natural function, CYP11B1 and CYP11B2 carry out the final steps in the biosynthesis of gluco- and mineralocorticoids. Dissociation constants for the new exogenous substrates were determined by a spectroscopic binding assay and demonstrated to be comparable to those of the natural substrates, 11-deoxycortisol and 11-deoxycorticosterone. Metabolites were produced at preparative scale with a CYP11B2-dependent *E. coli* whole-cell system and purified by HPLC. Using NMR spectroscopy, the metabolites of spironolactone were identified as 11β-OH-spironolactone, 18-OH-spironolactone and 19-OH-spironolactone. Canrenone was converted to 11β-OH-canrenone, 18-OH-canrenone as well as to the CYP11B2-specific product 11β,18-diOH-canrenone. Therefore a contribution of CYP11B1 and CYP11B2 to human pharmacokinetics should be taken into account and the metabolites should be tested for their potential toxic and pharmacological effects. A mineralocorticoid receptor transactivation assay in antagonist mode revealed 11β-OH-spironolactone as

pharmaceutically active metabolite, whereas all other hydroxylation products negate the antagonist properties of spironolactone and canrenone. Thus, human CYP11B1 and CYP11B2 turned out to metabolize steroid-based drugs additionally to the liver-dependent biotransformation of drugs. Compared with the action of the parental drug, changed properties of the metabolites at the target site have been observed.

Keywords: Aldosterone, hypertension, mineralocorticoid receptor antagonists, human CYP11B1/CYP11B2, xenobiotic metabolism

Abbreviations: AdR – adrenodoxin reductase, Adx – adrenodoxin, DOC – 11-deoxycorticosterone, E. coli – Escherichia coli, MR – mineralocorticoid receptor, MRA – mineralocorticoid receptor antagonist, RSS – 11-deoxycortisol (Reichstein's substance S)

Introduction

Aldosterone represents the main human mineralocorticoid. It regulates renal water and sodium retention as well as potassium secretion via the mineralocorticoid receptor (MR) and thereby controls water and electrolyte homeostasis, which directly projects onto blood pressure [1-4]. The MR is a ligand-activated transcription factor inducing genomic effects upon mineralocorticoid binding, subsequent translocation into the nucleus and regulation of transcription [2, 5]. In humans, aldosterone is synthesized from 11-deoxycorticosterone (DOC) in the *zona glomerulosa* of the adrenal cortex by a member of the cytochrome P450 superfamily (P450), CYP11B2, that catalyzes a reaction sequence consisting of 11β- and 18-hydroxylations followed by an 18-oxidation [6-10]. The other member of the human CYP11B subfamily, CYP11B1, which shares 93 % sequence identity with CYP11B2 on protein level, synthesizes the major human glucocorticoid cortisol in the adrenal *zona fasciculata* by 11β-hydroxylation of 11-deoxycortisol (RSS) [6-8, 11, 12]. Both CYP11B isoforms are located in the inner mitochondrial membrane and require NADPH as electron donor for their catalysis as well as an electron transfer system consisting of the NADPH-dependent adrenodoxin reductase (AdR) and adrenodoxin (Adx), which transfers electrons to the P450 for the activation of molecular oxygen [13].

Aldosterone induced activation of the MR is a key step in the development of hypertension and resultant cardiac and renal abnormalties [14]. In addition, MR activation in cardiac tissues also seems to play a direct role in cardiac fibrosis [3]. Consecutively, the MR represents an attractive drug target for blood pressure reducing therapies and mineralocorticoid receptor antagonists (MRAs) have been used

successfully for decades in the treatment of primary aldosteronism and essential hypertension as well as congestive heart failure [15-20]. Spironolactone, which has been clinically applied since the early 1960s, is still a widely used MRA and has been shown to reduce the risks of morbidity and mortality among patients with severe heart failure [17]. Upon administration, it is rapidly dethioacetylated to its principal pharmacologically active metabolite canrenone [21-23]. However, the use of spironolactone is limited by its non-selective receptor binding properties, which result in significant adverse effects, most of them being sex-related due to binding to the androgen receptor [24, 25]. As a consequence, the development of more selective MRAs, such as eplerenone, is of great interest to reduce steroid receptor cross-reactivity [18, 19, 26].

During the 1970s, several studies observed an interference of the MRAs spironolactone and canrenone with corticosteroid biosynthesis in rat adrenal tissue and mitochondrial preparations from bovine and human [27-29]. Furthermore, binding of both compounds to P450s leading to a type-I difference spectrum and the formation of hydroxylated MRA metabolites was described [28, 29]. As these studies were performed with tissue-derived preparations and the availability of high and pure quantities of each of the corticosteroid synthesizing P450s was not given at that time, neither the metabolites nor the particular P450s responsible for their formation could be identified. Metabolite identification and characterization is, however, crucial for drug design and development due to potential toxicity and pharmacological diversification. The recombinant expression of the 2 human mitochondrial steroidogenic P450s CYP11B1 and CYP11B2 in *E. coli* was finally achieved during the last years, which now enables an efficient purification and application for biotransformations in preparative scale [9, 11, 30]. According to the traditional P450 classification, these isozymes are counted as nondrug-metabolizing P450s and are presumed to display a narrow substrate spectrum, which is restricted to their natural function in steroid hormone biosynthesis [31, 32]. Nevertheless, very recently a few exogenous substrates were described for the CYP11B subfamily [33, 34] demonstrating their ability for the biotransformation of exogenous compounds. This study presents a new metabolic pathway for spironolactone and canrenone catalyzed by CYP11B1 and CYP11B2. Metabolism is demonstrated in a reconstituted in-vitro system and emerging metabolites were produced at preparative scale by E. coli based whole-cell bioconversions for structural elucidation by NMR. Alterations of their bioactivity concerning their antagonist properties for the MR were studied in a transactivation assay.

Material & Methods

Chemicals

All reagents were purchased from standard sources with the highest purity available. Steroids were purchased from Sigma-Aldrich.

Expression and purification of human CYP11B1 and CYP11B2

Human CYP11B1 and CYP11B2 were expressed in *E. coli* C43(DE3) using constructs described [9, 11]. The expression was performed in 2.8 L Fernbach flasks containing 400 mL TB medium (24 g technical yeast extract, 12 g peptone, 4 mL glycerol, 4.6 g KH_2PO_4, 25 g K_2HPO_4) supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin. The expression culture was inoculated with an overnight culture and incubated at 37 °C and 210 rpm until an $OD_{600\ nm}$ of 0.5 was reached. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, 4 mg/mL arabinose, 1 mM δ-aminolevulinic acid and 50 µg/ml ampicillin. Further incubation was carried out at 27.5 °C and 180 rpm for 24 h. Cells were harvested by centrifugation and sonicated in lysis buffer (50 mM potassium phosphate (pH 7.4), 20 % glycerol, 500 mM sodium acetate, 1.5 % sodium cholate, 1.5 % Tween 20, 0.1 mM phenylmethylsulfonylflourid, 0.1 mM dithiothreitol). Cell debris was removed by ultracentrifugation (30 min, 35.000 rpm, 4 °C) and the P450 was purified from the supernatant by immobilized metal ion affinity chromatography and cation exchange according protocols established previously [9, 11].

Concentrations of the purified cytochromes P450 were determined by reduced carbon-monoxide difference spectroscopy with an extinction coefficient ε_{450nm}=91 mM^{-1} cm^{-1} [35].

Purification of redox partners

Human AdR [36, 37] and Adx [38, 39] were expressed in *E. coli* and purified as previously described. Concentrations were determined with $\varepsilon_{450\ nm}$=11.3 mM^{-1} cm^{-1} mM for AdR and $\varepsilon_{414\ nm}$=9.8 mM^{-1} cm^{-1} for Adx.

In-vitro substrate conversion

For *in-vitro* substrate conversion assays, 400 µM of the respective MRA was incubated with the P450 system that was reconstituted with the natural human redox partners, AdR and Adx. The reaction mixture (0.5 mL) consisted of 0.5 µM CYP11B1 or CYP11B2, 0.5 µM AdR and 10 µM Adx in 50 mM HEPES buffer pH 7.4 supplemented with 20 % glycerol and 100 µM 1,2-Didodecanoyl-sn-glycero-3-phosphocholine. Furthermore, a NADPH-regeneration system consisting of 5 mM glucose-6-phosphate, 4 U/mL glucose-6-phosphate dehydrogenase and 1 mM $MgCl_2$ was added. The reaction was started by

adding 1 mM NADPH, incubated at 37 °C under shaking and stopped after 1 h by adding two reaction volumes of chloroform. For the conversion assays with natural substrates (RSS for CYP11B1 and DOC for CYP11B2) in the presence or absence of the MRAs, enzyme concentrations were down-scaled to 0.25 µM CYP11B1 or CYP11B2, 0.25 µM AdR and 5 µM Adx. Substrate and the respective MRA were added from stock solutions in ethanol with final concentrations of 20 µM substrate and 40 µM MRA and a final solvent concentration of 0.6 %. The reaction volume was increased to 750 µL to enable an appropriate detection of the steroids in the HPLC measurements. Reactions took place at 37 °C under shaking for two min. Progesterone was added as internal standard for quantification.

Reversed phase HPLC analysis

For the analysis of the product pattern via high performance liquid chromatography (HPLC), samples were extracted twice with two volumes chloroform whereupon the chloroform phase was evaporated and the remaining steroids were suspended in acetonitrile for HPLC analysis. Steroids were separated on a Jasco reversed phase HPLC system of the LC900 series (Jasco, Groß-Umstadt, Germany) using a 4.6 mm x 125 mm NucleoDur C18 Isis Reversed Phase column (Macherey-Nagel, Düren, Germany) with an acetonitrile/water gradient (Phase A: 10 % acetonitrile in water, Phase B: 100 % acetonitrile; 0 min 20 % B, 5 min 20 % B, 13 min 40 % B, 20 min 80 % B, 21 min 80 % B, 22 min 20 % B, 30 min 20 % B) at 40 °C and a flow rate of 0.8 ml/min. Steroids were detected by an UV/Vis detector (UV-2 075 Plus, Jasco) at 240 nm (for spironolactone) or 288 nm (for canrenone).

Spectroscopic binding assay

The determination of dissociation constants (K_d) was performed by difference spectroscopy in tandem cuvettes according to Schenkman [40]. Purified CYP11B1 or CYP11B2 were diluted in a buffer consisting of 50 mM potassium phosphate (pH 7.4), 20 % glycerol, 0.5 % sodium cholate and 0.05 % Tween 20 to a final concentration of 2 µM and were titrated with increasing amounts of the respective substrate dissolved in DMSO and difference spectra were recorded from 350 to 500 nm. To determine the K_d, the averaged peak-to-trough absorbance differences from three titrations were plotted against the substrate concentration and fitted with hyperbolic regression or tight binding quadratic equation.

Whole-cell conversion with recombinant E. coli and product purification

Preparative scale bioconversion was performed with whole *E. coli* cells containing a functional CYP11B2 system by recombinant expression of the P450 cDNA, bovine AdR and truncated bovine Adx_{1-108} from a tricistronic plasmid as previously described for CYP11B1 [30]. Protein expression was performed accordingly in 150 mL TB medium per 2 L-Erlenmeyer flask at 27.5 °C and 200 rpm, but

expression time was increased to 40 h. Subsequently, cultures were harvested by centrifugation (3200*g*, 10 min, 18 °C) and the cell pellet was washed in 50 mM potassium phosphate buffer (pH 7.4). For substrate conversion, cells were suspended in 125 ml of 50 mM potassium phosphate buffer (pH 7.4) and incubated in a 2 L- Erlenmeyer flask with addition of 1 mM isopropyl β-D-1-thiogalactopyranoside, 4mg/ml arabinose, 1 mM δ-aminolevulinic acid, 2 % glycerol and 200 µM substrate at 27.5 °C and 200 rpm for 24 h. Following the conversion time, steroids were extracted twice with 125 ml ethyl acetate. The organic phase containing the steroids was concentrated to dryness in a rotatory evaporator. The residues were dissolved in acetonitrile and filtered through a sterile syringe filter (0.45 µm). The products were separated by preparative HPLC (Jasco reversed phase HPLC system (Jasco, Groß-Umstadt, Germany), 4.6 mm x 250 mm NucleoDur C18ec column (Macherey-Nagel, Düren, Germany)) at 40 °C with acetonitrile/water gradients at 3 mL/min. Steroids were monitored by UV/Vis detection, fractions containing the desired products were collected, evaporated to dryness and analyzed by nuclear magnetic resonance (NMR) spectroscopy.

NMR analysis of metabolites

The NMR spectra were recorded in $CDCl_3$ with a Bruker Avance III 500 NMR spectrometer at 298 K using a CPTCI probe head. The chemical shifts were relative to $CHCl_3$ at δ 7.26 (^{1}H NMR) and $CDCl_3$ at δ 77.00 (^{13}C NMR) respectively using the standard δ notation in parts per million. The 1D NMR (^{1}H and ^{13}C NMR, DEPT135) and the 2D NMR spectra (gs-HH-COSY, gs-NOESY, gs-HSQCED, and gs-HMBC) were recorded using the BRUKER pulse program library. All assignments were based on extensive NMR spectral evidence.

11β-Hydroxyspironolactone (**1**). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.03 dd (J=11.9 and 3.8 Hz, H-9), 1.24 s (3xH-18), 1.41 m (H-14), 1.44 m (H-15a), 1.50 s (3xH-19), 1.53 m (H-12a), 1.58 m (H-15b), 1.71 dd (J= 13.7 and 2.8 Hz, H-12b), 1.78 m (H-16a), 1.82 m (H-1a), 1.93 ddd (J= 16.3, 9.3 and 7.3 Hz, H-20a), 2.24 m (H-1b), 2.28 m (2H, H-16b and H-20b), 2.34 m (H-6a), 2.33 s (3xH-24), 2.36 m (H-2a), 2.42 m (H-8), 2.45 m (H-21a), 2.47 m (H-2b), 2.55 ddd (J= 17.5, 9.3 and 6.5 Hz, H-21b), 2.94 ddd (J=14.8, 4.2 and 1.8 Hz, H-6b), 4.08 td (J= 4.2 and 2.8 Hz, H-7), 4.46 q (J=3.4, H-11 Hz) 5.64 s (H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 17.23 (CH3, C-18), 21.54 (CH3, C-19), 22.47 (CH2, C-15), 29.23 (CH2, C-21), 31.24 (CH2, C-20), 31.24 (CH3, C-24), 33.79 (CH2, C-2), 35.02 (CH2, C-16), 35.24 (CH2, C-1), 35.67 (CH, C-8), 39.38 (C, C-10), 39.51 (CH2, C-6), 40.90 (CH2, C-12), 44.74 (C, C-13), 45.98 (CH, C-7), 47.09 (CH, C-14), 52.53 (CH, C-9), 67.51 (CH, C-11), 96.02 (C, C-17), 125.41 (CH, C-4), 166.77 (C, C-5), 176.49 (C, C-22), 194.13 (C, C-23), 198.80 (C, C-3).

18-Hydroxyspironolactone (**2**). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.06 m (H-9), 1.12 m (H-12a), 1.23 s (3xH-19), 1.40 m (H-15a), 1.53 m (H-14), 1.55 (H-11a), 1.59 m (H-15b), 1.70 m (H-11b), 1.81 m (H-1a), 1.94 m (H-20a), 1.97 m (H-16a), 2.07 m (H-16b), 2.08 m (H-8), 2.23 m (H-1b), 2.24 m (2H, H-12b and H-20b), 2.34 s (3xH-24), 2.38 m (H-2a), 2.40 m (H-6a), 2.42 m (H-2b), 2.53 m (H-21a), 2.59 m (H-21b), 2.85 ddd (J=14.8, 4.2 and 2.0 Hz, H-6b), 3.76 d (J=12.5 Hz, H-18a), 3.92 d (J=12.5 Hz, H-18b), 3.96 td (J= 4.2 and 3.0 Hz, H-7), 5.71 d (J=1.8 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 17.70 (CH3, C-19), 20.23 (CH2, C-11), 22.56 (CH2, C-15), 26.62 (CH2, C-12), 28.34 (CH2, C-21), 31.04 (CH2, C-20), 31.34 (CH3, C-24), 33.88 (CH2, C-2), 35.53 (CH2, C-1), 35.62 (CH2, C-16), 38.52 (C, C-10), 38.60 (CH, C-8), 39.89 (CH2, C-6), 45.03 (CH, C-7), 45.90 (CH, C-14), 48.94 (C, C-13), 49.56 (CH, C-9), 60.94 (CH2, C-18), 96.94 (C, C-17), 127.07 (CH, C-4), 165.32 (C, C-5), 176.68 (C, C-22), 194.22 (C, C-23), 198.66 (C, C-3).

19-Hydroxyspironolactone (**3**). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.00 s (3xH-18), 1.09 m (H-9), 1.28 m (H-12a), 1.35 m (H-14), 1.39 m (H-15a), 1.56 m (H-15b), 1.59 (H-11a), 1.60 m (H-12b), 1.73 m (H-16a), 1.75 m (H-11b), 1.76 m (H-1a), 1.94 m (H-20a), 2.24 m (H-20b), 2.25 m (H-8), 2.32 m (2H, H-1b and H-16b), 2.34 s (3xH-24), 2.35 m (H-2a), 2.48 m (2H, H-6a and H-21a), 2.55 m (H-21b), 2.64 m (H-2b), 2.97 ddd (J=14.6, 4.2 and 2.0 Hz, H-6b), 3.95 d (J=10.3 Hz, H-19a), 4.01 td (J= 4.2 and 3.0 Hz, H-7), 4.04 d (J=10.3 Hz, H-19b), 5.90 d (J=1.8 Hz, H-4). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.70 (CH3, C-18), 20.90 (CH2, C-11), 22.19 (CH2, C-15), 29.18 (CH2, C-21), 31.15 (CH2, C-20), 31.35 (CH3, C-24), 31.59 (CH2, C-12), 33.63 (CH2, C-1), 34.71 (CH2, C-2), 35.20 (CH2, C-16), 39.78 (CH, C-8), 41.07 (CH2, C-6), 43.12 (C, C-10), 45.11 (CH, C-7), 45.60 (C, C-13), 46.51 (CH, C-14), 49.78 (CH, C-9), 66.40 (CH2, C-19), 95.50 (C, C-17), 129.58 (CH, C-4), 162.27 (C, C-5), 176.52 (C, C-22), 194.31 (C, C-23), 198.94 (C, C-3).

11β-Hydroxycanrenone (**5**). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.19 dd (J=10.3 and 3.2 Hz, H-9), 1.30 s (3xH-18), 1.32 m (H-14), 1.69 m (H-15a), 1.37 s (3xH-19), 1.56 dd (J= 13.7 and 3.0 Hz, H-12a), 1.89 m (H-15b), 1.73 dd (J= 13.7 and 2.8 Hz, H-12b), 1.83 m (H-1a), 1.87 m (H-16a), 1.92 m (H-20a), 2.23 ddd (J= 12.8, 5.3 and 2.3 Hz, H-1b), 2.34 m (H-16b), 2.47 m (H-2a), 2.76 t (J= 11.0 Hz, H-8), 2.48 dd (J= 9.3 and 7.5 Hz, H-21a), 2.56 dd (J= 9.3 and 6.3 Hz, H-21b), 2.63 ddd (J= 17.5, 14.5 and 5.3 Hz, H-2b), 2.27 ddd (J= 13.0, 6.9 and 2.9 Hz, H-20b), 4.08 td (J= 4.2 and 2.8 Hz, H-7), 4.47 q (J=3.4 Hz, H-11) 5.64 s (H-4), 6.14 dd (J=9.8 and 2.0 Hz, H-7), 6.16 brd (J=9.8 Hz, H-6). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 16.97 (CH3, C-18),18.97 (CH3, C-19), 22.66 (CH2, C-15), 29.27 (CH2, C-21), 31.52 (CH2, C-20), 33.19 (CH2, C-1), 33.86 (CH2, C-2), 34.09 (CH, C-8), 35.37 (CH2, C-16), 36.44 (C, C-10), 40.79 (CH2, C-12), 45.98 (C, C-13), 48.38 (CH, C-14), 53.33 (CH, C-9), 67.17 (CH, C-11), 95.77 (C, C-17), 122.88 (CH, C-4), 128.10 (CH2, C-6), 139.89 (CH, C-7), 164.06 (C, C-5), 176.51 (C, C-22), 199.55 (C, C-3).

18-Hydroxycanrenone (**7**). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.13 s (3xH-19), 1.18 td (J= 12.5 and 4.0 Hz, H-12a), 1.25 ddd (J=13.0, 10.3 and 3.7 Hz, H-9), 1.48 m (H-14), 1.53 (H-11a), 1.54 m (H-15a), 1.71 m (H-11b), 1.72 m (H-1a), 1.91 m (H-15b), 1.95 m (H-20a), 2.02 m (H-16a), 2.04 m (H-2a), 2.31 dt (J= 12.5 and 3.3 Hz, H-12b), 2.36 m (H-8), 2.40 m (H-20b), 2.41 m (H-16b), 2.46 m (H-2b), 2.55 m (H-21a), 2.58 m (H-1b), 2.60 m (H-21b), 3.84 d (J=12.5 Hz, H-18a), 3.95 d (J=12.5 Hz, H-18b), 5.69 s (H-4), 6.05 dd (J=9.8 and 2.0 Hz, H-7), 6.14 dd (J=9.8 and 2.7 Hz, H-6). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 16.66 (CH3, C-19), 19.85 (CH2, C-11), 22.71 (CH2, C-15), 26.61 (CH2, C-12), 28.45 (CH2, C-21), 31.14 (CH2, C-20), 33.92 (CH2, C-2), 33.96 (CH2, C-1), 35.89 (CH2, C-16), 36.11 (C, C-10), 37.41 (CH, C-8), 47.02 (CH, C-14), 49.67 (C, C-13), 50.53 (CH, C-9), 60.71 (CH2, C-18), 96.86 (C, C-17), 124.20 (CH, C-4), 128.70 (CH2, C-6), 138.71 (CH, C-7), 162.76 (C, C-5), 175.74 (C, C-22), 199.45 (C, C-3).

11β,18-Dihydroxycanrenone (**8**). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.18 ddd (J=β.0 and 2.7 Hz, H-9), 1.36 m (H-12a), 1.40 s (3xH-19), 1.47 m (H-14), 1.62 m (H-15a), 1.83 m (H-1a), 1.98 m (2H, H-15b and H-20a), 2.04 m (H-16a), 2.31 m (H-1b), 2.32 m (H-20b), 2.42 m (H-12b), 2.44 m (H-16b), 2.50 m (H-2a), 2.54 dd (J= 9.0 and 6.3 Hz, H-21a), 2.61 m (J= 9.0 and 7.5 Hz, H-21b), 2.65 ddd (J= 17.5, 14.5 and 5.4, H-2b), 2.89 t (J= 11.0 Hz, H-8), 3.97 m (J=12.0 Hz, H-18a), 4.13 d (J=12.0 Hz, H-18b), 4.43 q (J= 3.2 Hz, H-11), 5.64 s (H-4), 6.11 brd (J=9.8 Hz, H-7), 6.13 dd (J=9.8 and 2.0 Hz, H-6). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 18.68 (CH3, C-19), 22.79 (CH2, C-15), 28.72 (CH2, C-21), 31.42 (CH2, C-20), 33.22 (CH2, C-1), 33.84 (CH, C-8), 33.86 (CH2, C-2), 34.44 (CH2, C-12), 35.90 (CH2, C-16), 36.52 (C, C-10), 47.88 (CH, C-14), 49.20(C, C-13), 53.73 (CH, C-9), 62.89 (CH2, C-18), 66.02 (CH2, C-11), 96.61 (C, C-17), 123.08 (CH, C-4), 128.36 (CH2, C-6), 139.15 (CH, C-7), 163.84 (C, C-5), 175.84 (C, C-22), 199.64 (C, C-3).

MR transactivation assay

The cell culture-based reporter assay was performed using the Human MR Reporter Assay System (96-well Format Assays) from Indigo Biosciences. The assay was carried out according to the manufacturer's protocol (Technical manual (Version 7.1.)). For the antagonist mode, aldosterone was added in its median effective concentration (EC_{75}) of 333 pM. The test compounds were added in a concentration of 20 nM corresponding to the IC_{50} of spironolactone according to the manufacturer. The statistics were performed using student's t-test with unpaired samples as two sided test and a significance level of $p < 0.05$. For the agonist mode, all compounds were applied individually with a concentration of 333 pM (EC_{75} of aldosterone).

Results and discussion

CYP11B1- and CYP11B2-dependent in vitro-conversion of MRAs

In-vitro reconstitution assays were performed using the respective purified P450, human Adx and AdR in a molar ratio of 1:20:1 to ensure maximal enzyme activity [41-43]. Product formation was monitored by HPLC with UV/vis detection, whereby products with the same retention time were assumed to be the same compounds. Spironolactone and canrenone were successfully metabolized by CYP11B1 and CYP11B2 despite of their structural divergencies from the natural substrates, which comprise the carbolactone at C_{17} for both MRAs and the polar thioacetyl group at C_7 in α-orientation for spironolactone. The conversion of spironolactone by both isoforms led to similar product patterns showing one major metabolite (**1**) and two side products (metabolites **2** and **3**) (**Fig. 1A** and **B**). For canrenone, which is smaller than spironolcatone, due to the removal of the 7α-thioacetyl group, isoform-specific product formation can be observed. Canrenone is metabolized to three products by CYP11B1 and five products by CYP11B2 (**Fig. 1C** and **D**). Thereby, metabolites **5**, **6** and **7** are derived from both P450s, while metabolites **4** and **8** are specific for CYP11B2. This reflects the larger reaction capacity of CYP11B2 compared with CYP11B1 [8], which seems, however, to be restricted to smaller substrates.

No conversion of the third MRA, eplerenone, by any of the two isozymes could be observed (chromatogramm not shown). Additionally, spectroscopic binding assays revealed no spin state changes for CYP11B2 upon titration of eplerenone suggesting no binding to the active site (spectra not shown). We suppose that the 9,11α-epoxid of eplerenone hampers the binding and conversion of this MRA. The crystal structure of CYP11B2 revealed that proper binding of its substrates is ensured, besides hydrogen bonding, by hydrophobic interactions of the substrate's α-face with aromatic amino acid side chains lying opposite to the heme [44]. The presence of the α-epoxy group interferes with these interactions.

Taken together, our data show that the pharmaceutically relevant steroids spironolactone and canrenone can undergo biotransformation by CYP11B1 and CYP11B2, which strengthens the presumption of the participation of these adrenal P450s in xenobiotic metabolism. Hereby, the substrate acceptance depends on the conservation of key structures of the substrate for recognition and binding by the CYP11B isoenzymes like the 3-keto-Δ^4 motif and the degree and distribution of α-face polarity.

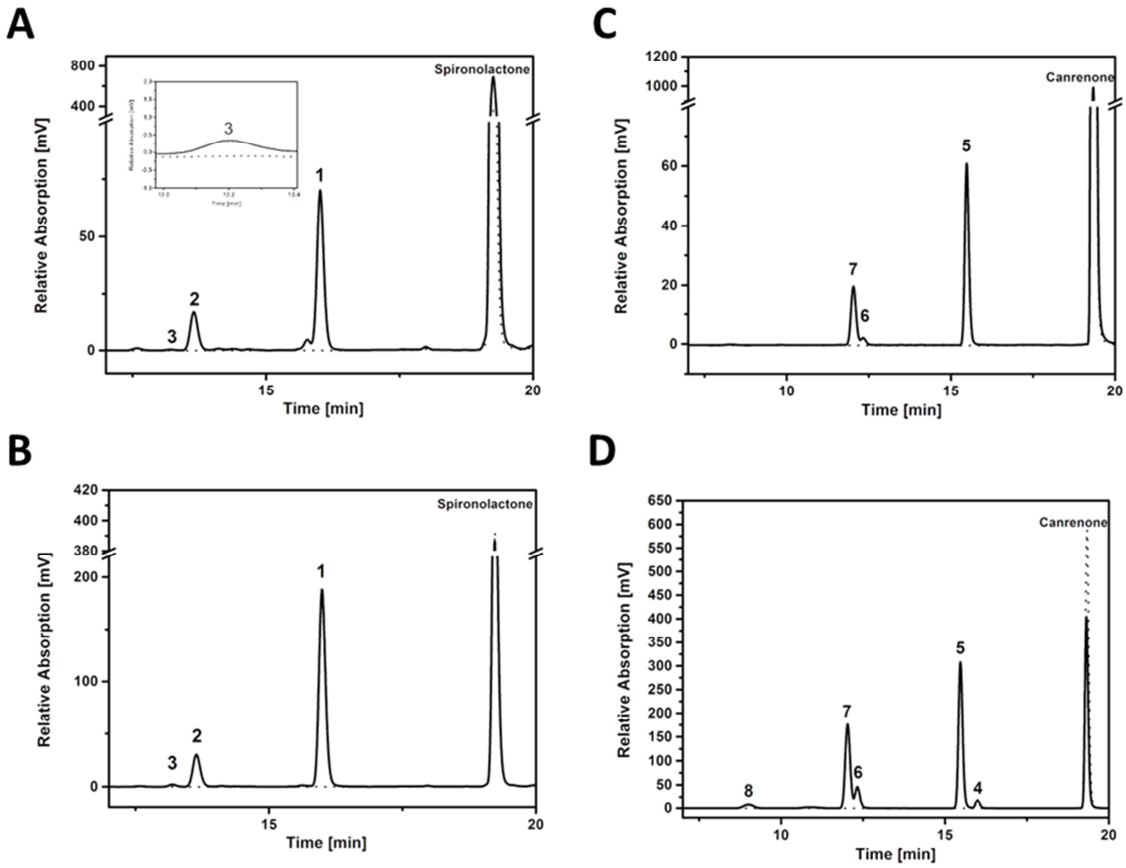

Fig. 1: HPLC chromatograms of the *in-vitro* conversion of spironolactone and canrenone by CYP11B1 and CYP11B2. A - spironolactone conversion by CYP11B1 with enlargement of metabolite **3** in the inset; **B** - spironolactone conversion by CYP11B2; **C** - canrenone conversion by CYP11B1; *D* - canrenone conversion by CYP11B2. The dotted lines show the respective negative control without NADPH. Reactions were performed for 1 h at 37 °C in 50 mM HEPES pH 7.4 supplemented with 20 % glycerol and 100 µM 1,2-didodecanoyl-sn-glycero-3-phosphocholine and the respective substrate in a concentration of 400 µM, using 0.5 µM CYP11B1 or CYP11B2, 0.5 µM AdR and 10 µM Adx. Extracted steroids were monitored at a wavelength of 240 nm or 288 nm for spironolactone and canrenone, respectively.

Determination of dissociation constants (K_d) of spironolactone and canrenone

For comparison of the binding affinities of the new exogenous substrates with the natural substrates of gluco- and mineralocorticoid biosynthesis, dissociation constants were determined by UV/vis-spectroscopy. All substances induce a high-spin shift of the heme iron (**Fig. 2, insets**), which enables the

determination of a K_d value using the type-I difference spectrum upon substrate titration to the P450. All values are summarized in **Table 1**.

Except for the binding of canrenone by CYP11B1, all K_d values for the investigated substrates are slightly higher but in the same order of magnitude than those for the natural substrates, DOC and RSS. The structural differences do, thus, not significantly influence the binding properties. CYP11B1 shows weaker binding of DOC than of RSS, which matches the deviations in the catalytic efficiency for the two substrates [11] and demonstrates a positive contribution of the 17α-OH group for substrate binding.

Although spironolactone is larger than canrenone and additionally differs from the natural substrates by the 7α-thioacetyl function, it exhibits a higher affinity for both CYP11B isoforms suggesting that either the size of the molecule promotes binding or the additional group itself enhances the affinity through new interactions. Furthermore, it is remarkable that CYP11B2 shows a higher affinity for both exogenous substrates than CYP11B1. This can be explained by the increased intrinsic flexibility and thus adaptability for foreign substrates, which has been postulated for CYP11B2 as a result of its isoform-specific residues around the H- and I-helices [44].

Table 1: Dissociation constants (K_d) of CYP11B1 and CYP11B2 for their endogenous and exogenous substrates. Values were determined by difference spectroscopy and regression of the absorbance shift as shown in **Fig. 2**. Regression coefficients (R^2) are indicated. n.d. = not determined.

	CYP11B1		**CYP11B2**	
Substrate	K_d [μM]	R^2	K_d [μM]	R^2
Canrenone	72.5 ± 9.2	0.99	5.5 ± 0.4	0.99
Spironolactone	9.3 ± 0.7	0.99	4.1 ± 0.4	0.98
DOC	8.1 ± 0.5	0.99	1.3 ± 0.1 [9]	0.99 [9]
RSS	6.6 ± 0.4	0.99	n.d.	n.d.
Corticosterone	n.d.	n.d.	115 ± 6 [9]	0.99 [9]

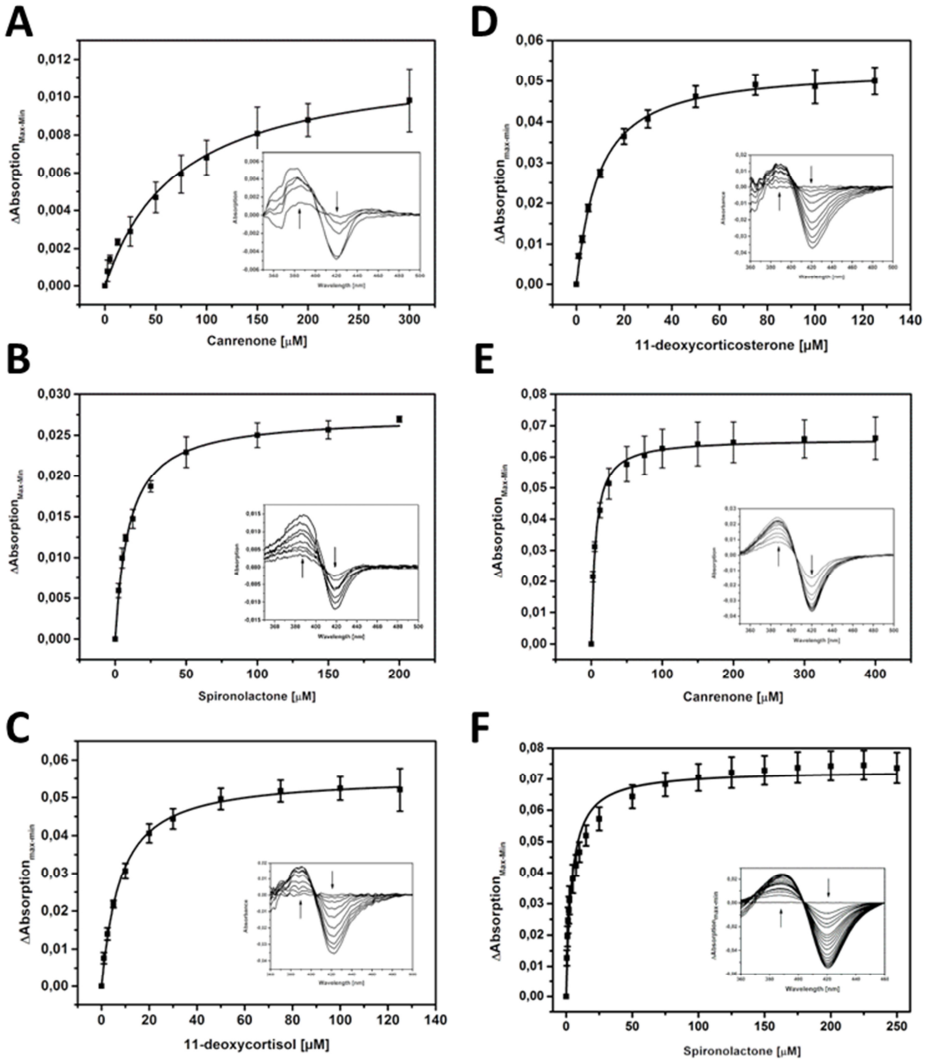

Fig. 2: Determination of CYP11B1 and CYP11B2 affinity for their substrates by difference spectroscopy and regression of the absorbance shift. CYP11B1 with canrenone (**A**), spironolactone (**B**), 11-deoxycortisol (**C**) and 11-deoxycorticosterone (**D**), CYP11B2 with canrenone (**E**) and spironolactone (**F**). 2 µM of the P450 were titrated in tandem cuvettes with increasing concentrations of the respective substrate from a stock solution in DMSO and difference spectra were recorded from 350-500 nm (**insets**). The peak-to-trough differences of the absorbance were plotted against the substrate concentrations and fitted as described in Material & Methods for the determination of the K_d.

Effect of spironolactone and canrenone on CYP11B1- and CYP11B2-dependent substrate conversions

The binding and conversion of spironolactone and canrenone by the two CYP11B isoforms suggests an effect on their natural function by a competition of the endogenous and exogenous substrates for the active site. We, therefore, evaluated the effect of spironolactone and canrenone on the conversion of RSS, the substrate for glucocorticoid synthesis, by CYP11B1 and the conversion of the aldosterone precursor DOC by CYP11B2 (**Fig. 3**). Natural substrate concentration was kept below the saturation at 20 µM and the respective MRA was added in two-fold excess, as plasma levels of spironolactone and canrenone can exceed RSS and DOC levels already after a single oral dose [22, 45-47]. Product formation from spironolactone and canrenone was not detectable due to the short reaction time. Spironolactone showed no significant effect on CYP11B2 activity, while it reduced CYP11B1-dependent conversion of RSS from 68 to 43 %. These differential effects on the two isoforms correlate well with their affinities for the natural substrates. CYP11B2 tightly binds DOC with a K_d of 1.3 µM, while the K_d for spironolactone is four-fold higher [9]. CYP11B1, in contrast, shows a higher K_d of 6.6 µM for its substrate RSS and a K_d of 9.3 µM for spironolactone and its conversion is, therefore, more susceptible to an interference by spironolactone. The presence of canrenone did not affect the activity of CYP11B1 thereby reflecting its high K_d value. Surprisingly, canrenone, whose K_d for CYP11B2 is higher when compared with spironolactone, exhibited an inhibitory effect on CYP11B2 and decreased DOC conversion from 97 to 73 %. This might be due to an additional non-competitive inhibitory effect of canrenone.

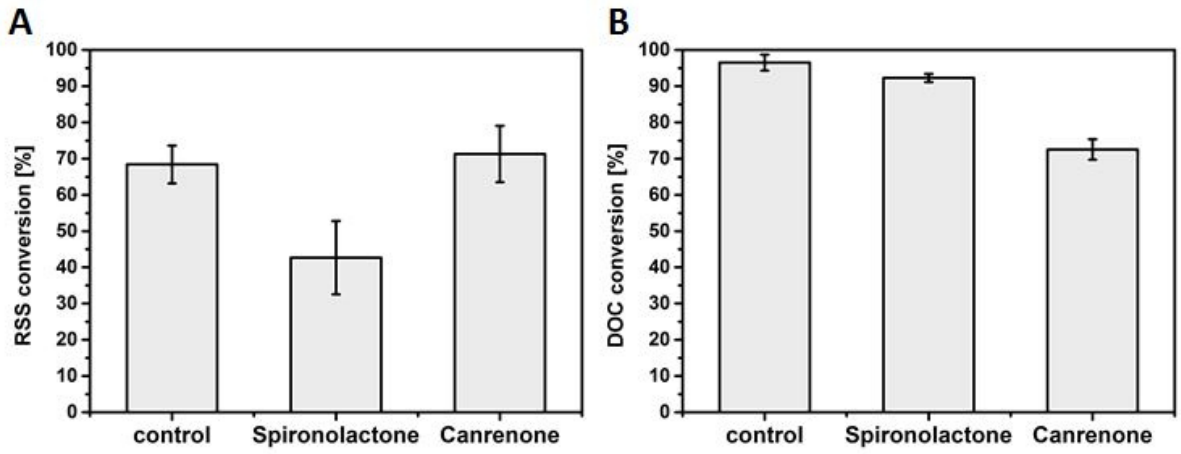

Fig. 3: Influence of spironolactone and canrenone on natural substrate conversion by CYP11B1 (A) and CYP11B2 (B) in the reconstituted *in-vitro* system. The system consisted of 0.25 µM CYP, 5 µM Adx and 0.25 µM AdR, 1 mM NADPH and an NADPH regenerating system and the reaction took place at 37 °C for two minutes. The substrate concentration was 20 µM to be below saturation and spironolactone or canrenone were added in two-fold excess (40 µM). Substrate conversion was determined by HPLC with progesterone as internal standard. Values represent the mean of three reactions with standard deviation.

Metabolite production by whole-cell catalysis with E. coli

Preparative scale production of the new MRA metabolites for purification and characterization was performed with recombinant *E. coli* cells containing a functional P450 system by co-expression of CYP11B2, bovine AdR and bovine Adx_{1-108} as previously described for CYP11B1 [30]. The auxiliary overexpression of the two *E. coli* chaperones *GroEL* and *GroES* ensured correct folding of the enzymes. Substrate conversion took place in potassium phosphate buffer after the transfer of the cells into a resting state for a better NADPH availability [48] and a reduction of the production of *E. coli* metabolites that could impede purification. HPLC analysis revealed the same product patterns of canrenone and spironolactone conversion as with the purified enzymes (see **Figs. 1** and **4**). For canrenone, the most polar metabolite **8** is produced in a greater portion than *in-vitro*, which can be a result of a prolonged functionality of the P450 system enabled by the cellular environment or a redox partner ratio, which is beneficial for the advancement of the reaction. For spironolactone, all three metabolites were purified. In case of canrenone, only the major metabolites **5**, **7** and **8** were produced in sufficient amounts for subsequent NMR studies.

Canrenone and spironolactone were converted with approximate volumetric productivities of 51 and 55 $mg*L^{-1}*d^{-1}$, respectively. The presented whole-cell system is thus an attractive tool to efficiently

produce the CYP11B2-derived metabolites on a preparative scale. As pharmaceutical steroids are widely applied for the treatment of multiple medicinal issues and an increasing number of them is identified as substrate for steroidogenic P450s, the preparation of their metabolites for the characterization of their bioactivity will gain increasing interest.

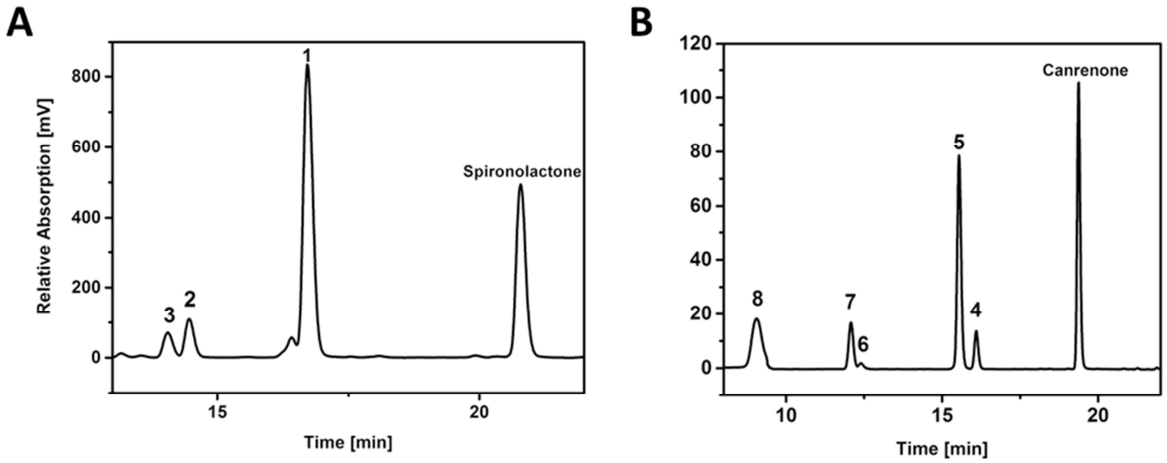

Fig. 4: HPLC chromatogram of the CYP11B2-dependent conversion of spironolactone (A) and canrenone (B) with an *E. coli* whole-cell system. *E. coli* C43(DE3) cells expressing human CYP11B2, bovine AdR and $Adx_{1\text{-}108}$ were employed in resting-cell modus for the bioconversion of 200 µM MRA in potassium phosphate buffer for 24 h.

Structure elucidation by NMR

Spironolactone was biotransformed into three products **1**-**3**. The NMR data of **1** revealed resonances for a secondary hydroxyl group (δH 4.46 q, J= 3.4 Hz; δC 67.51, CH). Its position at C-11 could be deduced from correlations of its proton with H-9 (1.03 dd, J=11.9 and 3.8 Hz), H-12a (1.53 m) and H-12b (J=1.71 dd, 13.7 and 2.8 Hz) in the HHCOSY. The coupling pattern of H-11 with coupling constants < 4 Hz gave hint to an equatorial position and H-11 is therefore β-oriented. As a consequence, the hydroxyl group at C-11 must be axial and in β-configuration. In contrast to spironolactone, its conversion products **2** and **3** both lacked one methyl group. Therefore, the resonances of a primary alcohol appeared in their NMR spectra (**2**: δH 3.76 and 3.92, both d, δC 60.94, CH2; **3**: δH 3.95 and 4.04, both d, δC 66.40, CH2). HMBC correlations between the alcoholic protons and their geminal and vicinal carbons revealed the presence of 18-OH-spironolactone for compound **2** and 19-OH-spironolactone for compound **3**.

The separation of the canrenone biotransformation products led to the isolation and structure elucidation of three conversion products, **5**, **7** and **8**, of which two were mono-alcohols and one was a

dihydroxylated compound. The NMR data of **5** resembled those of compound **1**, especially for the secondary alcohol function (δH 4.47 q, J= 3.4 Hz; δC 67.17, CH). This led to the structure of 11β-OH-canrenone for compound **5**, which could be proved by 2D NMR measurements. 11β-OH-canrenone is not known so far, in contrast to its 11β-epimer, which is the main product in the biotransformation of canrenone by Aspergillus ochraceus SIT34205 [49] and the key intermediate in the synthesis of the cardiovascular drug eplerenone [50]. The NMR spectra of **7** indicated the presence of 18-OH-canrenone. The former methyl group at C-18 of the substrate was replaced by a primary alcohol function. Its chemical shifts (δH 3.84 and 3.95, both d, δC 60.71, CH2) were very close to those of the correspondent spironolactone product **2**. Compound **8** was found to be a diol (δC 62.89, CH2; δC 66.02, CH). The proton shifts of the hydroxyl groups showed a combination of the properties as found in **5** and **7** and gave rise to a hydroxylation in position 11β (δH 4.43 q, J= 3.2 Hz,) and at C-18 (δH 3.97 and 4.13, both dd, both J=12.0 Hz). 2D NMR supported these findings and let to the full assignment of 11β,18-di-OH-canrenone.

Taken together, NMR analysis revealed that canrenone is metabolized to 11β- (metabolite **5**) and 18-OH-canrenone (metabolite **7**) by both isozymes and to the CYP11B2-specific metabolite 11β,18-di-OH-canrenone (**8**). Spironolactone is converted to three mono-hydroxylated metabolites: 11β-OH- (**1**), 18-OH- (**2**) and 19-OH-spironolactone (**3**). Reactions are illustrated in **Fig. 5**. They target the same positions as for the natural substrates DOC and RSS [8], which correlates with the steroidal nature of canrenone and spironolactone and the presence of the 3-keto-Δ^4 motif which is conserved among all known steroidal CYP11B substrates. These features ensure a binding mode of canrenone and spironolactone similar to that of the natural substrates. For both substrates, the major metabolite is the 11β-hydroxylated derivative, whose formation is also sterically and energetically favored for DOC [9, 44]. However, the total reaction repertoire differs between the two MRAs. Contrary to spironolactone, canrenone can also be di-hydroxylated by CYP11B2 and the total amount of products formed from canrenone, including the two side products, is higher than for spironolactone. The reduction in size of canrenone compared with spironolactone obviously enables a longer retention time and the movement in the active site and, thus, a second hydroxylation as well as the targeting of additional positions of the steran scaffold. For both MRAs, no oxidation of the hydroxyl to the corresponding aldehyde, as it is introduced by CYP11B2 at C_{18} during the formation of aldosterone, could, however, be demonstrated. This oxidation is assumed to occur processively and to require the retention of the intermediate [8, 9, 44], which seems not to be permitted for the MRAs.

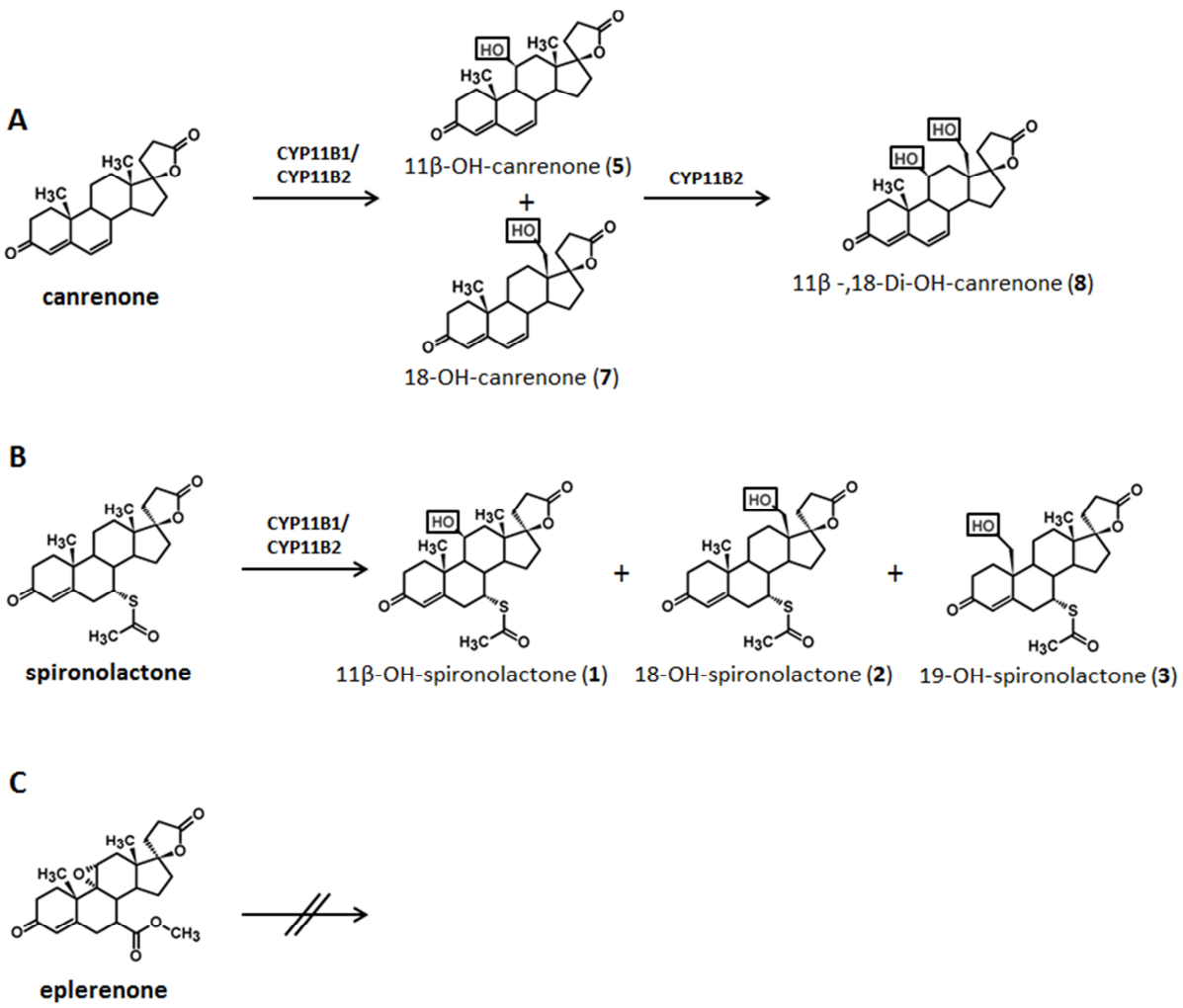

Fig. 5: Metabolism of MRAs catalyzed by CYP11B1 and CYP11B2. Metabolites were characterized by NMR after whole-cell production with recombinant *E. coli*.

Influence of MRA metabolites on MR transactivation

The metabolism of drugs can significantly alter or even reverse their bioactivity. Metabolite identification and studies of their toxicity and influence on the initial drug target are thus crucial for the development of efficient pharmaceuticals and the reduction of adverse effects. Spironolactone and canrenone target the MR, a key regulator of blood pressure, by blocking its aldosterone-mediated activation, which promotes their antihypertensive effects. MR activation depends on the development of a specific ligand-mediated hydrogen bond network, involving the residues Asn770 and Thr945, for the introduction of conformational changes inducing chaperone release, translocation into the nucleus and the transcription of target genes. The structure of the hydrogen bond network and, as a consequence, receptor activation or its prevention can be altered by oxofunctionalization of the ligands at positions C_3,

C_{11}, C_{17}, C_{18}, C_{20} and C_{21} [51-53]. Spironolactone is described as a "passive" MR antagonist. It binds inside the ligand binding pocket, but fails to build hydrogen bonds to Asn770 and Thr945 and in this way prevents the transition to the active conformation [51]. Therefore, we studied the MR antagonist potential of the newly identified MRA metabolites using a commercially available transactivation assay, which enables a relative quantification of the MR activity by luciferase derived luminescence. Aldosterone was applied in its EC_{75} and all test compounds were added in a concentration that corresponds to the IC_{50} of spironolactone (manufacturer's technical manual). Compared to the vehicle control, the presence of aldosterone alone leads to MR activation, which is specifically modulated by spironolactone, canrenone and their metabolites (**Fig. 6**). As can be expected according to the manual, spironolactone inhibits aldosterone-induced MR activation by approximately 50 %. The same extend of antagonist effect can be observed for canrenone. Interestingly, 11β-OH-spironolactone is the only metabolite that shows an antagonist effect comparable to the parent compounds and can be introduced as new putative pharmaceutically active metabolite of spironolactone. In case of canrenone, the 11β-hydroxylation abolishes the antagonist effect, which hints at a role of the combination with the opposite 7α-thioacetyl function for the antagonist retaining effect of the 11β-hydroxyl group. All other metabolites show a reduced MR antagonist activity, whereby the two 18-mono-hydroxylated metabolites, 18-OH-spironolactone and 18-OH-canrenone, even exhibit slight agonist properties. However, all compounds did not demonstrate MR activation when applied individually in an agonist mode assay at a concentration of the EC_{75} of aldosterone (data not shown). It is likely that the agonist effect of the 18-OH metabolites observed in the antagonist mode assay results from unspecific binding that supports aldosterone activity by long-range effects.

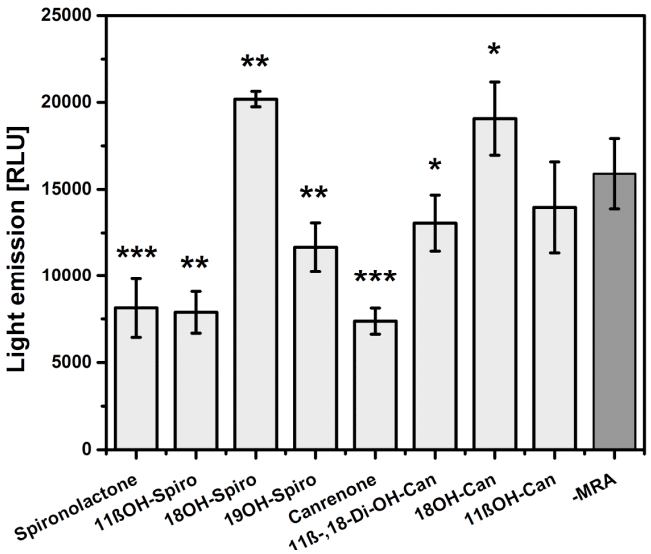

Fig. 6: Antagonist mode MR-transactivation assay with MRAs and the CYP11B-derived metabolites of spironolactone (Spiro) and canrenone (Can). Each compound was analyzed for its antagonist potential with INDIGO Bioscience Human Mineralocorticoid Receptor Reported Assay System following the manufacturer's protocol at a concentration of 20 nM (IC_{50} spironolactone), whereby aldosterone was added as agonist at its EC_{75} to all mixtures. Values represent the luminescence signal of MR-responsive luciferase reporter gene expression and were normalized to vehicle control. –MRA shows the aldosterone-mediated MR activation in absence of an antagonist. The assay was performed with n=5 for each compound. Statistical evaluation was performed using student's t-test with unpaired samples as two sided test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Conclusions

This work confirms the potential of the human steroid hormone synthesizing CYP11B1 and CYP11B2 to carry out the biotransformation of the xenobiotic steroids spironolactone and canrenone, which are frequently applied pharmaceuticals for the treatment of hypertension because of their MR antagonist features. The CYP11B-dependent metabolism of the MRAs abolishes this action, as all metabolites, except for 11β-OH-spironolactone, show reduced or completely voided MR antagonist properties. Therefore, we suggest that steroidogenic P450s should be considered, in addition to the liver isoforms, as drug-metabolizing enzymes, who can significantly modulate the bioactivity of a drug.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] S. Ardhanari, R. Kannuswamy, K. Chaudhary, W. Lockette, A. Whaley-Connell, Mineralocorticoid and Apparent Mineralocorticoid Syndromes of Secondary Hypertension, Advances in Chronic Kidney Disease, 22 (2015) 185-195.

[2] M. Funder, John W., Glucocorticoid and Mineralocorticoid Receptors: Biology and Clinical Relevance, Annual Review of Medicine, 48 (1997) 231-240.

[3] M.J. Young, J.W. Funder, Mineralocorticoid receptors and pathophysiological roles for aldosterone in the cardiovascular system, Journal of Hypertension, 20 (2002) 1465-1468.

[4] R. Bernhardt, M.R. Waterman, Cytochrome P450 and Steroid Hormone Biosynthesis, The Ubiquitous Roles of Cytochrome P450 Proteins, John Wiley & Sons, Ltd2007, pp. 361-396.

[5] F.M. Rogerson, F.E. Brennan, P.J. Fuller, Mineralocorticoid receptor binding, structure and function, Molecular and Cellular Endocrinology, 217 (2004) 203-212.

[6] M. Bureik, M. Lisurek, R. Bernhardt, The Human Steroid Hydroxylases CYP11B1 and CYP11B2, Biological Chemistry, 383 (2002) 1537-1551.

[7] M. Lisurek, R. Bernhardt, Modulation of aldosterone and cortisol synthesis on the molecular level, Molecular and Cellular Endocrinology, 215 (2004) 149-159.

[8] L. Schiffer, S. Anderko, F. Hannemann, A. Eiden-Plach, R. Bernhardt, The CYP11B subfamily, The Journal of Steroid Biochemistry and Molecular Biology, 151 (2015) 38-51.

[9] A. Hobler, N. Kagawa, M.C. Hutter, M.F. Hartmann, S.A. Wudy, F. Hannemann, R. Bernhardt, Human aldosterone synthase: Recombinant expression in E. coli and purification enables a detailed biochemical analysis of the protein on the molecular level, The Journal of Steroid Biochemistry and Molecular Biology, 132 (2012) 57-65.

[10] T. Kawamoto, Y. Mitsuuchi, T. Ohnishi, Y. Ichikawa, Y. Yokoyama, H. Sumimoto, K. Toda, K. Miyahara, I. Kuribayashi, K. Nakao, K. Hosoda, Y. Yamamoto, H. Imura, Y. Shizuta, Cloning and expression of a cDNA for human cytochrome P-450aldo as related to primary aldosteronism, Biochemical and Biophysical Research Communications, 173 (1990) 309-316.

[11] A. Zöllner, N. Kagawa, M.R. Waterman, Y. Nonaka, K. Takio, Y. Shiro, F. Hannemann, R. Bernhardt, Purification and functional characterization of human 11β hydroxylase expressed in Escherichia coli, FEBS Journal, 275 (2008) 799-810.

[12] K.M. Curnow, M.-T. Tusie-Luna, L. Pascoe, R. Natarajan, J.-L. Gu, J.L. Nadler, P.C. White, The Product of the CYP11B2 Gene Is Required for Aldosterone Biosynthesis in the Human Adrenal Cortex, Molecular Endocrinology, 5 (1991) 1513-1522.

[13] F. Hannemann, A. Bichet, K.M. Ewen, R. Bernhardt, Cytochrome P450 systems—biological variations of electron transport chains, Biochimica et Biophysica Acta (BBA) - General Subjects, 1770 (2007) 330-344.

[14] E. Davies, S.M. MacKenzie, E.M. Freel, S. Alvarez-Madrazo, R. Fraser, J.M.C. Connell, Altered corticosteroid biosynthesis in essential hypertension: A digenic phenomenon, Molecular and Cellular Endocrinology, 300 (2009) 185-191.

[15] W.I. Cranston, B.E. Juel-Jensen, The Effetcs of Spironolactone and Chlorthalidoneon arterial pressure, The Lancet, 279 1161-1164.

[16] R.L. Wolf, M. Mendlowitz, J. Roboz, G.H. Styan, P. Kornfeld, A. Weigl, Treatment of hypertension with spironolactone: Double-blind study, The journal of the American Medical Association, 198 (1966) 1143-1149.

[17] B. Pitt, F. Zannad, W.J. Remme, R. Cody, A. Castaigne, A. Perez, J. Palensky, J. Wittes, The Effect of Spironolactone on Morbidity and Mortality in Patients with Severe Heart Failure, New England Journal of Medicine, 341 (1999) 709-717.

[18] B. Pitt, G. Williams, W. Remme, F. Martinez, J. Lopez-Sendon, F. Zannad, J. Neaton, B. Roniker, S. Hurley, D. Burns, R. Bittman, J. Kleiman, The EPHESUS Trial: Eplerenone in Patients with Heart Failure Due to Systolic Dysfunction Complicating Acute Myocardial Infarction, Cardiovasc Drugs Ther, 15 (2001) 79-87.

[19] B. Pitt, W. Remme, F. Zannad, J. Neaton, F. Martinez, B. Roniker, R. Bittman, S. Hurley, J. Kleiman, M. Gatlin, Eplerenone, a Selective Aldosterone Blocker, in Patients with Left Ventricular Dysfunction after Myocardial Infarction, New England Journal of Medicine, 348 (2003) 1309-1321.

[20] G. Colussi, C. Catena, L.A. Sechi, Spironolactone, eplerenone and the new aldosterone blockers in endocrine and primary hypertension, Journal of Hypertension, 31 (2013) 3-15.

[21] C.M. Kagawa, D.J. Bouska, M.L. Anderson, Oral absorption with various preparations of spironolactone in dogs, Journal of Pharmaceutical Sciences, 53 (1964) 450-451.

[22] W. Sadée, M. Dagcioglu, R. Schröder, Pharmacokinetics of Spironolatone, Canrenone and Canrenoate-K in humans, Journal of Pharmacology and Experimental Therapeutics, 185 (1973) 686-695.

[23] D.L. Phelps, A. Karim, Spironolactone: Relationship between concentrations of dethioacetylated metabolite in human serum and milk, Journal of Pharmaceutical Sciences, 66 (1977) 1203-1203.

[24] S.M. Rifka, J.C. Pita, R.A. Vigersky, Y.A. Wilson, D.L. Loriaux, Interaction of Digitalis and Spironolactone with Human Sex Steroid Receptors, The Journal of Clinical Endocrinology & Metabolism, 46 (1978) 338-344.

[25] S.M. Garthwaite, E.G. McMahon, The evolution of aldosterone antagonists, Molecular and Cellular Endocrinology, 217 (2004) 27-31.

[26] M.H. Weinberger, B. Roniker, S.L. Krause, R.J. Weiss, Eplerenone, a selective aldosterone blocker, in mild-to-moderate hypertension, American Journal of Hypertension, 15 (2002) 709-716.

[27] H.C. Erbler, Stimulation of aldosterone production in vitro and its inhibition by spironolactone, Naunyn-Schmiedeberg's Arch. Pharmacol., 273 (1972) 366-375.

[28] H.C. Erbler, On the mechanism of the inhibitory action of the spirolactone SC 9376 (aldadiene) on the production of corticosteroids in rat adrenals in vitro, Naunyn-Schmiedeberg's Arch. Pharmacol., 277 (1973) 139-149.

[29] S.C. Cheng, K. Suzuki, W. Sadee, B.W. Harding, Effects of Spironolactone, Canrenone and Canrenoate-K on Cytochrome P450, and 11β- and 18-Hydroxylation in Bovine and Human Adrenal Cortical Mitochondria1, Endocrinology, 99 (1976) 1097-1106.

[30] L. Schiffer, S. Anderko, A. Hobler, F. Hannemann, N. Kagawa, R. Bernhardt, A recombinant CYP11B1 dependent Escherichia coli biocatalyst for selective cortisol production and optimization towards a preparative scale, Microbial Cell Factories, 14 (2015) 25.

[31] U.M. Zanger, M. Schwab, Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation, Pharmacology & Therapeutics, 138 (2013) 103-141.

[32] S. Rendic, F.P. Guengerich, Survey of Human Oxidoreductases and Cytochrome P450 Enzymes Involved in the Metabolism of Xenobiotic and Natural Chemicals, Chemical Research in Toxicology, 28 (2015) 38-42.

[33] M.K. Parr, A. Zöllner, G. Fußhöller, G. Opfermann, N. Schlörer, M. Zorio, M. Bureik, W. Schänzer, Unexpected contribution of cytochrome P450 enzymes CYP11B2 and CYP21, as well as CYP3A4 in xenobiotic androgen elimination – Insights from metandienone metabolism, Toxicology Letters, 213 (2012) 381-391.

[34] B.-O. Lund, J. Lund, Novel Involvement of a Mitochondrial Steroid Hydroxylase (P450c11) in Xenobiotic Metabolism, Journal of Biological Chemistry, 270 (1995) 20895-20897.

[35] T. Omura, R. Sato, The Carbon Monoxide-binding Pigment of Liver Microsomes: II. Solubilization, Purification and Properties, Journal of Biological Chemistry, 239 (1964) 2379-2385.

[36] T. Sugiyama, T. Yamano, Purification and crystallization of NADPH-adrenodoxin reductase from bovine adrenocortical mitochondria, FEBS Letters, 52 (1975) 145-148.

[37] Y. Sagara, A. Wada, Y. Takata, M.R. Waterman, K. Sekimizu, T. Horiuchi, Direct expression of adrenodoxin reductase in Escherichia coli and the functional characterization, Biol Pharm Bull, 16 (1993) 627-630.

[38] H. Uhlmann, V. Beckert, D. Schwarz, R. Bernhardt, Expression of bovine adrenodoxin in E. coli and site-directed mutagenesis of /2 FE・2S/ cluster ligands, Biochemical and Biophysical Research Communications, 188 (1992) 1131-1138.

[39] B. Schiffler, A. Zöllner, R. Bernhardt, Stripping Down the Mitochondrial Cholesterol Hydroxylase System, a Kinetics Study, Journal of Biological Chemistry, 279 (2004) 34269-34276.

[40] J.B. Schenkman, Studies on the nature of the type I and type II spectral changes in liver microsomes, Biochemistry, 9 (1970) 2081-2091.

[41] J.D. Lambeth, D. Seybert, J. Lancaster, Jr., J. Salerno, H. Kamin, Steroidogenic electron transport in adrenal cortex mitochondria, Mol Cell Biochem, 45 (1982) 13-31.

[42] D.W. Seybert, J.R. Lancaster, J.D. Lambeth, H. Kamin, Participation of the membrane in the side chain cleavage of cholesterol. Reconstitution of cytochrome P-450scc into phospholipid vesicles, Journal of Biological Chemistry, 254 (1979) 12088-12098.

[43] D.W. Seybert, J.D. Lambeth, H. Kamin, The participation of a second molecule of adrenodoxin in cytochrome P-450-catalyzed 11beta hydroxylation, Journal of Biological Chemistry, 253 (1978) 8355-8358.

[44] N. Strushkevich, A.A. Gilep, L. Shen, C.H. Arrowsmith, A.M. Edwards, S.A. Usanov, H.-W. Park, Structural Insights into Aldosterone Synthase Substrate Specificity and Targeted Inhibition, Molecular Endocrinology, 27 (2013) 315-324.

[45] N. Gochman, C.L. Gantt, A fluorimetric method for the determination of a major spironolactone (Aldactone) metabolite in human plasma, Journal of Pharmacology and Experimental Therapeutics, 135 (1962) 312-316.

[46] P. Gardiner, K. Schrode, D. Quinlan, B.K. Martin, D.R. Boreham, M.S. Rogers, K. Stubbs, M. Smith, A. Karim, Spironolactone Metabolism: Steady-State Serum Levels of the Sulfur-Containing Metabolites, The Journal of Clinical Pharmacology, 29 (1989) 342-347.

[47] A. Jankowski, A. Skorek-Jankowska, H. Lamparczyk, Simultaneous determination of spironolactone and its metabolites in human plasma, Journal of Pharmaceutical and Biomedical Analysis, 14 (1996) 1359-1365.

[48] A.Z. Walton, J.D. Stewart, Understanding and Improving NADPH-Dependent Reactions by Nongrowing Escherichia coli Cells, Biotechnology Progress, 20 (2004) 403-411.

[49] S.F. Rong, X.L. Zhang, B.M. Ding, Y.F. Wang, X.H. Pan, J. Zhang, Y. Shen, Structural characterization of 1β,11α-dihydroxycanrenone biotrans-formed from canrenone by Aspergillus ochraceus SIT34205, Chinese Chemical Letters, 23 (2012) 313-316.

[50] N. Kaushik, S. Biswas, J. Singh, Biocatalysis and Biotransformation Processes – An Insight, The Scitech Journal 1(2014) 12-22.

[51] R.K. Bledsoe, K.P. Madauss, J.A. Holt, C.J. Apolito, M.H. Lambert, K.H. Pearce, T.B. Stanley, E.L. Stewart, R.P. Trump, T.M. Willson, S.P. Williams, A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor, The Journal of biological chemistry, 280 (2005) 31283-31293.

[52] Y. Li, K. Suino, J. Daugherty, H.E. Xu, Structural and Biochemical Mechanisms for the Specificity of Hormone Binding and Coactivator Assembly by Mineralocorticoid Receptor, Molecular Cell, 19 (2005) 367-380.

[53] P.J. Fuller, Y. Yao, J. Yang, M.J. Young, Mechanisms of ligand specificity of the mineralocorticoid receptor, Journal of Endocrinology, 213 (2012) 15-24.

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Review

The CYP11B subfamily

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ABSTRACT

The biosynthesis of steroid hormones is dependent on P450-catalyzed reactions. In mammals, cholesterol is the common precursor of all steroid hormones, and its conversion to pregnenolone is the initial and rate-limiting step in hormone biosynthesis in steroidogenic tissues such as gonads and adrenal glands. The production of glucocorticoids and mineralocorticoids takes place in the adrenal gland and the final steps are catalyzed by 2 mitochondrial cytochromes P450, CYP11B1 (11β-hydroxylase or P45011β) and CYP11B2 (aldosterone synthase or P450aldo). The occurrence and development of these 2 enzymes in different species, their contribution to the biosynthesis of steroid hormones as well as their regulation at different levels (gene expression, cellular regulation, regulation on the level of proteins) is the topic of this chapter.

Contents

1. Diversity on organismic level: function, expression and evolution of the CYP11B subfamily

CYP11B1 and CYP11B2 are members of the cytochrome P450 (P450) superfamily. P450s belong to a highly conserved class of enzymes that occur in every domain of life. They catalyze various reactions which are indispensable for many species like detoxification, defense and biosynthesis of endogenous compounds such

Abbreviations: A, aldosterone; B, corticosterone; DOC, 11-deoxycorticosterone; E, cortisone; F, cortisol; S, 11-deoxycortisol.

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as steroid hormones. As external monooxygenases they are able to utilize the activation of molecular oxygen for substrate hydroxylation. But their range of possible reactions is much higher comprising almost 30 different reaction types [1–3]. Despite their highly conserved structural properties, the amino acid composition differs from enzyme to enzyme which contributes to a high diversity among the superfamily, consisting of more than 21,000 genes (http://drnelson.uthsc.edu/CytochromeP450.html). An outstanding example for enzyme diversity in contempt of their evolutionary conserved function and highly similar structure is given by enzymes belonging to the CYP11B subfamily. These proteins are located in the inner mitochondrial membrane where they are supplied with electrons from NADPH via 2 electron transfer proteins, the adrenodoxin reductase (AdR) and adrenodoxin (Adx).

In the following subchapters, the CYP11B interspecies diversity, their role and regulation within mammalian endocrine systems as well as their divergence during evolution and species dependent endocrine functions will be discussed.

1.1. Mammalian CYP11B isoforms – evolution, interspecies diversity and expression pattern

Steroid hormone synthesizing P450s like the CYP11B subfamily members seem to originate from early detoxifying variants and there is evidence for a co-evolutionary process with their particular steroid substrates [4]. Recent phylogenetic studies revealed a common ancestor of mitochondrial cytochromes P450 which evolved by duplication events into the enzymes of the CYP11A and the CYP11B subfamily [5]. In human, 2 CYP11B isoforms exist which are involved in the biosynthesis of 2 of the most important steroid hormones (steroids are abbreviated as defined on page 1): CYP11B1 (11β-hydroxylase) catalyzes the final step of F synthesis by a one-step reaction from S, whereas A is synthesized via 3 steps by CYP11B2 (aldosterone synthase) from DOC [6–8]. The distinct reactions will be discussed more detailed later in this review. Human CYP11B enzymes show an amino acid similarity of 93% and their genes are tandemly arranged on chromosome 8 with a distance of 40 kbp. Evidently, the possession of 2 distinct enzymes is restricted to mammals, whereby the CYP11B2 homologs show a higher similarity towards each other than CYP11B1 homologs do, which proofs the evolution of CYP11B2 by a gene duplication of CYP11B1 [9]. Comparably to humans, two functionally distinct CYP11B isoforms can be found in mouse [10], guinea pig [9,11], baboon [12,13] and hamster [14]. Rats exhibit even 3 enzyme variants, CYP11B1, CYP11B2 and CYP11B3, whereupon the CYP11B3 isoform is only expressed for a few days during postnatal development [15]. In rat, B is the main glucocorticoid. Interestingly, cattle (*Bos taurus)* possesses 2 variants of a CYP11B enzyme, commonly assigned as CYP11B1, that perform both F [16] and A [16–21] biosynthesis. Cattle CYP11B1 has been extensively studied and much of our present knowledge on the CYP11B subfamily has been derived from this enzyme. In total, the bovine genome encodes 5 CYP11B1 genes [22]. Three of these represent pseudogenes [22,23]. The 2 functional genes encode 2 distinct, but very similar CYP11B1 variants with only slight functional varieties [18,24,25]. Moreover, structural diversity of bovine CYP11B1 is enhanced by the occurrence of polymorphisms [26,27]. Gluco- and mineralocorticoid synthesis is also catalyzed by a single enzyme in porcine [19], frog [28] and sheep [29].

CYP11B enzymes have to be strictly regulated on transcriptional, translational and post-translational level because of the higher demand of glucocorticoids compared to mineralocorticoids (Fig. 1). Therefore, a complex network of mechanisms exists starting with different gene expression patterns in adrenal tissues. In human as well as in rat it was demonstrated that the expression of

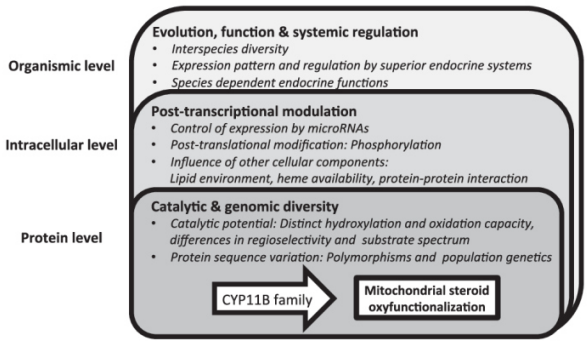

Fig. 1. Levels of CYP11B diversity discussed in this review.

CYP11B1 and CYP11B2 in the adrenal glands is spatially separated. While expression of CYP11B1 takes place in the *zona reticularis/fasciculata*, CYP11B2 expression and A synthesis are restricted to the *zona glomerulosa* [30–34]. F production by CYP11B1 is, however, limited to the *zona fasciculata* because of a 3β-hydroxysteroid dehydrogenase deficiency in the *zona reticularis* [35]. Recent studies revealed that in human adults CYP11B2 expression and CYP11B2 catalyzed A production can also occur in so-called subcapsular aldosterone-producing cell clusters (APCCs) of non-pathologic origin whose functional role and formation are not clear yet [36,37]. In cattle, A and F production are also limited to the respective adrenal zone, but due to the fact that CYP11B1 is localized in all 3 zones of the adrenal cortex [38] and synthesizes both steroids, this zonation cannot be explained by a different expression pattern and needs to be further investigated. Since bovine CYP11B1 is able under *in vitro* conditions to produce F and A, factors that suppress A formation in the *zona glomerulosa* need to be identified. However, until now, all attempts to identify such specific factors were unsuccessful. Although it was shown that angiotensin II could support A production in fetal bovine adrenal cells, an analysis of the promoter regions did not give an insight into zone-dependent A and F formation in cattle [32,39]. Current studies focusing on epigenetic aspects of CYP11B expression support the idea that a gene silencing of the particular CYP11B-variant by a hypermethylation of the gene in the respective tissue could lead to adrenal zonation [40]. Therefore, epigenetics is a promising approach for further elucidation of adrenal zonation and CYP11B regulation mechanisms, especially in case of cattle.

Another important aspect is the expression of the CYP11B enzymes in extra-adrenal tissues. There is evidence for an expression in rat and human brain but the physiological function remains unclear [41,42]. The CYP11B2 expression in heart is controversial [43–45]. It is however a fact that A can lead to cardiac fibrosis and heart insufficiency [46,47].

The distinctly controlled and induced production of gluco- and mineralocorticoids is a result of a highly sensible interaction between crucial components of the hypothalamic–pituitary–adrenal axis (HPA) and the renin–angiotensin-system (RAS). CYP11B2 expression and A secretion are mainly controlled by angiotensin II and potassium which cause an increase of the cellular calcium level, leading to the activation of calmodulin and of calmodulin-dependent kinases, subsequently. It is assumed that the activated kinases phosphorylate both activating transcription factors and members of the CRE-binding protein (CREB, cAMP-regulated binding protein) family which bind to 5′ flanking promoter regions of the CYP11B2 gene and trigger gene transcription in the *zona glomerulosa* [32,48,49]. Studies revealed that the adrenocorticotropic hormone (ACTH) can also influence

CYP11B2 expression. However, the effect distinguishes itself by a short-term activating and long-term suppressing effect on A secretion, which is elaborated in detail in a recent publication [50]. In the *zona fasciculata,* CYP11B1 expression and F (or B in case of rat) secretion are controlled by ACTH and a cAMP regulated signaling pathway, which also involves the CREB protein family. Rainey and Omura described in detail the different regulatory mechanisms of CYP11B1 and CYP11B2 gene transcription [32,51]. Gene regulation of the respective isoforms of CYP11B takes place by distinct mechanisms to ensure the right ratio of gluco- to mineralocorticoids. Considering the highly conserved core promoter regions of each enzyme across species, there are significant differences in the 5′ upstream region between CYP11B1 and CYP11B2 which gives an explanation for the specific transcriptional regulation mechanisms [32]. Recently, also the role of transposable elements which could contribute to intra- and interspecies evolutionary diversification of the CYP11B enzymes was described. It was shown that Alu and L1 elements located 5′ upstream of the core promoter sequence can modulate the transcription of both genes while Alu has an enhancing effect on both genes and L1 elements can block the effect of Alu on CYP11B1 transcription [52].

In the next section adrenal CYP11B expression will be discussed in the context of complex endocrine systems in mammals.

1.2. The role of the endocrine system for CYP11B expression

The high conservation of CYP11B proteins can be explained by the outstanding role of their end products for the development of terrestrial life. Throughout all evolutionary processes crucial metabolic and physiological pathways have been highly conserved despite divergence events and subsequent species diversity. One essential pathway for survival is the HPA axis which occurs exclusively in vertebrate species. This complex network consists of different peptides, receptors and steroid hormones and its action is highly diverse. Various stimuli like stress, illness or the circadian rhythm activate the HPA via the release of CRH (corticotropin-releasing hormone) in the hypothalamus which leads to a stimulation of the anterior pituitary gland and a subsequent release of ACTH [53]. ACTH binds its receptor in the adrenal glands and induces CYP11B1 expression and, therefore, the secretion of F. F subsequently evokes systemic stress responses through glucocorticoid receptor interaction and activation of various targets involved in energy mobilization, glucose homeostasis, immune response and behavior [54–57]. Besides, F is involved in ontogenetic processes, such as sex differentiation and the development from embryonic to adult organisms [58] and it can also regulate, in coexistence with the appropriate receptor, the mineral balance in organisms which are not able to produce A like some fish species. This highlights the promiscuity of corticosteroid action within diverse metabolic and physiological processes [59]. A feedback regulatory effect of F represses CRH release and ensures the fine-tuning of HPA activity by avoiding an excess of circulating glucocorticoids [60,61].

A dysregulation of this accurately synchronized network can lead to physical disorders, but also to mental illness, such as depressions, an exploding social phenomenon in our times [62,63]. Protein constituents of the HPA like nuclear receptors or peptide hormones, e.g., ACTH, are highly conserved within metazoa and seem to have evolved from the same ancestors emphasizing their crucial role in vertebrate development and terrestrial life [64]. An exciting observation occurring upon HPA evolution is the co-evolution of nuclear receptors and the corresponding steroids which is leading to a refinement of the various physiological responses [4]. Although the classical vertebrate HPA is not found in invertebrates like ascidiens because of an early divergence, many HPA components and homologous synthesis islands occur individually which hints at a possible bridging between the vertebrate and invertebrate endocrine systems [65]. This phenomenon can be further elucidated with the help of suitable model organisms like zebra fish [66].

Higher vertebrates acquired the capability to synthesize A which regulates the salt and water homeostasis and allowed the evolvement of terrestrial life by sophisticated water reabsorption strategies. In contrast to the biosynthesis of F, A production and thus water reabsorption, osmotic regulation and blood pressure are mainly regulated by the RAS which controls adrenocortical CYP11B2 expression [67]. An overproduction of A is associated with hypertension and accordingly, CYP11B2 is a promising target in drug development, as selective inhibitors could decrease A level and blood pressure [68–70]. The independent regulation of the CYP11B enzymes by different superior endocrine systems guarantees their expression in accordance with the physiological need for their respective products, F and A.

1.3. Low vertebrate CYP11B isoforms – evolution and species dependent endocrine functions

CYP11B orthologous genes have been identified in over 25 species ranging from teleosts to mammals (http://www.ncbi.nlm.nih.gov/gene/?Term=ortholog_gene_1584 [group]). Because of the high conservation of stress axis mechanisms during the evolution of vertebrates, great efforts were made over the last years to identify and characterize conserved components including enzymes of the CYP11B subfamily in lower vertebrates, with focus on fish, which represent the lowest species among vertebrates. Several new members of the CYP11B subfamily (CYP11B isoforms from fish are also termed CYP11C) were discovered and are reviewed in [71]. While in mammals the synthesis of F and A represents the main function of CYP11B enzymes, the synthesis of 11-oxygenated androgens is an additional crucial function of CYP11B enzymes in fish, which lack A, except for a few species, and use F as main gluco- and mineralocorticoid, which is produced in the head kidney [72]. Fish CYP11B enzymes also catalyze the 11β-hydroxylation of testosterone to 11β-hydroxytestosterone in gonads. 11β-hydroxytestosterone is the direct precursor of the testicular androgen 11-ketotestosterone [73], which has been described for a long time as sex determining male hormone in fish [73]. More recent studies could now establish the direct relationship between CYP11B expression and testicular development and differentiation in sea bass [74] and pejerry [75], male morph-specification in vocal fish [76], spermatogenesis in *rainbow trout* and *nile tilapia* [77,78] and sex changes to a male phenotype in hermaphroditic teleosts [77,79].

The identification of new enzyme subfamily members from evolutionary lower species contributes to our understanding of evolution and divergence within the group of CYP11B enzymes. There is evidence from gene cluster analysis that CYP11B1 seems to have evolved from fish CYP11C1 [5]. CYP11C1 of zebra fish is catalyzing the formation of the main glucocorticoid F. Therefore, zebra fish represents a promising and genetically amenable model organism with conserved endocrine mechanisms compared to higher vertebrates. A further potential key player for the elucidation of CYP11B evolution might be the functionally not identified CYP11-like gene sequence from lancelet, which represents an important transitional fossil at the border between invertebrates and vertebrates [80].

In addition to interspecies diversity and functional diversity induced by the endocrine system, CYP11B systems were discovered to be modulated by several factors on intracellular level. Furthermore, diversity and promiscuity can be found on protein level represented in variations of the catalytic potential and

protein sequence variation with distinct population genetics. These two levels (Fig. 1) will be discussed in the next chapters.

2. Modulation of CYP11B systems on intracellular level

2.1. MicroRNAs in post-transcriptional regulation

The transcriptional control mechanisms by the CYP11B promoters [32,48,52,81,82] and the influence of polymorphisms in the CYP11B genes [83–86] on transcription have been described by several studies. In addition to the topic of transcriptional regulation, which is not the focus of this review, the increasing knowledge about the role of microRNAs (miRNAs) opens up new perspectives on a further regulation level for the CYP11B1 and CYP11B2 genes as well as for the derived proteins and steroid hormone production.

Post-transcriptional regulation by miRNAs, which have emerged as key regulatory molecules that control ~30% of all mammalian genes [87], is a hitherto less studied issue in the field of steroid biosynthesis. MiRNAs are endogenous single-stranded noncoding RNA molecules of ~22 nucleotides that can play an important regulatory role by targeting specific sequences on mRNAs of protein-coding genes to direct their post-transcriptional repression [88]. So far, the few studies investigating the role of miRNAs in steroid hormone biosynthesis documented an influence on adrenal cell development and survival [89], dysregulation in adrenal carcinomas [90] and regulation of A production [91]. Recently, the role of miRNAs was investigated with particular focus on the genes of the human CYP11B subfamily [92]. It was shown that the expression of CYP11B1 and CYP11B2 in a human adrenocortical cell line depends on the presence of *Dicer1*, a key enzyme in miRNA maturation, and on the function of microRNA miR-24, for which binding sites in the 3′ untranslated region of CYP11B1 and CYP11B2 mRNAs were identified. The miR-24 regulates both genes in a canonical miRNA manner and seems to mediate their repression to a similar degree.

With the knowledge of this new post-transcriptional regulation level, it has to be considered whether siRNAs can be applied for the silencing of e.g., CYP11B2, which might be a novel approach to treat metabolic diseases like hyperaldosteronism, aldosterone caused hypertension and congestive heart failure.

2.2. The role of phosphorylation in regulating CYP11B-dependent activities

Although a wealth of interesting and promising results on the regulation of mitochondrial steroid hydroxylase systems on the transcriptional level have been obtained, far less attention has been paid to investigate post-translational modifications of the CYP11B electron-transfer systems, consisting of AdR, Adx and the respective P450 (Fig. 2). In this context, studies concentrated on protein phosphorylation, which is likely to alter the steric, electrostatic, and hydrogen bonding properties of the proteins. This is of particular relevance for cytochrome P450 systems because the introduction of a charged phosphate group might significantly influence the redox properties of the involved proteins or their binding behavior during the redox complex association and dissociation.

Early investigations of the phosphorylation state of rat adrenal mitochondrial proteins indicated that ACTH stimulated both phosphorylation and dephosphorylation of adrenal proteins and increased the B production 6-fold [93–95]. However, a specific target could not be identified in these early studies. The question whether mitochondrial cytochromes P450 represent regulation targets for phosphorylation or not continued to remain unclear in the following studies. On the one hand, experiments provided

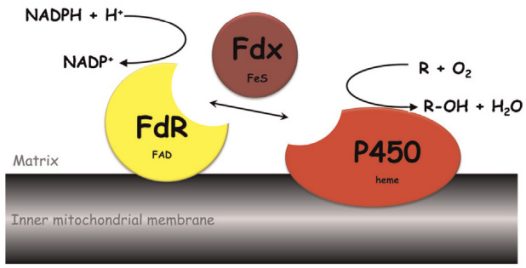

Fig. 2. The mitochondrial electron transfer system. Electrons are delivered along the inner mitochondrial membrane from NADPH via the redox proteins AdR, a membrane associated ferredoxin reductase (FdR), and the soluble ferredoxin (Fdx) Adx to members of the membrane incorporated CYP11B subfamily. The redox centers, flavin adenine dinucleotide (FAD), iron-sulfur cluster ([2Fe2S]) and Fe-protoporphyrin IX (heme) are indicated.

evidence that a crude preparation of cytochrome P450 from bovine corpus luteum mitochondria was phosphorylated *in vitro* in a cAMP-dependent reaction and consequently stimulated steroidogenesis [96], on the other hand, studies performed with preparations from rat adrenals could not confirm the stimulating effect [97]. In contrast to that, direct evidence has been provided that CYP11B1, purified from bovine adrenal cortex, can be phosphorylated using the cAMP-dependent protein kinase subunit C [98]. This phosphorylation increased the CYP11B1-Adx affinity, which was discussed to be of potential physiological importance for the steroidogenesis under Adx limited conditions.

Beside the CYP11B enzymes, the electron shuttle proteins of P450 systems attracted attention as phosphorylation target. It was demonstrated that renodoxin, the renal ferredoxin (analogous to Adx) [99], can be phosphorylated and following studies revealed that renodoxin and its phosphorylated form have a differential regulatory effect on the activity of the 1α- and 24-hydroxylases, CYP27B1 and CYP24A1, respectively [100,101]. In case of the bovine adrenal ferredoxin, *in vitro* phosphorylation experiments documented that the protein can be selectively modified at residue Thr-71 using the protein kinase CK2 [102] and at residue Ser-88[1] using the cAMP-dependent protein kinase (PKA) [103]. Adx phosphorylated by PKA increased the activity of reconstituted CYP11A1 and CYP11B1 systems, whereas Adx phosphorylated at residue Thr-71 increased only substrate conversions catalyzed by CYP11A1. Since both phosphorylation positions are located within the redox partner interaction domain of Adx it was supposed that steroid hydroxylase activity can be modulated on the post-translational level by influencing the protein interactions between the redox-partners of the P450 system [102,104]. Due to only very recent availability of human CYP11B1 and CYP11B2 for *in vitro* studies [105,106], corresponding studies on the effect of Adx phosphorylation on their activities have not been performed so far.

However, in order to establish the significance of such post-translational modifications in cell cultures kinase inhibitor studies were performed in V79 lung fibroblast cells [107], which expressed human CYP11B1 and CYP11B2 constitutively. Applications of kinase inhibitors indicated differential changes of the steroid hydroxylation activity of CYP11B1 and CYP11B2 depending on the inhibitor used. This supported the hypothesis that CYP11B enzyme systems are specifically regulated by phosphorylation processes and points to a modulation of the CYP11B activity by phosphorylation *in vivo*. In order to prove this hypothesis further studies are greatly needed to verify the phosphorylation targets, the natural

[1] Later studies revealed that PKA phosphorylation does not take place in this position (Bernhardt unpublished results).

regulatory mechanisms and the physiological meaning for the synthesis of F and A.

2.3. Modulation of CYP11B1 and CYP11B2 activities by cellular components

After their synthesis, CYP11B enzymes are transported to the inner membrane of the mitochondria. Once integrated in the phospholipid bilayer, the membrane and its associated components provide the environment to maintain the full enzyme function and may influence the catalytic and physico–chemical properties of these enzymes [108] (Fig. 2). One factor that is influencing enzyme activity on this level is the presence and composition of phospholipids. It was described that phospholipids extracted from adrenocortical mitochondria remarkably enhanced the CYP11B activity in reconstituted systems [109]. This effect was analyzed in more detail in studies indicating that the stimulation is depending on the presence of neutral or acidic phospholipids and on the type of fatty acyl substituents of the lipids [110,111].

A second CYP11B activity influencing factor is the availability of heme, which is essentially required for the assembly of a functional cytochrome P450. This is in agreement with the finding that activation of adrenal cytochromes in the adrenocortical cell line H295R by ACTH induces simultaneously the heme biosynthesis [112]. It was also shown that the administration of hemin stimulated B and A production in rat adrenal homogenates and in primary cultures of the calf adrenal *zona glomerulosa* in a dose-dependent fashion, whereas 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC), an inhibitor of heme synthesis, partially blocked ACTH-mediated steroid hormone synthesis [113,114]. These studies indicate that an influence on the activity of CYP11B enzymes can also take place on the level of protein folding depending on the synthesis and proper incorporation of heme into CYP11B enzymes.

Other factors modulate the activity of CYP11B enzymes by affecting redox partner-interactions. Calmodulin and another mitochondrial P450, CYP11A1, were described to influence the CYP11B affinity to electron transport partners as well as substrates. The adrenocortical form of calmodulin, a small, versatile and ubiquitous intracellular trigger protein, was detected to interact with bovine CYP11B1 in the presence of Adx modulating the activity of A synthesis [115]. The study described that the calmodulin effect is Ca^{2+} dependent and led to a decreased rate of A production, while it increased the rate of 18OH-B formation. Another protein that influences CYP11B activity is CYP11A1, the cholesterol side chain cleavage enzyme, which was intensively investigated with respect to its modulation of CYP11B enzymes. On the one hand, competition for reducing equivalents was observed between rat CYP11A1 and CYP11B1 *in vitro* [116] as well as in COS-1 cell cultures expressing human and bovine enzymes [117] which results in a decrease of F and A synthesis under conditions with limited resources of reducing equivalents. On the other hand, results indicated that bovine CYP11A1 interacted specifically with CYP11B1 and stimulated its 11β-hydroxylation activity while it decreased 18OH-B and A formation in reconstituted liposomal membranes [118,119] and COS-1 cells [117]. It has to be mentioned that an allosteric influence of CYP11A1 on the product pattern of CYP11B enzymes seems to be species-dependent, because such an effect has not been observed for the human enzymes in COS-1 cells [117].

In addition to CYP11A1, the efficiency of interaction with the redox partner Adx is also of high impact for CYP11B activities, although the role of Adx is often underestimated [120]. Adx as essential part of the NADPH-dependent redox system, delivers electrons to all mitochondrial cytochromes P450 (Fig. 2). It has been investigated with regard to both binding and electron transfer to various mitochondrial cytochromes P450 as well as to its influence on substrate binding and activity modulation. Several defined residues and protein domains were shown to be necessary to form a functional Adx-P450 complex, whereby overlapping but not identical binding sites for different P450s (CYP11B1, CYP11A1) have been identified using bovine proteins [121,122]. Moreover, it has been observed that enzyme activities were low in recombinant systems using COS-1, V79 cells or *fission yeast* if only the cytochrome P450 was expressed using the autologous redox partners and can be significantly stimulated upon cotransfection of an Adx gene [26,123–126]. This shows the importance of the Adx concentration and of efficient redox partner interactions with mitochondrial P450s for their activity. Interestingly, a recently performed study using reconstituted systems containing purified human CYP11B proteins only confirmed an Adx-dependent stimulation for CYP11B2 but not CYP11B1 [127]. In contrast, a stimulatory effect was caused by N- and C-terminally truncated forms of Adx with respect to B formation by bovine CYP11B1 [128]. In addition, a shift of the product equilibrium towards 18OH-B and A was observed in reactions of bovine CYP11B1 and human CYP11B2 studied in COS-1 cells when using Adx species displaying a higher efficiency of electron transfer to bovine CYP11B1 and human CYP11B2 [129]. This observation is consistent with Biacore and stopped-flow measurements, which indicated that complex rearrangements or conformational gating, induced by Adx might be necessary before an efficient electron transfer can take place in the complex with human CYP11B1 [106]. These results, together with the observation that a proteolytic digestion of the C-terminal part of Adx in the bovine adrenal gland produced several truncated Adx forms [130–132], led to the assumption that Adx expression level in general and its potential proteolytic modification in particular may regulate steroid hormone biosynthesis [133].

3. Diversity of CYP11B enzymes on protein level

3.1. Variation of catalytic potential and product spectrum between CYP11B isoforms

CYP11B isozymes are well known to be responsible for the final steps in the biosynthesis of gluco- and mineralocorticoids by catalyzing the formation of F and A from S and DOC, which are produced from their respective precursors by CYP21A2. However, the spectrum of substrates that are converted by CYP11B isozymes and the distinct functions fulfilled by the different enzymes from various organisms demonstrate a clear diversity between isozymes of the CYP11B subfamily. The on-going exploitation of their substrate spectrum ascribes them additional roles in biotransformations other than F and A synthesis. These aspects of functional diversity and promiscuity will be elucidated in the following section by taking examples of the CYP11B isozymes from bovine, human and rat as organisms with 1, 2 and 3 functional CYP11B enzymes, respectively. Fig. 3 sums up the respective activities of the discussed isozymes in transformations of the A and F precursors DOC and S.

3.1.1. Rat CYP11B enzymes

Rattus norvegicus expresses 3 different CYP11B enzymes [134,135] that have evolved with clearly defined functions in steroid hydroxylation and oxidation within the synthesis of mineralo- and glucocorticoids.

The predominant function of the first rat isozyme, CYP11B1, is the 11β-hydroxylation of DOC and S to B and F, respectively [7,136–139]. One should remember at this point that in rat B represents the major glucocorticoid, and not F like in cattle or human [30]. Besides the 11β-hydroxylation, CYP11B1 displays minor activities as 18- and 19-hydroxylase. The respective

products occur as side-products in the conversion of DOC [7,136–138], while 18OH-S is formed as a side product from S [139]. Additionally, the products of the 11β-hydroxylation, B and F, can further serve as substrates for the 18-hydroxylase reaction yielding 18OH-B [137,138] and 18OH-F [139], respectively. Comparable to the human CYP11B1, which will be discussed later in this section, the rat isoform is not capable of performing additional reactions on 18OH-DOC [137]. Thus, for the synthesis of 18OH-B by CYP11B1, the 11β-hydroxylation seems to be required to precede the hydroxylation in position 18. In addition to its functions as hydroxylase, rat CYP11B1 is able to perform oxidase reactions toward position 11 and 19. 19OH-DOC, which occurs as side-product during the conversion of DOC, can be oxidized to the 19-oxo product [137,138] and F, as the 11β-

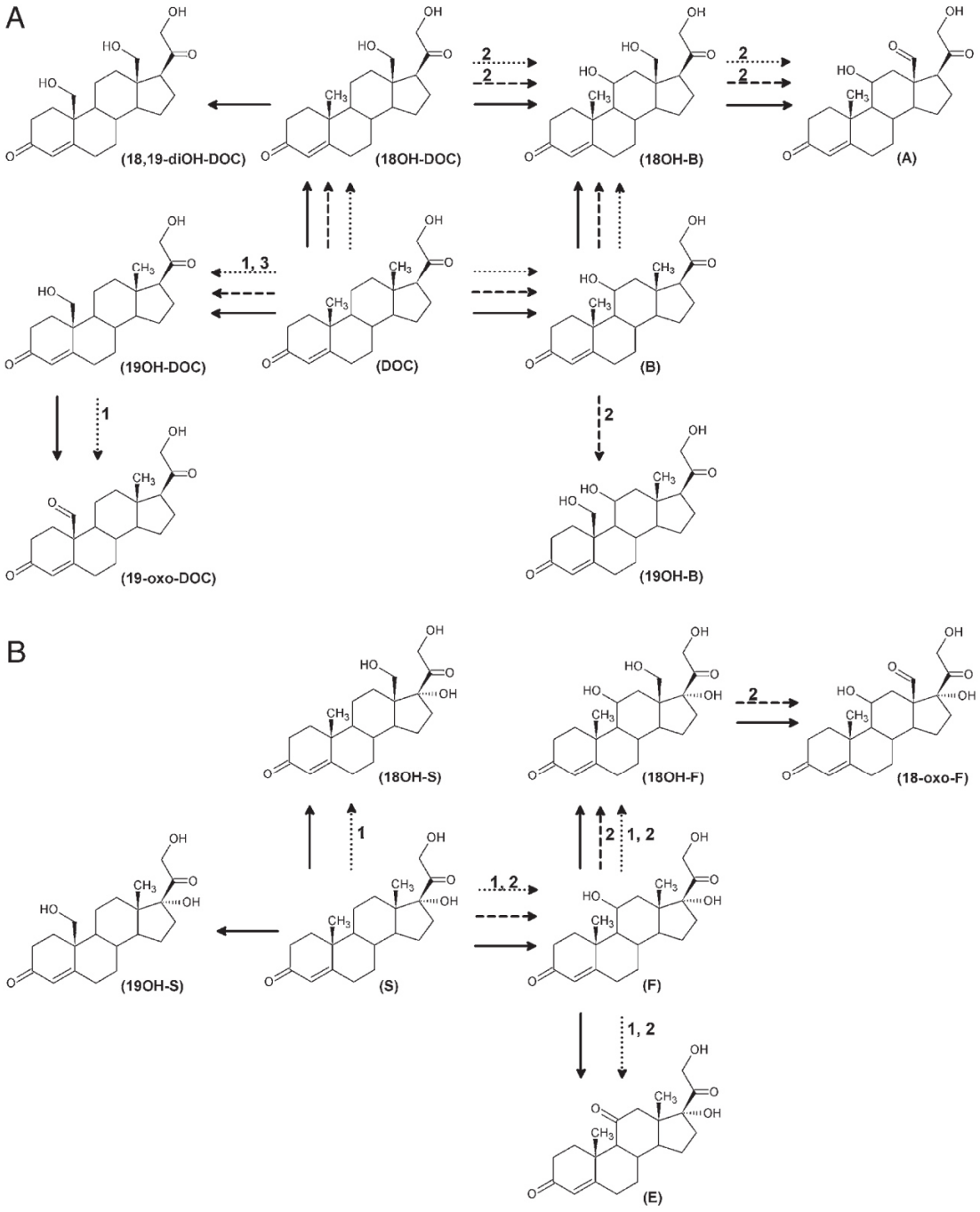

Fig. 3. Reactions catalyzed by different CYP11B isozymes from rat (dotted arrows), bovine (solid arrows) and human (dashed arrows) catalyzed from DOC (A) and S (B). For human and rat the number next to the respective arrow represents the responsible isozyme, if the reaction is not performed by all isozymes of the organism. Steroids are abbreviated as defined on page 1.

hydroxylated product of S, can subsequently be oxidized in small quantities in position 11 leading on to E [139]. Thus, rat CYP11B1 can be described as an 11β-hydroxylase with slight side activities as 18- and 19-hydroxylase as well as 11- and 19-oxidase, but it lacks an 18-oxidase function and consequently the capability of A formation.

This reaction is carried out in rat by CYP11B2, which is the only rat CYP11B isozyme that combines 11β- and 18-hydroxylase activities with an 18-oxidase activity. It forms A from DOC via the intermediates B and 18OH-B along with the formation of 18OH-DOC as side product [140]. *In vitro* activity studies indicate the existence of a distinct chronological pathway of intermediate formation, which is required for A formation by rat CYP11B2: if 18OH-DOC is used as a substrate, it is converted to 18OH-B [137,138], which is contrary to rat CYP11B1 but in agreement with the results for human CYP11B2 [127,141]. However, no consecutive oxidation to A by CYP11B2 can be observed under these conditions. This gives rise to the hypothesis, that A synthesis by rat CYP11B2 demands processive 18-hydroxylation followed by 18-oxidation. Considering S transformation by rat CYP11B2, the same products as for the CYP11B1 isozyme (F, E and 18OH-F) are formed except for 18OH-S [139]. Although no information about the involvement of CYP11B2 in 18-oxo-F synthesis is available so far, its participation in this reaction is very likely as the formation of this steroid by the rat adrenal has been observed [142].

The third rat isozyme, CYP11B3, is a pure hydroxylase without any oxidase activity. It converts DOC to B and 18OH-DOC [15,143] as well as B to 18OH-B [15]. However, in doing so the ratio of 18OH-DOC formation to B formation reveals a clear preference for 18- over 11β-hydroxylation [143], which distinguishes rat CYP11B3 from the CYP11B1 isozyme. Summarizing, it can be stated that in rat the 3 different CYP11B isozymes have distinct preferences for hydroxylation and oxidation although they display overlapping activities.

3.1.2. The bovine CYP11B1 enzyme

In *Bos taurus*, a single CYP11B enzyme, termed CYP11B1, was described to integrate an efficient 11β-hydroxylase activity with hydroxylation and oxidation activity towards position 18. It performs both F [16] as well as A [16–21] biosynthesis from S and DOC, respectively. However, more recent studies on bovine CYP11B1 activity including the purification of CYP11B1 from bovine adrenocortical mitochondria [18] and systematic analysis of cDNA libraries of the bovine adrenal gland [24,25] revealed 2 distinct variants of bovine CYP11B1 and 3 pseudogenes. Although the enzymatic characterization of the 2 variants after purification from the adrenal cortex [18] or in recombinant COS-7 cells [25] revealed slight differences in A and 18OH-B formation, both forms catalyze the same reactions and variations in activity are not necessarily considered in other studies. Most of the investigations were carried out with simple protein preparations from bovine adrenocortical mitochondria during the early stages of research on mammalian mitochondrial P450 systems.

Besides the efficient production of F and A, bovine CYP11B1 demonstrates an immense versatility in additional steroid oxyfunctionalization. Comparable to A synthesis, F can also undergo CYP11B1-catalyzed 18-hydroxylation and -oxidation, but to a lesser extent than B [144]. As CYP11B1 also functions as 19-hydroxylase, A and F production is accompanied by the formation of 18- and 19-hydroxy derivatives of the precursors DOC and S [16,21,145], but 11β-hydroxylation is significantly preferred over hydroxylation at positions 18 or 19 [16]. 18OH-DOC can further be converted by bovine CYP11B1 to 18OH-B or 18,19-dihydroxy-DOC by respective hydroxylation at positions 11β or 19 [146,147].

Besides the 18-oxidase activity that enables A formation, the enzyme also owns an oxidase activity towards positions 11 and 19 and can oxidize F to E as well as 19OH-DOC [21] and 19OH-androstenedione [148] to the corresponding 19-oxo products. 19-Oxo-androstenedione can consecutively be transformed to estrone and 19-norandrostenedione [148–150], which ascribes bovine CYP11B1 an additional aromatase and a nonaromatizing 10-demethylase function. Moreover, androgens like testosterone and androstenedione can be hydroxylated in position 11β and 19 [151]. Another class of steroids, which are subject to transformation by bovine CYP11B1, are 19-norsteroids. 19-nortestosterone has been shown to be converted into 2 products [152] and 19-norandrostenedione [150] is hydroxylated in positions 11β, 18 and 6. In summary, bovine CYP11B1 can be regarded as a highly promiscuous CYP11B isoform as it accepts a broad range of substrates including C21 steroids with and without a hydroxyl group in position 17 as well as C19 and C18 steroids. It is a multifunctional enzyme with 11β, 18- and 19-hydroxylase activity as well as oxidase activity towards all these positions. Additionally, aromatizing and non-aromatizing 10-demethylase reactions are carried out [148–150].

3.1.3. Human CYP11B enzymes

In contrast to cattle, human possess 2 CYP11B isozymes with distinct functions in the synthesis of corticosteroids. The major function of human CYP11B1 (11β-hydroxylase) is the 11β-hydroxylation of corticosteroids and thereby it is primarily responsible for the formation of F from S [108,127,133,153,154]. However, DOC also represents a good substrate for this reaction [127,153–155] but S is the preferred substrate with regard to catalytic efficiency [106,127]. The respective products of this reaction, F and B, can then be hydroxylated by CYP11B1 at position 18 yielding 18OH-F [127,154] and 18OH-B [106,127,154,155]. 18OH-B formation by CYP11B1 is in agreement with the activity of rat CYP11B1, but in contrast to the CYP11B1 isozyme from guinea pig, which is not capable of performing any further reaction on B [11]. Moreover, 18OH-DOC and 19OH-DOC are formed as side-products during the conversion of DOC by CYP11B1 [127,155]. Human CYP11B1 is thus a steroid 11β, 18- and 19-hydroxylase with the following regioselectivity: 11β >> 18 > 19.

While CYP11B1 owns no oxidase activity, human CYP11B2, is capable of carrying out an oxidase reaction towards position 18 and is responsible for the production of the mineralocorticoid A from DOC, via B and 18OH-B [127,153,154,156]. A constant but low A production is ensured by a low processivity with high intermediate release [105]. Besides this additional function, CYP11B2 also differs from CYP11B1 regarding substrate specificity, efficiency and selectivity of its hydroxylase activities. It hydroxylates S and DOC in position 11β, but less efficient than CYP11B1 [127,156]. In guinea pig, the catalytic efficiency of 11β-hydroxylation is distributed inversely and CYP11B2 has been described to convert S, DOC and androstenedione more efficiently compared with CYP11B1 [9]. As for human CYP11B1, 18OH-DOC and 19OH-DOC appear as side-products in the conversion of DOC by human CYP11B2 [105,127,156]. In addition, 2 further monohydroxylated DOC-products are formed, which have not been identified so far [105]. These results are in good agreement with the binding mode of DOC in the active site of CYP11B2, which was obtained by the recent resolution of the crystal structure of this complex [127]. It shows a rather loose coordination of DOC in the hydrophobic cavity of the active site via a hydrogen bond formed by the C21-hydroxyl group and a potential hydrogen bond formed by the 3-keto group. Hydrophobic interactions with aromatic residues along the I-helix guarantee binding from the α-face and render C11, C18 and C19 accessible for hydroxylation over the heme. Although C19 is the closest to the heme iron, C11 represents the position of steric and energetically most favorable hydroxylation [105,127], which is reflected in the corresponding catalytic efficiencies.

The results of different *in vitro* studies employing the intermediates of A formation as substrates give insight into the sequential reaction mechanism, that needs to be carried out for A formation. If 18OH-DOC, which emerges as minor product in DOC conversion, is applied, it can further be converted to 18OH-B by CYP11B2 [127,141], which represents a common feature with bovine CYP11B1. This reaction is not supported by human CYP11B1 [127,141]. Thus, CYP11B2 can form 18OH-B via 2 different pathways carrying out chronology-independent 11β and 18-hydroxylation, while CYP11B1 requires 11β-hydroxylation as the first step. However, A formation from 18OH-DOC via 18OH-B is not likely to be relevant in man [141]. When B is available, CYP11B2 produces 18OH-B and subsequently A as well as 19OH-B as a side-product [105]. The 19-hydroxylase activity of CYP11B1 is, in contrast, restricted to DOC. The deployment of 18OH-B as substrate in a reconstituted *in vitro* system with a concentration of 400 μM does not result in the formation of A and titration of CYP11B2 with increasing 18OH-B concentrations up to 1 mM does not induce a high spin shift of the heme iron [105]. This hints at a requirement of complete processivity of 18-hydroxylation and oxidation for A formation at naturally occurring concentrations. However, the mechanisms of intermediate turnover need further investigations comprising a wider range of concentrations as the binding affinity of intermediates is significantly reduced during the reaction sequence. Processivity of A formation in bovine is still a point of discussion as bovine CYP11B1 is able to oxidize free 18OH-B [109], although the main route of A formation is realized from DOC or B as a substrate, whereby A is formed successively [157]. Whether this need for processivity is substrate related or a general aspect of the 18-oxidase function can be investigated by studying the metabolism of F in more detail as F represents the second substrate that has so far been described for CYP11B2-dependent 18-hydroxylase and -oxidase activity [154,158]. The structural basis for the differentiated processivity and selectivity of both human isoforms was discussed in Ref. [127]. The authors compared the results of several previous studies, performed with the intention to identify function-specific residues in CYP11B enzymes (for review see Ref. [133]) with data of the human CYP11B2 structure and came to the conclusion that functional differences in gluco- and mineralocorticoid synthesis are mainly due to enhanced protein dynamics in CYP11B2 compared with CYP11B1 modulating the binding of substrates and the dissociation rates of hydroxylated intermediates.

In addition to their role in gluco- and mineralocorticoid synthesis, both human CYP11B enzymes have recently been proofed to be involved in transformations of progesterone and the 2 androgenic steroids, testosterone and androstenedione [127,159]. Their substrate spectrum, which has previously been regarded as rather narrow compared to e.g., liver P450s, therefore includes C21 steroids with and without 17- and 21-hydroxy groups as well as C19 steroids. The only shared structural element is the 3-keto-Δ4 motif of the A-ring and no metabolism of 3OH-Δ5 steroids has been observed so far. This restriction of structural motifs seems physiologically necessary, because it prevents direct metabolism of CYP11A1 products in the mitochondria. Hence, human CYP11B enzymes show additional, diverse functions in biotransformations of steroid hormones and a role in the derivatization of androgens, gestagens and F is indicated. Their 19-hydroxylase activity suggests a contribution to the biosynthesis of 19-normineralocorticoids by delivering 19-hydroxylated precursors. The finding that both CYP11B enzymes are also able to metabolize metandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-on; 1,2-dehydromethyltestosterone) [160,161], a synthetic steroid that is frequently abused by professional and amateur sportsmen for doping purposes because of its anabolic effects, raises the question whether human CYP11B enzymes and adrenocortical P450s, in general, are involved in biotransformations of exogenous steroid-like compounds. The contribution of CYP11B1 in the biotransformation of methylsulfonyl-DDE (3-methylsulfonyl-2,2-bis-(4-chlorophenyl)-1,1-dichlorethen), a metabolite of the insecticide DDT (1,1,1-trichlor-2,2-bis-(4-chlorophenyl)ethan; dichlordiphenyltrichlorethan), in mice [162] even expands this aspect to nonsteroid-like molecules.

Taken together, the presented examples for CYP11B enzymes from different organisms reveal that the biosynthesis of gluco- and mineralocorticoids can be successfully performed by functional promiscuity of an individual enzyme, like in cattle, as well as by the evolvement of clear functional diversity between multiple isozymes of one organism, as presented for rat and human. The elucidation of the substrate space for the different enzymes supports the idea that there is no strict relationship between a steroid hydroxylase and its potential substrates as their interaction does not follow a simple lock-key principal.

3.2. Protein sequence variation and population genetics of human CYP11B enzymes

Already with the first publications about successful cloning and characterization of human CYP11B isozymes by different laboratories in the late 1980s and early 1990s [155,156,163], differences between the reported sequences on DNA and protein level could be discerned. Ever since the number of known sequence variants greatly increased, as major progress in the fields of DNA sequencing technologies and subsequent bioinformatical evaluation could be achieved, which enables today's systematic high-throughput analysis of human genes. Human CYP11B1 and CYP11B2 have been the target of such approaches in numerous studies as their essential role for the regulation of human metabolic and electrolyte homeostasis and immune response possibly results, in case of altered function and/or activity, in causal effects for common, especially cardiovascular, diseases. Thus, to understand the relationship between their genomic variation and disease can be a key in diagnosis, treatment and discovery of predisposition. The following section will briefly summarize the current state of our knowledge about sequence variation of these genes. Within that context we will focus on exonic, non-synonymous polymorphisms, in order to elucidate structural diversity on the protein level. Polymorphisms are defined as natural variants, that occur with an allele frequency of >1%. Individual, random mutations which are directly associated with disorders in steroidogenesis will not be included, as the purpose of this article is to highlight natural diversity and promiscuity of functional 11B enzymes.

Among human genes in general as well as in the group of genes encoding human steroidogenic cytochromes P450, the 2CYP11B genes belong to those with elevated genetic diversity. They display a variation frequency with a mean of about 1 single nucleotide polymorphism (SNP) per 100 base pairs in coding and non-coding regions, which represents about the 3-fold compared to the average SNP frequency of other genes involved in common diseases and regulation of blood pressure [164,165]. CYP21A2 is the only P450 involved in steroid biosynthesis, that shows a higher frequency of genetic variation (approximately 1 SNP per 80 bp) than CYP11B1 and CYP11B2 [164].

Table 1A and B summarizes the most frequent coding, non-synonymous polymorphisms occurring for human CYP11B1 and CYP11B2 and provide additional information about their localization in the protein molecule as well as results from *in vitro* activity studies [105,127,166]. The indicated allele frequencies are derived from the online browser of the 1000 genomes project (http://browser.1000genomes.org), the currently most stringent project for the identification of genetic variants in populations from

Table 1
Summary of the most common polymorphisms of human CYP11B1 (A) and CYP11B2 (B) including their respective average frequency in global population (1000 genomes browser), localization in the protein structure, indication of a potential origin in a gene conversion event with the other CYP11B isoform, and activity alterations in *in vitro* systems.

A – CYP11B1

Polymorphism	Average frequency (%)	Localization	Gene conversion	Effect on activity *in vitro*	Test system
R43Q	14	A-helix, outer surface	Yes	11β-Hydroxylation decreased to 50–30% with stronger effect on DOC conversion	Recomb. JEG-3 cells [181], substrates DOC and S
A386V	13	β1-4, outer surface	Yes	n.d.	–

B – CYP11B2

Polymorphism	Average frequency (%)	Localization	Gene conversion	Effect on activity *in vitro*			Test system
				11β-Hydroxylation	18-Hydroxylation	18-Oxidation	
K173R	36	D-helix, outer surface	No	Unaltered			Recomb. COS-7 cells [172], substrate DOC
I339T	12	J-helix, outer surface	No	Unaltered for DOC and S	Unaltered for DOC		Recomb. COS-1 cells [182], substrates DOC and S*
V386A	4	β1-4, outer surface	Yes	Unaltered	Decreased	Unaltered	Recomb. COS cells [179], substrate DOC
				Efficiency generally decreased			Recomb. COS-7 cells [172], substrate DOC
				Increased	Decreased	Unaltered	Recomb. *fission yeast* [177], substrate DOC
G435S	15	Loop between K′- and L-helix, outer surface	No	Unaltered for DOC	Decreased for B unaltered for DOC	Abolished	Purified protein from recomb. COS-7 [178], substrates DOC and B
				Unaltered	Decreased	Unaltered	Recomb. *fission yeast* [177], substrate DOC

* Analyzed for a CYP11B1/2 hybrid enzyme carrying the I339T exchange in comparison to CYP11B2 wt.

origins distributed all over the world, and represent the average frequencies in all individuals studied worldwide. However, for several polymorphisms characteristic allele distributions over populations with different ancestry can be observed, which nicely demonstrates, how classical evolutionary mechanisms, like genetic drifts and founder effects, reinforce biological diversity. While CYP11B1 R43Q and CYP11B2 G435S appear with an increased frequency of 43 and 48%, respectively, in populations from East Asian ancestry, CYP11B1 A386V and CYP11B2 I339T seem to be primarily restricted to populations with African ancestry, showing an elevated frequency of 38 and 44%, respectively, in these populations (for comparison with the corresponding mean values of frequency worldwide, see Table 1). The most common polymorphism in human CYP11B isozymes is the exchange of lysine in position 173 of CYP11B2 to arginine (K173R). In European populations, both alleles are almost equally distributed (K 52%, R 48%), but lysine is significantly more frequent in populations of African ancestry (81%). Iberian populations in Spain are the only European subpopulation exposing an increased frequency of 79% for the lysin allele, which has also been reported to occur predominantly in black individuals outside of Africa [167]. This might reflect African origin. Although the role of this polymorphism in primary aldosteronism and the development of hypertension has been intensively discussed, it turned out that the attribution of the K allele with hypertension results from a linkage disequilibrium between K173R and the -334C/T polymorphism in the promoter region, which alters gene expression and, in consequence, A level [168–171]. The amino acid exchange itself does not significantly affect CYP11B2 activity [172]. In addition to these well-known common polymorphisms, further benign variants of lower frequency can be discovered by screening small, unselected populations. Holloway et al. could identify 11 additional coding, non-synonymous polymorphisms of CYP11B2 with frequencies up to 4% by screening a number of 69 healthy, normotensive subjects. Eight of these (R87G, N281T, G288S, K296N, D335N, Q404R, A414P, H439Y) were investigated for B and A formation from DOC in a recombinant JEG-3 cell culture and all showed altered activity for at least one of the functions [173].

When considering the amino acid exchanges introduced by the quoted common polymorphisms it becomes obvious that the types of substitutions are very diverse with a range from conservative exchanges within the same class of amino acids (CYP11B1: A386V; CYP11B2: K173R and V386A) to more radical changes of the residue's chemical nature with the introduction of polar groups (CYP11B2: I339T and G435S) or the removal of a charge (CYP11B1: R43Q), which would be expected to threaten structural integrity of the enzyme. Despite alterations in catalytic activities for several polymorphisms (e.g., CYP11B1: R43Q; CYP11B2: V386A, G435S), that were observed by *in vitro* activity studies, these variants appear in healthwise unobtrusive subjects [172–176]. This leads to the hypothesis that human physiology tolerates CYP11B enzyme diversity concerning amino acid sequence up to a level, which might even lead to functional variations *in vivo*. Interestingly, individual amino acid exchanges seem to modulate the different reactions carried out by the CYP11B isozymes in distinct ways. For example, the V386A variant of CYP11B2, when expressed in fission yeast, exposes an increased activity for 11β-hydroxylation but a reduced activity for the respective reaction towards position 18, while the 18-oxidation activity remains unaltered [177]. Differentiated studies of enzyme variants *in vitro* can thus be an essential help to understand structure/function relation. However, limitations of *in vitro* systems in mimicking an authentic environment to a recombinant protein lead to disagreements between the results from different models, as it is the case for the data published about the effect of the V386A and G435R polymorphisms on CYP11B2 activity [172,177–179]. Still though, all studies support the thesis, that both variants especially reduce the efficiency of 18-hydroxylation.

When it comes to classifying the severity of the observed CYP11B diversity on protein level, 2 additional facts need to be taken into account. Firstly, the vast majority of polymorphisms concerns residues that are located on the protein surface

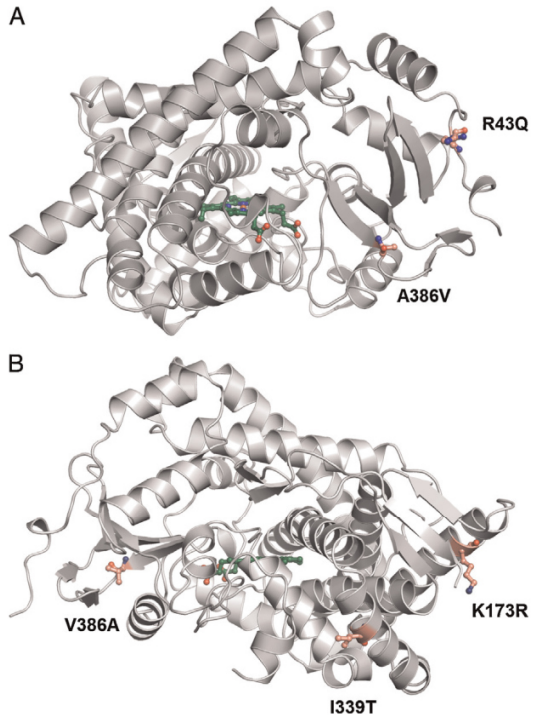

Fig. 4. Mapping of polymorphic residues in structure models of human CYP11B1 (A) and CYP11B2 (B). For the depiction of the CYP11B1 and CYP11B2 molecule the latest homology model [105] and the crystal structure [127] were used, respectively. For CYP11B2, G435S could not be mapped because of a gap in the structure.

(see Table 1 and CYP11B2: D335N, Q404R, A414P, H439Y described by Holloway et al. [173]) and consecutively do not have direct effects on essential events of the catalytic cycle in the active site (Fig. 4). However, changes in the flexibility of individual protein domains may affect the hydroxylation activity or selectivity. Secondly, a big number of polymorphisms (CYP11B1: R43Q, and A386V; CYP11B2: V386A and 9 out of 11 described by Refs. [173,180]) represent a substitution with an amino acid, that is present in the respective position of the other, highly homologous CYP11B isozyme, and presumably results from conversion events between the 2 genes. This might contribute to the maintenance of functionality despite the exchange, as long as key residues, which distinguish gluco- from mineralocorticoid synthesizing functions, are not involved. Therefore, natural diversity of human CYP11B isozymes on protein level can be ranged in as rather frequent, but generally leads to an only moderate degree of structural variation, which ensures functional integrity of the enzyme.

4. Conclusions

The diversity of CYP11B enzymes is omnipresent on multiple levels ranging from structural diversity of genes and proteins via specific transcriptional, translational and post-translational regulation mechanisms up to functional diversity and catalytic capacities. However, the underlying structural features and detailed regulatory mechanisms are still not sufficiently understood. Regarding the aspect of expression restriction to distinct zones of the adrenal cortex, which ensures a tissue specific production of gluco- and mineralocorticoids, modern techniques in epigenetics and the field of microRNA are promising novel approaches to get deeper insight into this challenging phenomenon. The expression of CYP11B isozymes in several organs other than the adrenals raises the question of a potential paracrine function in distinct extra-adrenal tissues. Zebra fish, whose role as model species in developmental studies is greatly increasing due to its genetic amenability and a highly conserved stress axis when compared to vertebrates, can serve as valuable tool with a completely revealed CYPome [183] for studies of stress-regulated steroidogenic mechanisms [184,185]. Although there are multiple indices for a determining role of post-translational modifications and especially phosphorylations for a modulation of the activity and product pattern of adrenal steroids, progress in this area is still limited and certainly needs further investigation. On the protein level, many open questions remain with regard to the isozyme specific catalytic properties. Structural characteristics and key residues that determine distinct functional specialization of different CYP11B enzymes and especially the capability to form A by an 18-oxidation, are not fully identified. Residues, which contribute to the differentiation of activity, could be narrowed down by mutagenesis studies [154,186,187] and the crystal structure of human CYP11B2 hints at a possible role of differences in conformational flexibility, but the detailed relations are not finally understood. In humans, all isozyme specific amino acids that have been studied and proofed to influence functionality are located between positions 284 and 339. However, in an alignment of this protein region with other CYP11B enzymes from different organisms expressing 1 or 2 isozymes (Fig. 5) no clear relationship between the encoded amino acids and functionality can be deduced. The only clear difference is glycine in position 288, which is present in all CYP11B2 structures as well as in the CYP11B proteins catalyzing F and A formation, but is absent in CYP11B1. This supports a hypothesis of a combinatorial effect of different

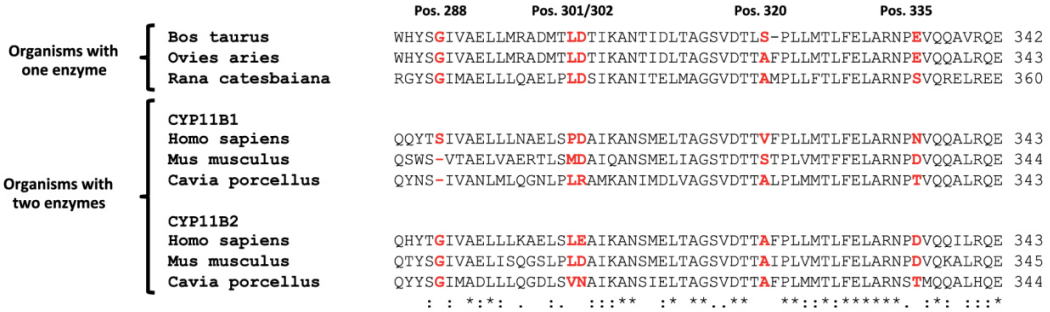

Fig. 5. Alignment of CYP11B isoforms of 6 organisms (amino acid residues 284–342) containing either one or 2 enzymes. Residues identified as relevant for isozyme specific activity by mutagenesis studies with the human enzymes (*Homo sapiens*) are marked in red and were compared with the sequences of cattle (*Bos taurus*), sheep (*Ovies aries*), bullfrog (*Rana catesbaiana*), mouse (*Mus musculus*) and guinea pig (*Cavia porcellus*). Alignment was performed with the ClustalW2 multiple sequence alignment tool of the EMBL-EBI website.

positions on the 18-oxidation ability. Further investigations following structural alignments of functionally diverse CYP11B isozymes are hampered by the absence of 3D structures other than human CYP11B2. The structural resolution of a CYP11B1 isozyme without A forming activity as well as the resolution of a bifunctional isozyme, like the bovine one, able to produce F as well as A could raise new hints.

Additionally, the detailed reaction mechanisms and dynamics of A synthesis still need to be further elucidated in order to understand the integration of intermediate release and the need for processivity. Nevertheless, as human CYP11B1 and CYP11B2 are available in a purified form in larger amounts only since 2008 and 2012 [105,106], respectively, our understanding of the enzymatic function is still developing and certainly will lead to new insights into this demanding and important group of enzymes within the next few years.

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References

[1] P.R. Ortiz de Montellano, The 1994 Bernard B. Brodie Award Lecture. Structure, mechanism, and inhibition of cytochrome P450, Drug Metab. Dispos. 23 (1995) 1181–1187.
[2] R. Bernhardt, Cytochrome P450: structure, function, and generation of reactive oxygen species, Rev. Physiol. Biochem. Pharmacol. 127 (1996) 137–221.
[3] R. Bernhardt, V.B. Urlacher, Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations, Appl. Microbiol. Biotechnol. 98 (2014) 6185–6203.
[4] M.E. Baker, Origin and diversification of steroids: co-evolution of enzymes and nuclear receptors, Mol. Cell. Endocrinol. 334 (2011) 14–20.
[5] D.R. Nelson, J.V. Goldstone, J.J. Stegeman, The cytochrome P450 genesis locus: the origin and evolution of animal cytochrome P450s, Philos. Trans. R. Soc. Lond. B Biol. Sci. 368 (2013) 1–22.
[6] K.M. Curnow, M.T. Tusie-Luna, L. Pascoe, R. Natarajan, J.L. Gu, J.L. Nadler, P.C. White, The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex, Mol. Endocrinol. 5 (1991) 1513–1522.
[7] T. Ogishima, F. Mitani, Y. Ishimura, Isolation of aldosterone synthase cytochrome P-450 from zona glomerulosa mitochondria of rat adrenal cortex, J. Biol. Chem. 264 (1989) 10935–10938.
[8] T. Ogishima, H. Shibata, H. Shimada, F. Mitani, H. Suzuki, T. Saruta, Y. Ishimura, Aldosterone synthase cytochrome P-450 expressed in the adrenals of patients with primary aldosteronism, J. Biol. Chem. 266 (1991) 10731–10734.
[9] H.E. Bulow, R. Bernhardt, Analyses of the CYP11B gene family in the guinea pig suggest the existence of a primordial CYP11B gene with aldosterone synthase activity, Eur. J. Biochem. 269 (2002) 3838–3846.
[10] L.J. Domalik, D.D. Chaplin, M.S. Kirkman, R.C. Wu, W.W. Liu, T.A. Howard, M.F. Seldin, K.L. Parker, Different isozymes of mouse 11 beta-hydroxylase produce mineralocorticoids and glucocorticoids, Mol. Endocrinol. 5 (1991) 1853–1861.
[11] H.E. Bulow, K. Mobius, V. Bahr, R. Bernhardt, Molecular cloning and functional expression of the cytochrome P450 11B-hydroxylase of the guinea pig, Biochem. Biophys. Res. Commun. 221 (1996) 304–312.
[12] M. Hampf, A.C. Swart, P. Swart, Sequence of the 11 beta-hydroxylase gene from the Cape baboon (*Papio ursinus*), Endocr. Res. 22 (1996) 495–499.
[13] A.C. Swart, N. Brown, N. Kolar, P. Swart, Baboon cytochrome P450c11 is encoded by more than one gene, Endocr. Res. 26 (2000) 1011–1018.
[14] S. Veronneau, H. Bernard, M. Cloutier, J. Courtemanche, L. Ducharme, A. Lefebvre, J.I. Mason, J.G. LeHoux, The hamster adrenal cytochrome P450c11 has equipotent 11beta-hydroxylase and 19-hydroxylase activities, but no aldosterone synthase activity, J. Steroid. Biochem. Mol. Biol. 57 (1996) 125–139.
[15] S.H. Mellon, S.R. Bair, H. Monis, P450c11B3 mRNA, transcribed from a third P450c11 gene, is expressed in a tissue-specific, developmentally, and hormonally regulated fashion in the rodent adrenal and encodes a protein with both 11-hydroxylase and 18-hydroxylase activities, J. Biol. Chem. 270 (1995) 1643–1649.
[16] H. Sato, N. Ashida, K. Suhara, E. Itagaki, S. Takemori, M. Katagiri, Properties of an adrenal cytochrome P-450 (P-45011beta) for the hydroxylations of corticosteroids, Arch. Biochem. Biophys. 190 (1978) 307–314.
[17] S. Takemori, H. Sato, T. Gomi, K. Suhara, M. Katagiri, Purification and properties of cytochrome P-450(11beta) from adrenocortical mitochondria, Biochem. Biophys. Res. Commun. 67 (1975) 1151–1157.
[18] T. Ogishima, F. Mitani, Y. Ishimura, Isolation of two distinct cytochromes P-45011 beta with aldosterone synthase activity from bovine adrenocortical mitochondria, J. Biochem. 105 (1989) 497–499.
[19] K. Yanagibashi, M. Haniu, J.E. Shively, W.H. Shen, P. Hall, The synthesis of aldosterone by the adrenal cortex. Two zones (fasciculata and glomerulosa) possess one enzyme for 11 beta-, 18-hydroxylation, and aldehyde synthesis, J. Biol. Chem. 261 (1986) 3556–3562.
[20] A. Lombardo, G. Defaye, C. Guidicelli, N. Monnier, E.M. Chambaz, Integration of purified adrenocortical cytochrome P-450(11) beta into phospholipid vesicles, Biochem. Biophys. Res. Commun. 104 (1982) 1638–1645.
[21] M. Ohta, S. Fujii, A. Wada, T. Ohnishi, T. Yamano, M. Okamoto, Production of 19-hydroxy-11-deoxycorticosterone and 19-oxo-11-deoxycorticosterone from 11-deoxycorticosterone by cytochrome P-450(11) beta, J. Steroid. Biochem. 26 (1987) 73–81.
[22] S. Kirita, T. Hashimoto, M. Kitajima, S. Honda, K. Morohashi, T. Omura, Structural analysis of multiple bovine P-450(11 beta) genes and their promoter activities, J. Biochem. 108 (1990) 1030–1041.
[23] T. Hashimoto, K. Morohashi, T. Omura, Cloning and characterization of bovine cytochrome P-450(11 beta) genes, J. Biochem. 105 (1989) 676–679.
[24] S. Kirita, K. Morohashi, T. Hashimoto, H. Yoshioka, Y. Fujii-Kuriyama, T. Omura, Expression of two kinds of cytochrome P-450(11 beta) mRNA in bovine adrenal cortex, J. Biochem. 104 (1988) 683–686.
[25] K. Morohashi, Y. Nonaka, S. Kirita, O. Hatano, A. Takakusu, M. Okamoto, T. Omura, Enzymatic activities of P-450(11 beta)s expressed by two cDNAs in COS-7 cells, J. Biochem. 107 (1990) 635–640.
[26] P.A. Mathew, J.I. Mason, J.M. Trant, D. Sanders, M.R. Waterman, Amino acid substitutions Phe66—Leu and Ser126—pro abolish cortisol and aldosterone synthesis by bovine cytochrome P450(11) beta, J. Biol. Chem. 265 (1990) 20228–20233.
[27] M. Okamoto, Y. Nonaka, H. Takemori, J. Doi, Molecular identity and gene expression of aldosterone synthase cytochrome P450, Biochem. Biophys. Res. Commun. 338 (2005) 325–330.
[28] Y. Nonaka, H. Takemori, S.K. Halder, T. Sun, M. Ohta, O. Hatano, A. Takakusu, M. Okamoto, Frog cytochrome P-450 (11 beta,aldo), a single enzyme involved in the final steps of glucocorticoid and mineralocorticoid biosynthesis, Eur. J. Biochem. 229 (1995) 249–256.
[29] W.C. Boon, P.J. Roche, V.E. Hammond, K. Jeyaseelan, R.J. Crawford, J.P. Coghlan, Cloning and expression analysis of a cytochrome P-450(11 beta) cDNA in sheep, Biochim. Biophys. Acta 1260 (1995) 109–112.
[30] G.P. Vinson, Glomerulosa function and aldosterone synthesis in the rat, Mol. Cell. Endocrinol. 217 (2004) 59–65.
[31] A.E. Rudolph, E.R. Blasi, J.A. Delyani, Tissue-specific corticosteroidogenesis in the rat, Mol. Cell. Endocrinol. 165 (2000) 221–224.
[32] W.E. Rainey, Adrenal zonation: clues from 11beta-hydroxylase and aldosterone synthase, Mol. Cell. Endocrinol. 151 (1999) 151–160.
[33] T. Ogishima, H. Suzuki, J. Hata, F. Mitani, Y. Ishimura, Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-45011 beta in rat adrenal cortex: histochemical basis for the functional zonation, Endocrinology 130 (1992) 2971–2977.
[34] R. Bernhardt, M.R. Waterman, Cytochrome P450 and steroid hormone biosynthesis, in: A. Sigel, H. Sigel, R.K.O. Sigel (Eds.), The Ubiquitous Roles of Cytochrome P450 Proteins, John Wiley & Sons, Chichester, UK, 2007 pp. 361–369.
[35] J.S. Gell, B.R. Carr, H. Sasano, B. Atkins, L. Margraf, J.I. Mason, W.E. Rainey, Adrenarche results from development of a 3beta-hydroxysteroid dehydrogenase-deficient adrenal reticularis, J. Clin. Endocrinol. Metab. 83 (1998) 3695–3701.
[36] M. Aiba, M. Fujibayashi, Alteration of subcapsular adrenocortical zonation in humans with aging: the progenitor zone predominates over the previously well-developed zona glomerulosa after 40 years of age, J. Histochem. Cytochem. 59 (2011) 557–564.
[37] K. Nishimoto, K. Nakagawa, D. Li, T. Kosaka, M. Oya, S. Mikami, H. Shibata, H. Itoh, F. Mitani, T. Yamazaki, T. Ogishima, M. Suematsu, K. Mukai, Adrenocortical zonation in humans under normal and pathological conditions, J. Clin. Endocrinol. Metab. 95 (2010) 2296–2305.
[38] F. Mitani, T. Shimizu, R. Ueno, Y. Ishimura, S. Izumi, N. Komatsu, K. Watanabe, Cytochrome P-45011 beta and P-450scc in adrenal cortex: zonal distribution and intramitochondrial localization by the horseradish peroxidase-labeled antibody method, J. Histochem. Cytochem. 30 (1982) 1066–1074.
[39] W.E. Rainey, I.M. Bird, J.I. Mason, Angiotensin-II-directed glomerulosa cell function in fetal adrenal cells, J. Steroid. Biochem. Mol. Biol. 43 (1992) 847–854.
[40] M. Demura, F. Wang, T. Yoneda, M. Kometani, T. Sawamura, Y. Cheng, K. Ono, Y. Nakamura, H. Sasano, K. Saijoh, Y. Takeda, Differential DNA methylation of the CYP11B1 and CYP11B2 genes in association with human adrenal zonation, Endocr. Abstr. 29 (2012) 35.
[41] S.M. MacKenzie, C.J. Clark, R. Fraser, C.E. Gomez-Sanchez, J.M. Connell, E. Davies, Expression of 11beta-hydroxylase and aldosterone synthase genes in the rat brain, J. Mol. Endocrinol. 24 (2000) 321–328.
[42] B. Erdmann, H. Gerst, A. Lippoldt, H. Bulow, D. Ganten, K. Fuxe, R. Bernhardt, Expression of cytochrome P45011B1 mRNA in the brain of normal and hypertensive transgenic rats, Brain Res. 733 (1996) 73–82.
[43] M. Yoshimura, S. Nakamura, T. Ito, M. Nakayama, E. Harada, Y. Mizuno, T. Sakamoto, M. Yamamuro, Y. Saito, K. Nakao, H. Yasue, H. Ogawa, Expression of aldosterone synthase gene in failing human heart: quantitative analysis using modified real-time polymerase chain reaction, J. Clin. Endocrinol. Metab. 87 (2002) 3936–3940.

[44] P. Ye, C.J. Kenyon, S.M. MacKenzie, A.S. Jong, C. Miller, G.A. Gray, A. Wallace, A. S. Ryding, J.J. Mullins, M.W. McBride, D. Graham, R. Fraser, J.M. Connell, E. Davies, The aldosterone synthase (CYP11B2) and 11beta-hydroxylase (CYP11B1) genes are not expressed in the rat heart, Endocrinology 146 (2005) 5287–5293.
[45] S.M. MacKenzie, J.M. Connell, E. Davies, Non-adrenal synthesis of aldosterone: a reality check, Mol. Cell. Endocrinol. 350 (2012) 163–167.
[46] C. Heymes, A. Garnier, S. Fuchs, J.K. Bendall, J. Nehme, M.L. Ambroisine, E. Robidel, B. Swynghedauw, P. Milliez, C. Delcayre, Aldosterone-synthase overexpression in heart: a tool to explore aldosterone's effects, Mol. Cell. Endocrinol. 217 (2004) 213–219.
[47] C. Delcayre, J.S. Silvestre, A. Garnier, A. Oubenaissa, S. Cailmail, E. Tatara, B. Swynghedauw, V. Robert, Cardiac aldosterone production and ventricular remodeling, Kidney Int. 57 (2000) 1346–1351.
[48] M.H. Bassett, P.C. White, W.E. Rainey, The regulation of aldosterone synthase expression, Mol. Cell. Endocrinol. 217 (2004) 67–74.
[49] E.F. Nogueira, W.E. Rainey, Regulation of aldosterone synthase by activator transcription factor/cAMP response element-binding protein family members, Endocrinology 151 (2010) 1060–1070.
[50] N.G. Hattangady, L.O. Olala, W.B. Bollag, W.E. Rainey, Acute and chronic regulation of aldosterone production, Mol. Cell. Endocrinol. 350 (2012) 151–162.
[51] T. Omura, K. Morohashi, Gene regulation of steroidogenesis, J. Steroid Biochem. Mol. Biol. 53 (1995) 19–25.
[52] L.C. Cheng, T.W. Pai, L.A. Li, Regulation of human CYP11B1 and CYP11B2 promoters by transposable elements and conserved cis elements, Steroids 77 (2012) 100–109.
[53] S.R. Bornstein, W.C. Engeland, M. Ehrhart-Bornstein, J.P. Herman, Dissociation of ACTH and glucocorticoids, Trends Endocrinol. Metab. 19 (2008) 175–180.
[54] R.J. Denver, Structural and functional evolution of vertebrate neuroendocrine stress systems, Ann. N. Y. Acad. Sci. 1163 (2009) 1–16.
[55] L. Panagiotakopoulos, G.N. Neigh, Development of the HPA axis: where and when do sex differences manifest? Front Neuroendocrinol. 35 (2014) 285–302.
[56] M.A. Bellavance, S. Rivest, The HPA – immune axis and the immunomodulatory actions of glucocorticoids in the Brain, Front Immunol. 5 (2014) 136.
[57] A.J. Dunn, The HPA axis and the immune system: a perspective, in: G.P.C. Adriana del Rey, O.B. Hugo (Eds.), NeuroImmune Biology, Elsevier, 2007, 2014 pp. 3–15.
[58] H. Wada, Glucocorticoids: mediators of vertebrate ontogenetic transitions, Gen. Comp. Endocrinol. 156 (2008) 441–453.
[59] D. Bradshaw, S. McCormick, Hormonal control of salt and water balance in vertebrates – a symposium, Gen. Comp. Endocrinol. 147 (2006) 1–2 (Epub 2005 Nov 2008).
[60] C. Tsigos, G.P. Chrousos, Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress, J. Psychosom. Res. 53 (2002) 865–871.
[61] T.M. Buckley, A.F. Schatzberg, On the interactions of the hypothalamic–pituitary–adrenal (HPA) axis and sleep: normal HPA axis activity and circadian rhythm exemplary sleep disorders, J. Clin. Endocrinol. Metab. 90 (2005) 3106–3114.
[62] M.F. Juruena, Early-life stress and HPA axis trigger recurrent adulthood depression, Epilepsy Behav. 19 (2013) 1–12.
[63] A.P. Harris, M.C. Holmes, E.R. de Kloet, K.E. Chapman, J.R. Seckl, Mineralocorticoid and glucocorticoid receptor balance in control of HPA axis and behaviour, Psychoneuroendocrinology 38 (2013) 648–658.
[64] G.V. Markov, R. Tavares, C. Dauphin-Villemant, B.A. Demeneix, M.E. Baker, V. Laudet, Independent elaboration of steroid hormone signaling pathways in metazoans, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 11913–11918.
[65] R.K. Campbell, N. Satoh, B.M. Degnan, Piecing together evolution of the vertebrate endocrine system, Trends Genet. 20 (2004) 359–366.
[66] D. Alsop, M.M. Vijayan, Development of the corticosteroid stress axis and receptor expression in zebrafish, Am. J. Physiol. Regul. Integr. Comp. Physiol. 294 (2008) R711–719.
[67] A.H. Gradman, Evolving understanding of the renin-angiotensin-aldosterone system: pathophysiology and targets for therapeutic intervention, Am. Heart J. 157 (2009) 1–6.
[68] T. Hakki, K. Hubel, H. Waldmann, R. Bernhardt, The development of a whole-cell based medium throughput screening system for the discovery of human aldosterone synthase (CYP11B2) inhibitors: old drugs disclose new applications for the therapy of congestive heart failure, myocardial fibrosis and hypertension, J. Steroid Biochem. Mol. Biol. 125 (2011) 120–128.
[69] Q. Hu, L. Yin, R.W. Hartmann, Aldosterone synthase inhibitors as promising treatments for mineralocorticoid dependent cardiovascular and renal diseases, J. Med. Chem. 57 (2014) 5011–5022.
[70] I. Schuster, R. Bernhardt, Inhibition of cytochromes p450: existing and new promising therapeutic targets, Drug Metab. Rev. 39 (2007) 481–499.
[71] T. Uno, M. Ishizuka, T. Itakura, Cytochrome P450 (CYP) in fish, Environ. Toxicol. Pharmacol. 34 (2012) 1–13.
[72] H.A. Bern, S.S. Madsen, A selective survey of the endocrine system of the rainbow trout (*Oncorhynchus mykiss*) with emphasis on the hormonal regulation of ion balance, Aquaculture 100 (1992) 237–262.
[73] B. Borg, Androgens in teleost fishes, Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 109 (1994) 219–245.
[74] S. Socorro, R.S. Martins, L. Deloffre, C.C. Mylonas, A.V. Canario, A cDNA for European sea bass (*Dicentrachus labrax*) 11beta-hydroxylase: gene expression during the thermosensitive period and gonadogenesis, Gen. Comp. Endocrinol. 150 (2007) 164–173.
[75] M. Blasco, J.I. Fernandino, L.G. Guilgur, C.A. Strussmann, G.M. Somoza, D. Vizziano-Cantonnet, Molecular characterization of cyp11a1 and cyp11b1 and their gene expression profile in pejerrey (*Odontesthes bonariensis*) during early gonadal development, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156 (2010) 110–118.
[76] A.S. Arterbery, D.L. Deitcher, A.H. Bass, Divergent expression of 11beta-hydroxysteroid dehydrogenase and 11beta-hydroxylase genes between male morphs in the central nervous system, sonic muscle and testis of a vocal fish, Gen. Comp. Endocrinol. 167 (2010) 44–50.
[77] J.F. Liu, Y. Guiguen, S.J. Liu, Aromatase (P450arom) and 11beta-hydroxylase (P45011beta) genes are differentially expressed during the sex change process of the protogynous rice field eel, *Monopterus albus*, Fish Physiol. Biochem. 35 (2009) 511–518.
[78] W.L. Zhang, L.Y. Zhou, B. Senthilkumaran, B.F. Huang, C.C. Sudhakumari, T. Kobayashi, Y. Nagahama, D.S. Wang, Molecular cloning of two isoforms of 11beta-hydroxylase and their expressions in the Nile tilapia, *Oreochromis niloticus*, Gen. Comp. Endocrinol. 165 (2010) 34–41.
[79] S. Miura, R. Horiguchi, M. Nakamura, Immunohistochemical evidence for 11β-hydroxylase (P45011β) and androgen production in the gonad during sex differentiation and in adults in the protandrous anemonefish *Amphiprion clarkii*, Zool. Sci. 25 (2008) 212–219.
[80] O. Gotoh, Evolution of cytochrome p450 genes from the viewpoint of genome informatics, Biol. Pharm. Bull. 35 (2012) 812–817.
[81] K. Takayama, K. Morohashi, S. Honda, N. Hara, T. Omura, Contribution of Ad4BP, a steroidogenic cell-specific transcription factor, to regulation of the human CYP11A and bovine CYP11B genes through their distal promoters, J. Biochem. 116 (1994) 193–203.
[82] M.H. Bassett, Y. Zhang, P.C. White, W.E. Rainey, Regulation of human CYP11B2 and CYP11B1: comparing the role of the common CRE/Ad1 element, Endocr. Res. 26 (2000) 941–951.
[83] S. Alvarez-Madrazo, S.M. Mackenzie, E. Davies, R. Fraser, W.K. Lee, M. Brown, M.J. Caulfield, A.F. Dominiczak, M. Farrall, M. Lathrop, T. Hedner, O. Melander, P.B. Munroe, N. Samani, P.M. Stewart, B. Wahlstrand, J. Webster, C.N. Palmer, S. Padmanabhan, J.M. Connell, Common polymorphisms in the CYP11B1 and CYP11B2 genes: evidence for a digenic influence on hypertension, Hypertension 61 (2013) 232–239.
[84] E. Davies, C.D. Holloway, M.C. Ingram, G.C. Inglis, E.C. Friel, C. Morrison, N.H. Anderson, R. Fraser, J.M. Connell, Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene CYP11B2, Hypertension 33 (1999) 703–707.
[85] C. Newton-Cheh, T. Johnson, V. Gateva, M.D. Tobin, M. Bochud, L. Coin, S.S. Najjar, J.H. Zhao, S.C. Heath, S. Eyheramendy, K. Papadakis, B.F. Voight, L.J. Scott, F. Zhang, M. Farrall, T. Tanaka, C. Wallace, J.C. Chambers, K.T. Khaw, P. Nilsson, P. van der Harst, S. Polidoro, D.E. Grobbee, N.C. Onland-Moret, M.L. Bots, L.V. Wain, K.S. Elliott, A. Teumer, J. Luan, G. Lucas, J. Kuusisto, P.R. Burton, D. Hadley, W.L. McArdle, M. Brown, A. Dominiczak, S.J. Newhouse, N.J. Samani, J. Webster, E. Zeggini, J.S. Beckmann, S. Bergmann, N. Lim, K. Song, P. Vollenweider, G. Waeber, D.M. Waterworth, X. Yuan, L. Groop, M. Orho-Melander, A. Allione, A. Di Gregorio, S. Guarrera, S. Panico, F. Ricceri, V. Romanazzi, C. Sacerdote, P. Vineis, I. Barroso, M.S. Sandhu, R.N. Luben, G.J. Crawford, P. Jousilahti, M. Perola, M. Boehnke, L.L. Bonnycastle, F.S. Collins, A. U. Jackson, K.L. Mohlke, H.M. Stringham, T.T. Valle, C.J. Willer, R.N. Bergman, M.A. Morken, A. Doring, C. Gieger, T. Illig, T. Meitinger, E. Org, A. Pfeufer, H.E. Wichmann, S. Kathiresan, J. Marrugat, C.J. O'Donnell, S.M. Schwartz, D.S. Siscovick, I. Subirana, N.B. Freimer, A.L. Hartikainen, M.I. McCarthy, P.F. O'Reilly, L. Peltonen, A. Pouta, P.E. de Jong, H. Snieder, W.H. van Gilst, R. Clarke, A. Goel, A. Hamsten, J.F. Peden, et al., Genome-wide association study identifies eight loci associated with blood pressure, Nat. Genet. 41 (2009) 666–676.
[86] D. Levy, G.B. Ehret, K. Rice, G.C. Verwoert, L.J. Launer, A. Dehghan, N.L. Glazer, A.C. Morrison, A.D. Johnson, T. Aspelund, Y. Aulchenko, T. Lumley, A. Kottgen, R.S. Vasan, F. Rivadeneira, G. Eiriksdottir, X. Guo, D.E. Arking, G.F. Mitchell, F.U. Mattace-Raso, A.V. Smith, K. Taylor, R.B. Scharpf, S.J. Hwang, E.J. Sijbrands, J. Bis, T.B. Harris, S.K. Ganesh, C.J. O'Donnell, A. Hofman, J.I. Rotter, J. Coresh, E.J. Benjamin, A.G. Uitterlinden, G. Heiss, C.S. Fox, J.C. Witteman, E. Boerwinkle, T. J. Wang, V. Gudnason, M.G. Larson, A. Chakravarti, B.M. Psaty, C.M. van Duijn, Genome-wide association study of blood pressure and hypertension, Nat. Genet. 41 (2009) 677–687.
[87] B.P. Lewis, C.B. Burge, D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, Cell 120 (2005) 15–20.
[88] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[89] C.C. Huang, H.H. Yao, Inactivation of *Dicer1* in Steroidogenic factor 1-positive cells reveals tissue-specific requirement for *Dicer1* in adrenal, testis, and ovary, BMC Dev. Biol. 10 (66) (2010) 1–10.
[90] P.S. Soon, L.J. Tacon, A.J. Gill, C.P. Bambach, M.S. Sywak, P.R. Campbell, M.W. Yeh, S.G. Wong, R.J. Clifton-Bligh, B.G. Robinson, S.B. Sidhu, miR-195 and miR-483-5p identified as predictors of poor prognosis in adrenocortical cancer, Clin. Cancer Res. 15 (2009) 7684–7692.
[91] D.G. Romero, M.W. Plonczynski, C.A. Carvajal, E.P. Gomez-Sanchez, C.E. Gomez-Sanchez, Microribonucleic acid-21 increases aldosterone secretion and proliferation in H295R human adrenocortical cells, Endocrinology 149 (2008) 2477–2483.
[92] S. Robertson, S.M. MacKenzie, S. Alvarez-Madrazo, L.A. Diver, J. Lin, P.M. Stewart, R. Fraser, J.M. Connell, E. Davies, MicroRNA-24 is a novel regulator of

aldosterone and cortisol production in the human adrenal cortex, Hypertension 62 (2013) 572–578.
[93] T.M. Koroscil, S. Gallant, On the mechanism of action of adrenocorticotropic hormone. The role of ACTH-stimulated phosphorylation and dephosphorylation of adrenal proteins, J. Biol. Chem. 255 (1980) 6276–6283.
[94] T.M. Koroscil, S. Gallant, The phosphorylation of adrenal proteins in response to adrenocorticotropic hormone, J. Biol. Chem. 256 (1981) 6700–6707.
[95] G. Bhargava, E. Schwartz, S.B. Koritz, The stimulation by the adrenocorticotropic hormone of the phosphorylation of an adrenal mitochondrial preparation, Proc. Soc. Exp. Biol. Med. 158 (1978) 183–186.
[96] M.G. Caron, S. Goldstein, K. Savard, J.M. Marsh, Protein kinase stimulation of a reconstituted cholesterol side chain cleavage enzyme system in the bovine corpus luteum, J. Biol. Chem. 250 (1975) 5137–5143.
[97] K. Hofmann, J.J. Kim, F.M. Finn, The role of protein kinases in ACTH-stimulated steroidogenesis, Biochem. Biophys. Res. Commun. 84 (1978) 1136–1143.
[98] G. Defaye, N. Monnier, C. Guidicelli, E.M. Chambaz, Phosphorylation of purified mitochondrial cytochromes P-450 (cholesterol desmolase and 11 beta-hydroxylase) from bovine adrenal cortex, Mol. Cell. Endocrinol. 27 (1982) 157–168.
[99] N. Siegel, N. Wongsurawat, H.J. Armbrecht, Parathyroid hormone stimulates dephosphorylation of the renoredoxin component of the 25-hydroxyvitamin D3-1 alpha-hydroxylase from rat renal cortex, J. Biol. Chem. 261 (1986) 16998–17003.
[100] R. Nemani, J.G. Ghazarian, B. Moorthy, N. Wongsurawat, R. Strong, H.J. Armbrecht, Phosphorylation of ferredoxin and regulation of renal mitochondrial 25-hydroxyvitamin D-1 alpha-hydroxylase activity in vitro, J. Biol. Chem. 264 (1989) 15361–15366.
[101] M.L. Mandel, B. Moorthy, J.G. Ghazarian, Reciprocal post-translational regulation of renal 1 alpha- and 24-hydroxylases of 25-hydroxyvitamin D3 by phosphorylation of ferredoxin. mRNA-directed cell-free synthesis and immunoisolation of ferredoxin, Biochem. J. 266 (1990) 385–392.
[102] M. Bureik, A. Zollner, N. Schuster, M. Montenarh, R. Bernhardt, Phosphorylation of bovine adrenodoxin by protein kinase CK2 affects the interaction with its redox partner cytochrome P450scc (CYP11A1), Biochemistry 44 (2005) 3821–3830.
[103] N. Monnier, G. Defaye, E.M. Chambaz, Phosphorylation of bovine adrenodoxin. Structural study and enzymatic activity, Eur. J. Biochem. 169 (1987) 147–153.
[104] A. Zollner, M.A. Pasquinelli, R. Bernhardt, D.N. Beratan, Protein phosphorylation and intermolecular electron transfer: a joint experimental and computational study of a hormone biosynthesis pathway, J. Am. Chem. Soc. 129 (2007) 4206–4216.
[105] A. Hobler, N. Kagawa, M.C. Hutter, M.F. Hartmann, S.A. Wudy, F. Hannemann, R. Bernhardt, Human aldosterone synthase: recombinant expression in *E. coli* and purification enables a detailed biochemical analysis of the protein on the molecular level, J. Steroid Biochem. Mol. Biol. 132 (2012) 57–65.
[106] A. Zollner, N. Kagawa, M.R. Waterman, Y. Nonaka, K. Takio, Y. Shiro, F. Hannemann, R. Bernhardt, Purification and functional characterization of human 11beta hydroxylase expressed in *Escherichia coli*, FEBS J. 275 (2008) 799–810.
[107] M. Bureik, A. Zeeh, R. Bernhardt, Modulation of steroid hydroxylase activity in stably transfected V79MZh11B1 and V79MZh11B2 cells by PKC and PKD inhibitors, Endocr. Res. 28 (2002) 351–355.
[108] M. Bureik, M. Lisurek, R. Bernhardt, The human steroid hydroxylases CYP1B1 and CYP11B2, Biol. Chem. 383 (2002) 1537–1551.
[109] A. Wada, T. Ohnishi, Y. Nonaka, M. Okamoto, T. Yamano, Synthesis of aldosterone by a reconstituted system of cytochrome P-45011 beta from bovine adrenocortical mitochondria, J. Biochem. 98 (1985) 245–256.
[110] T. Ohnishi, A. Wada, Y. Nonaka, M. Okamoto, T. Yamano, Effect of phospholipid on aldosterone biosynthesis by a cytochrome P-450(11) beta-reconstituted system, Biochem. Int. 9 (1984) 715–723.
[111] D.W. Seybert, Lipid regulation of bovine cytochrome P450(11) beta activity, Arch. Biochem. Biophys. 279 (1990) 188–194.
[112] C.N. Martini, D.G. Romero, L.L. Yanes, C. Vila Mdel, Induction of 5-aminolevulinate synthase by activators of steroid biosynthesis, Life Sci. 81 (2007) 19–25.
[113] E.N. Cozza, M.C. Vila, C.E. Gomez-Sanchez, Stimulation of aldosterone production by hemin in calf adrenal glomerulosa cell cultures, Steroids 58 (1993) 384–386.
[114] C.N. Martini, S.G. Vaena de Avalos, D.G. Romero, L. San Martin de Viale, C. Vila Mde, Heme availability affects corticosterone and aldosterone biosynthesis in rat adrenal, Steroids 62 (1997) 767–770.
[115] T. Ohnishi, A. Wada, Y. Nonaka, T. Sugiyama, T. Yamano, M. Okamoto, Effect of calmodulin on aldosterone synthesis by a cytochrome P-45011 beta-reconstituted system from bovine adrenocortical mitochondria, J. Biochem. 100 (1986) 1065–1076.
[116] T. Yamazaki, B.C. McNamara, C.R. Jefcoate, Competition for electron transfer between cytochromes P450scc and P45011 beta in rat adrenal mitochondria, Mol. Cell. Endocrinol. 95 (1993) 1–11.
[117] P.R. Cao, R. Bernhardt, Interaction of CYP11B1 (cytochrome P-45011 beta) with CYP11A1 (cytochrome P-450scc) in COS-1 cells, Eur. J. Biochem. 262 (1999) 720–726.
[118] S. Ikushiro, S. Kominami, S. Takemori, Adrenal P-450scc modulates activity of P-45011 beta in liposomal and mitochondrial membranes. Implication of P-450scc in zone specificity of aldosterone biosynthesis in bovine adrenal, J. Biol. Chem. 267 (1992) 1464–1469.
[119] S. Kominami, D. Harada, S. Takemori, Regulation mechanism of the catalytic activity of bovine adrenal cytochrome P-450(11) beta, Biochim. Biophys. Acta 1192 (1994) 234–240.
[120] R. Bernhardt, The role of adrenodoxin in adrenal steroidogenesis, Curr. Opin. Endocrinol. Diabetes Obes. 7 (2000) 109–115.
[121] A.V. Grinberg, F. Hannemann, B. Schiffler, J. Muller, U. Heinemann, R. Bernhardt, Adrenodoxin: structure, stability, and electron transfer properties, Proteins 40 (2000) 590–612.
[122] K.M. Ewen, M. Kleser, R. Bernhardt, Adrenodoxin: the archetype of vertebrate-type [2Fe-2S] cluster ferredoxins, Biochim. Biophys. Acta 1814 (2011) 111–125.
[123] M.X. Zuber, J.I. Mason, E.R. Simpson, M.R. Waterman, Simultaneous transfection of COS-1 cells with mitochondrial and microsomal steroid hydroxylases: incorporation of a steroidogenic pathway into nonsteroidogenic cells, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 699–703.
[124] D. LaSala, Y. Shibanaka, A.Y. Jeng, Coexpression of CYP11B2 or CYP11B1 with adrenodoxin and adrenodoxin reductase for assessing the potency and selectivity of aldosterone synthase inhibitors, Anal. Biochem. 394 (2009) 56–61.
[125] M. Bureik, B. Schiffler, Y. Hiraoka, F. Vogel, R. Bernhardt, Functional expression of human mitochondrial CYP11B2 in fission yeast and identification of a new internal electron transfer protein, etp1, Biochemistry 41 (2002) 2311–2321.
[126] T. Hakki, S. Zearo, C.A. Dragan, M. Bureik, R. Bernhardt, Coexpression of redox partners increases the hydrocortisone (cortisol) production efficiency in CYP11B1 expressing fission yeast *Schizosaccharomyces pombe*, J. Biotechnol. 133 (2008) 351–359.
[127] N. Strushkevich, A.A. Gilep, L. Shen, C.H. Arrowsmith, A.M. Edwards, S.A. Usanov, H.W. Park, Structural insights into aldosterone synthase substrate specificity and targeted inhibition, Mol. Endocrinol. 27 (2013) 315–324.
[128] H. Uhlmann, R. Kraft, R. Bernhardt, C-terminal region of adrenodoxin affects its structural integrity and determines differences in its electron transfer function to cytochrome P-450, J. Biol. Chem. 269 (1994) 22557–22564.
[129] P.R. Cao, R. Bernhardt, Modulation of aldosterone biosynthesis by adrenodoxin mutants with different electron transport efficiencies, Eur. J. Biochem. 265 (1999) 152–159.
[130] M. Tanaka, M. Haniu, K.T. Yasunobu, T. Kimura, The amino acid sequence of bovine adrenodoxin, J. Biol. Chem. 248 (1973) 1141–1157.
[131] A. Hiwatashi, N. Sakihama, M. Shin, Y. Ichikawa, Heterogeneity of adrenocortical ferredoxin, FEBS Lett. 209 (1986) 311–315.
[132] N. Sakihama, A. Hiwatashi, A. Miyatake, M. Shin, Y. Ichikawa, Isolation and purification of mature bovine adrenocortical ferredoxin with an elongated carboxyl end, Arch. Biochem. Biophys. 264 (1988) 23–29.
[133] M. Lisurek, R. Bernhardt, Modulation of aldosterone and cortisol synthesis on the molecular level, Mol. Cell. Endocrinol. 215 (2004) 149–159.
[134] M. Nomura, K. Morohashi, S. Kirita, Y. Nonaka, M. Okamoto, H. Nawata, T. Omura, Three forms of rat CYP11B genes: 11 beta-hydroxylase gene, aldosterone synthase gene, and a novel gene, J. Biochem. 113 (1993) 144–152.
[135] K. Mukai, M. Imai, H. Shimada, Y. Ishimura, Isolation and characterization of rat CYP11B genes involved in late steps of mineralo- and glucocorticoid syntheses, J. Biol. Chem. 268 (1993) 9130–9137.
[136] M. Lauber, J. Muller, Purification and characterization of two distinct forms of rat adrenal cytochrome P450(11) beta: functional and structural aspects, Arch. Biochem. Biophys. 274 (1989) 109–119.
[137] Y. Nonaka, M. Okamoto, Functional expression of the cDNAs encoding rat 11 beta-hydroxylase [cytochrome P450(11 beta)] and aldosterone synthase [cytochrome P450(11 beta, aldo)], Eur. J. Biochem. 202 (1991) 897–902.
[138] Y. Nonaka, T. Fujii, N. Kagawa, M.R. Waterman, H. Takemori, M. Okamoto, Structure/function relationship of CYP11B1 associated with Dahl's salt-resistant rats – expression of rat CYP11B1 and CYP11B2 in *Escherichia coli*, Eur. J. Biochem. 258 (1998) 869–878.
[139] M. Okamoto, Y. Nonaka, Molecular biology of rat steroid 11 beta-hydroxylase [P450(11 beta)] and aldosterone synthase [P450(11 beta, aldo)], J. Steroid Biochem. Mol. Biol. 41 (1992) 415–419.
[140] N. Matsukawa, Y. Nonaka, Z. Ying, J. Higaki, T. Ogihara, M. Okamoto, Molecular cloning and expression of cDNAS encoding rat aldosterone synthase: variants of cytochrome P-450(11 beta), Biochem. Biophys. Res. Commun. 169 (1990) 245–252.
[141] A. Fisher, E.C. Friel, R. Bernhardt, C. Gomez-Sanchez, J.M. Connell, R. Fraser, E. Davies, Effects of 18-hydroxylated steroids on corticosteroid production by human aldosterone synthase and 11beta-hydroxylase, J. Clin. Endocrinol. Metab. 86 (2001) 4326–4329.
[142] C.E. Gomez-Sanchez, M.F. Foecking, C.H. Shackleton, M.R. Chavarri, E.P. Gomez-Sanchez, 18-Hydroxy-11-deoxycortisol: a new steroid isolated from incubations of the adrenal with 11-deoxycortisol, J. Steroid Biochem. 26 (1987) 105–111.
[143] M.Y. Zhou, E.P. Gomez-Sanchez, M.F. Foecking, C.E. Gomez-Sanchez, Cloning and expression of the rat adrenal cytochrome P-450 11B3 (CYP11B3) enzyme cDNA: preferential 18-hydroxylation over 11 beta-hydroxylation of DOC, Mol. Cell. Endocrinol. 114 (1995) 137–145.
[144] E.N. Cozza, M.R. Chavarri, M.F. Foecking, C.E. Gomez-Sanchez, Synthesis of 18-hydroxycortisol and 18-oxocortisol in bovine adrenal zona glomerulosa mitochondria, Proc. Soc. Exp. Biol. Med. 203 (1993) 317–322.
[145] M. Ohta, S. Fujii, R. Miura, Y. Nonaka, M. Okamoto, Bovine adrenal cytochrome P-450(11 beta)-mediated conversion of 11-deoxycortisol to 18- and 19-hydroxy derivatives; structural analysis by 1H-NMR, J. Steroid Biochem. Mol. Biol. 39 (1991) 911–920.

[146] K. Momoi, M. Okamoto, S. Fujii, C.Y. Kim, Y. Miyake, T. Yamano, 19-Hydroxylation of 18-hydroxy-11-deoxycorticosterone catalyzed by cytochrome P-45011 beta of bovine adrenocortex, J. Biol. Chem. 258 (1983) 8855–8860.
[147] M. Okamoto, K. Momoi, S. Fujii, T. Yamano, 18,19-dihydroxydeoxycorticosterone; a novel product of cytochrome P-45011 beta-catalyzed reaction, Biochem. Biophys. Res. Commun. 109 (1982) 236–241.
[148] K. Suhara, K. Takeda, M. Katagiri, P-45011 beta-dependent conversion of cortisol to cortisone, and 19-hydroxyandrostenedione to 19-oxoandrostenedione, Biochem. Biophys. Res. Commun. 136 (1986) 369–375.
[149] K. Suhara, K. Ohashi, K. Takahashi, M. Katagiri, Aromatase and nonaromatizing 10-demethylase activity of adrenal cortex mitochondrial P-450(11) beta, Arch. Biochem. Biophys. 267 (1988) 31–37.
[150] K. Suhara, K. Ohashi, K. Takeda, M. Katagiri, P-450(11beta)-dependent conversion of androgen to estrogen, the aromatase reaction, Biochem. Biophys. Res. Commun. 140 (1986) 530–535.
[151] B. Sato, Y. Miyashita, Y. Nishizawa, K. Noma, S. Kishimoto, H. Mori, K. Matsumoto, Biological and biochemical characterization of estrogen-dependent mouse Leydig cell tumors, J. Steroid Biochem. 27 (1987) 421–429.
[152] K. Suhara, M. Yamamoto, M. Katagiri, Hydroxylation of 19-norandrostenedione by adrenal cortex mitochondrial P-450(11) beta, J. Steroid Biochem. 36 (1990) 361–367.
[153] K. Denner, J. Doehmer, R. Bernhardt, Cloning of CYP11B1 and CYP11B2 from normal human adrenal and their functional expression in COS-7 and V79 Chinese hamster cells, Endocr. Res. 21 (1995) 443–448.
[154] P. Mulatero, K.M. Curnow, B. Aupetit-Faisant, M. Foekling, C. Gomez-Sanchez, F. Veglio, X. Jeunemaitre, P. Corvol, L. Pascoe, Recombinant CYP11B genes encode enzymes that can catalyze conversion of 11-deoxycortisol to cortisol, 18-hydroxycortisol, and 18-oxocortisol, J. Clin. Endocrinol. Metab. 83 (1998) 3996–4001.
[155] T. Kawamoto, Y. Mitsuuchi, K. Toda, K. Miyahara, Y. Yokoyama, K. Nakao, K. Hosoda, Y. Yamamoto, H. Imura, Y. Shizuta, Cloning of cDNA and genomic DNA for human cytochrome P-45011 beta, FEBS Lett. 269 (1990) 345–349.
[156] T. Kawamoto, Y. Mitsuuchi, T. Ohnishi, Y. Ichikawa, Y. Yokoyama, H. Sumimoto, K. Toda, K. Miyahara, I. Kuribayashi, K. Nakao, et al., Cloning and expression of a cDNA for human cytochrome P-450aldo as related to primary aldosteronism, Biochem. Biophys. Res. Commun. 173 (1990) 309–316.
[157] T. Imai, T. Yamazaki, S. Kominami, Kinetic studies on bovine cytochrome p45011 beta catalyzing successive reactions from deoxycorticosterone to aldosterone, Biochemistry 37 (1998) 8097–8104.
[158] E.M. Freel, L.A. Shakerdi, E.C. Friel, A.M. Wallace, E. Davies, R. Fraser, J.M. Connell, Studies on the origin of circulating 18-hydroxycortisol and 18-oxocortisol in normal human subjects, J. Clin. Endocrinol. Metab. 89 (2004) 4628–4633.
[159] L. Schloms, K.H. Storbeck, P. Swart, W.C. Gelderblom, A.C. Swart, The influence of *Aspalathus linearis* (*Rooibos*) and dihydrochalcones on adrenal steroidogenesis: quantification of steroid intermediates and end products in H295R cells, J. Steroid Biochem. Mol. Biol. 128 (2012) 128–138.
[160] M.K. Parr, A. Zollner, G. Fussholler, G. Opfermann, N. Schlorer, M. Zorio, M. Bureik, W. Schanzer, Unexpected contribution of cytochrome P450 enzymes CYP11B2 and CYP21, as well as CYP3A4 in xenobiotic androgen elimination – insights from metandienone metabolism, Toxicol. Lett. 213 (2012) 381–391.
[161] A. Zollner, M.K. Parr, C.A. Dragan, S. Dras, N. Schlorer, F.T. Peters, H.H. Maurer, W. Schanzer, M. Bureik, CYP21-catalyzed production of the long-term urinary metandienone metabolite 17beta-hydroxymethyl-17 alpha-methyl-18-norandrosta-1,4,13-trien-3-one: a contribution to the fight against doping, Biol. Chem. 391 (2010) 119–127.
[162] B.O. Lund, J. Lund, Novel involvement of a mitochondrial steroid hydroxylase (P450c11) in xenobiotic metabolism, J. Biol. Chem. 270 (1995) 20895–20897.
[163] E. Mornet, J. Dupont, A. Vitek, P.C. White, Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-450(11) beta), J. Biol. Chem. 264 (1989) 20961–22067.
[164] M. Cargill, D. Altshuler, J. Ireland, P. Sklar, K. Ardlie, N. Patil, N. Shaw, C.R. Lane, E.P. Lim, N. Kalyanaraman, J. Nemesh, L. Ziaugra, L. Friedland, A. Rolfe, J. Warrington, R. Lipshutz, G.Q. Daley, E.S. Lander, Characterization of single-nucleotide polymorphisms in coding regions of human genes, Nat. Genet. 22 (1999) 231–238.
[165] M.K. Halushka, J.B. Fan, K. Bentley, L. Hsie, N. Shen, A. Weder, R. Cooper, R. Lipshutz, A. Chakravarti, Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis, Nat. Genet. 22 (1999) 239–247.
[166] N.V. Belkina, M. Lisurek, A.S. Ivanov, R. Bernhardt, Modelling of three-dimensional structures of cytochromes P450 11B1 and 11B2, J. Inorg. Biochem. 87 (2001) 197–207.
[167] H. Zhu, G.A. Sagnella, Y. Dong, M.A. Miller, A. Onipinla, N.D. Markandu, G.A. MacGregor, Contrasting associations between aldosterone synthase gene polymorphisms and essential hypertension in blacks and in whites, J. Hypertens. 21 (2003) 87–95.
[168] J.M. Connell, R. Fraser, S.M. MacKenzie, E.C. Friel, M.C. Ingram, C.D. Holloway, E. Davies, The impact of polymorphisms in the gene encoding aldosterone synthase (CYP11B2) on steroid synthesis and blood pressure regulation, Mol. Cell. Endocrinol. 217 (2004) 243–247.
[169] I. Komiya, T. Yamada, M. Takara, T. Asawa, M. Shimabukuro, T. Nishimori, N. Takasu, Lys(173) Arg and -344T/C variants of CYP11B2 in Japanese patients with low-renin hypertension, Hypertension 35 (2000) 699–703.
[170] H. Tanahashi, T. Mune, Y. Takahashi, M. Isaji, T. Suwa, H. Morita, N. Yamakita, K. Yasuda, T. Deguchi, P.C. White, J. Takeda, Association of Lys173Arg polymorphism with CYP11B2 expression in normal adrenal glands and aldosterone-producing adenomas, J. Clin. Endocrinol. Metab. 90 (2005) 6226–6231.
[171] P.C. White, L. Slutsker, Haplotype analysis of CYP11B2, Endocr. Res. 21 (1995) 437–442.
[172] S. Portrat-Doyen, J. Tourniaire, O. Richard, P. Mulatero, B. Aupetit-Faisant, K.M. Curnow, L. Pascoe, Y. Morel, Isolated aldosterone synthase deficiency caused by simultaneous E198D and V386A mutations in the CYP11B2 gene, J. Clin. Endocrinol. Metab. 83 (1998) 4156–4161.
[173] C.D. Holloway, S.M. MacKenzie, R. Fraser, S. Miller, M. Barr, D. Wilkinson, G.H. Forbes, E. Friel, J.M. Connell, E. Davies, Effects of genetic variation in the aldosterone synthase (CYP11B2) gene on enzyme function, Clin. Endocrinol. (Oxf.) 70 (2009) 363–371.
[174] B. Chen, S. Nie, Z. Yue, W. Shou, C. Xiao, Haplotype-based case–control study of the human CYP11B2 gene and essential hypertension in Yi and Hani minorities of China, Biochem. Genet. 49 (2011) 122–137.
[175] D.P. Merke, T. Tajima, A. Chhabra, K. Barnes, E. Mancilla, J. Baron, G.B. Cutler Jr., Novel CYP11B1 mutations in congenital adrenal hyperplasia due to steroid 11 beta-hydroxylase deficiency, J. Clini. Endocrinol. Metab. 83 (1998) 270–273.
[176] M.E. Montasser, L.C. Shimmin, D. Gu, J. Chen, C. Gu, T.N. Kelly, C.E. Jaquish, T.K. Rice, D.C. Rao, J. Cao, D.P. Liu, P.K. Whelton, L.L. Hamm, J. He, J.E. Hixson, Variation in genes that regulate blood pressure are associated with glomerular filtration rate in Chinese, PLoS One 9 (2014) 1–7.
[177] M.K. Tin, T. Hakki, R. Bernhardt, Fission yeast *Schizosaccharomyces pombe* as a new system for the investigation of corticosterone methyloxidase deficiency-causing mutations, J. Steroid Biochem. Mol. Biol. 124 (2011) 31–37.
[178] I. Kuribayashi, H. Kuge, R.J. Santa, A.Z. Mutlaq, N. Yamasaki, T. Furuno, A. Takahashi, S. Chida, T. Nakamura, F. Endo, Y. Doi, S. Onishi, Y. Shizuta, A missense mutation (GGC[435Gly]–>AGC[Ser]) in exon 8 of the CYP11B2 gene inherited in Japanese patients with congenital hypoaldosteronism, Horm. Res. 60 (2003) 255–260.
[179] L. Pascoe, K.M. Curnow, L. Slutsker, A. Rosler, P.C. White, Mutations in the human CYP11B2 (aldosterone synthase) gene causing corticosterone methyloxidase II deficiency, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 4996–5000.
[180] C.E. Fardella, D.W. Hum, H. Rodriguez, G. Zhang, F.L. Barry, A. Ilicki, C.A. Bloch, W.L. Miller, Gene conversion in the CYP11B2 gene encoding P450c11AS is associated with, but does not cause, the syndrome of corticosterone methyloxidase II deficiency, J. Clin. Endocrinol. Metab. 81 (1996) 321–326.
[181] M. Barr, S.M. MacKenzie, D.M. Wilkinson, C.D. Holloway, E.C. Friel, S. Miller, T. MacDonald, R. Fraser, J.M. Connell, E. Davies, Functional effects of genetic variants in the 11beta-hydroxylase (CYP11B1) gene, Clin. Endocrinol. (Oxf.) 65 (2006) 816–825.
[182] S. Portrat, P. Mulatero, K.M. Curnow, J.L. Chaussain, Y. Morel, L. Pascoe, Deletion hybrid genes, due to unequal crossing over between CYP11B1 (11beta-hydroxylase) and CYP11B2(aldosterone synthase) cause steroid 11beta-hydroxylase deficiency and congenital adrenal hyperplasia, J. Clin. Endocrinol. Metab. 86 (2001) 3197–3201.
[183] J.V. Goldstone, A.G. McArthur, A. Kubota, J. Zanette, T. Parente, M.E. Jonsson, D. R. Nelson, J.J. Stegeman, Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish, BMC Genomics 11 (643) (2010) 1–21.
[184] D. Nesan, M.M. Vijayan, Role of glucocorticoid in developmental programming: evidence from zebrafish, Gen. Comp. Endocrinol. 181 (2013) 35–44.
[185] J. Tokarz, G. Moller, M.H. de Angelis, J. Adamski, Zebrafish and steroids: what do we know and what do we need to know? J. Steroid Biochem. Mol. Biol. 137 (2013) 165–173.
[186] B. Bottner, H. Schrauber, R. Bernhardt, Engineering a mineralocorticoid- to a glucocorticoid-synthesizing cytochrome P450, J. Biol. Chem. 271 (1996) 8028–8033.
[187] K.M. Curnow, P. Mulatero, N. Emeric-Blanchouin, B. Aupetit-Faisant, P. Corvol, L. Pascoe, The amino acid substitutions Ser288Gly and Val320Ala convert the cortisol producing enzyme, CYP11B1, into an aldosterone producing enzyme, Nat. Struct. Biol. 4 (1997) 32–35.

3. Discussion & conclusions

3.1 Insights into the pharmacokinetic potential of steroidogenic P450s

3.1.1 Selected AAS are metabolized by particular steroidogenic P450s.

In this work, the potential of steroidogenic P450s to convert exogenous compounds was initially studied by taking the example of anabolic androgenic steroids (AAS), which is presented in publication 2.3. Because of their performance enhancing effects via the activation of the androgen receptor (AR), anabolic agents, and in particular AAS, are widely misused for doping purposes in all sports by professional and recreational athletes. In doping controls, they represent the most frequently detected class of substances prohibited by the World Anti-Doping Agency (WADA) (WADA, 2015). AAS are synthetic derivatives of the natural androgen testosterone, which are chemically modified to favor anabolic effects, reduce androgenic effects and alter distribution and metabolism (Fragkaki et al., 2009). After intake and resorption, AAS as well as other drugs or xenobiotics undergo extensive phase I- and phase II-metabolism in the liver, which includes biotransformations by hepatic, microsomal P450s of the CYP1, 2 and 3 families. Therefore, structural modifications were developed to reduce the first-pass metabolism of AAS in the liver and provide oral availability and prolongation of the anabolic effect (Fragkaki et al., 2009). Thereby, 17α-alkylation is an efficient modification to enhance the stability towards hepatic metabolism. All three compounds tested in this work (oral-turinabol (OT), oxandrolone and stanozolol) carry a 17α-methyl group as can be seen from the structural formula presented in **Figure 3.1**. Additionally, the A-ring is modified by the introduction of a double bond and a substituent (oral-turinabol, OT), heteroatom replacement (oxandrolone), junction with a pyrazole ring (stanozolol) and reduction of the Δ^4 double bond to 5α configuration (oxandrolone, stanozolol), which enhances AR binding and hence anabolic activity. The reduced first-pass metabolism in the liver, conclusively, leads to high levels of the parent drug in the blood and raises the question for alternative metabolic paths at extrahepatic sites. In this context, P450s of the CYP11, 17, 19 and 21 families, which contribute to the biosynthesis of steroid hormones in the adrenal cortex and reproductive tissues, are interesting target enzymes for further investigations due to the structural similarity of their natural substrates to AAS. All six steroidogenic P450s were hence assayed in this work for the conversion of the three selected AAS.

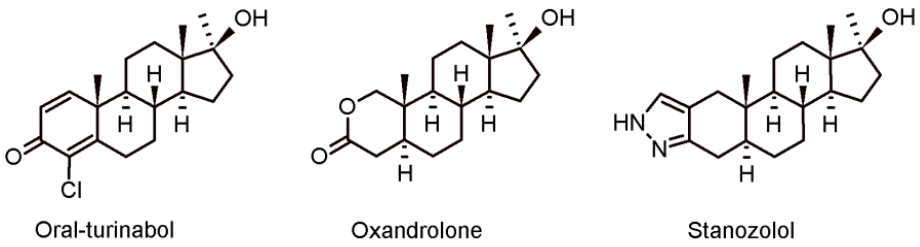


Figure 3.1: Structural formula of AAS tested for metabolism by human steroidogenic P450s.

OT was demonstrated to be converted by all three human mitochondrial steroidogenic P450s (CYP11A1, CYP11B1 and CYP11B2), whereby 2-OH-OT (suggested to be 2β-OH-OT), 11β-OH-OT, 11β,18-diOH-OT and 11β-OH-OT-18-al emerged as new OT derivatives that have to date not been described in the literature and, thus, not been tested for bioactivity and analytical application. No metabolism of OT by CYP17A1, CYP19A1 and CYP21A2 could be observed. Oxandrolone and stanozolol were not metabolized by any of the P450s. This leads to the conclusion that steroidogenic P450s are capable of converting xenobiotic steroids, but catalysis requires the conservation of general and P450-specific core motifs to enable the binding of the steroid in the active site and to present a target atom for oxygen incorporation in close proximity over the heme. These are a 3-keto-Δ^4 or 3-OH-Δ^5 motif, which are present in all natural steroid intermediates and permit hydrogen bonding to specific residues in the active site (Ghosh et al., 2009; Strushkevich et al., 2011; Strushkevich et al., 2013; Petrunak et al., 2014; Pallan et al., 2015). For oxandrolone and stanozolol the absence of such a motif seems to prevent catalysis. The crystal structures of human CYP17A1 and CYP21A1 revealed a perpendicular binding of the natural substrates relative to the heme, which renders the C_{17} and C_{20} or C_{21} atom available over the heme iron for oxygen incorporation (Petrunak et al., 2014; Pallan et al., 2015). Due to its 3-keto-Δ^4 motif, OT is likely to bind in a mode comparable to the natural substrates, but it lacks the C_{20}-C_{21} side chain in β position and is, thus, placed too far from the heme to interact with the activated oxygen. This is consistent with the results published by Parr *et al*., who did not observe a conversion of metandienone, another 17β-OH-17α-methyl AAS, by CYP17A1 and CYP21A2 (Parr et al., 2012). In case of CYP19A1, the reaction mechanism is proposed to be initiated by the abstraction of the hydrogen in position 2β and 2,3-enolization of the substrate (Ghosh et al., 2009). The absence of a hydrogen in position 2β and the presence of Cl at position 4 in the same plane as the 3-keto group might hinder this initiation. Taken together, the insights from crystal structures explain that CYP11B1 and CYP11B2 have the greatest potential for the conversion of AAS and similar exogenous steroids. The crystal structure of CYP11B2 in complex with DOC revealed a binding of the substrate parallel to the heme (Strushkevich et al., 2013). A comparable orientation of the substrate in the active site of CYP11B1 can be assumed due to the high sequence identity to CYP11B2 and their consistent

selectivity. The parallel binding mode makes the substrate likely to be placed close enough over the heme for catalysis independent from its size.

3.1.2 OT products derived from metabolism by the CYP11 family constitute interesting compounds for preventive doping research.

As introduced above, anabolic agents are prohibited, but popular performance enhancing drugs and constitute, with 48 % in 2014, the most adverse analytical findings (WADA, 2015) reported by WADA accredited laboratories. The evidence of a doping delict is commonly provided by the detection of urinary metabolites by chromatographic/mass spectrometric techniques (Geyer et al., 2014; Schänzer and Thevis, 2015). However, the prolongation of the detection window, which is required for a better retrospective conviction, is a great challenge. It can be approached by an improvement of the analytical screening procedure and by the discovery of new long-term metabolites (LTMs), whose incorporation into routine screening regularly leads to great increases in the number of adverse analytical findings (Geyer et al., 2014). The search for new metabolites is commonly conducted by chemical synthesis of reference compounds and comparison with the metabolic profile of biological matrices after administration by mass spectrometric methods. An alternative, which was chosen for this work, is the incubation of the parent compound with a system containing the human enzymes suggested to contribute to metabolism. These can be human tissue homogenates, whose availability is, however, limited, recombinant expression systems like insect cell cultures (Rendic et al., 1999), microorganisms (Zöllner et al., 2010b) or purified enzymes. The utilization of recombinant systems additionally enables the identification of the biocatalyst responsible for the transformation, which gives basic insights into pharmacokinetics and enzymatic properties. Studies with recombinant insect cells and purified enzymes could identify CYP3A4 as 6β-hydroxylase of OT (Rendic et al., 1999). A variety of AAS modifications occurring during the phase-I metabolism have been elucidated since the 1960s. Hydroxylation in positions 6β, 16α, 16β and 12 are described (Schänzer and Donike, 1993; Schänzer et al., 1995; Schänzer, 1996; Schänzer et al., 1996), whereby the 6β-hydroxylation seems to be the major metabolic pathway for OT (Schänzer et al., 1995). CYP11A1, which was identified in this work as additional putative 6- and 16-hydroxylase for OT, could contribute to the production of these metabolites, as it is expressed in all steroidogenic tissues, but also in the gastrointestinal tract (Guo et al., 2003), where it comes into contact with orally administered compounds. Subsequent to 16-hydroxylation, an oxidation to the 16-ketone can take place. Further modifications are a 3α-/3β-hydroxy-reduction of the 3-ketone, whereby the 3α-isomer emerges for all 17β-hydroxy-17α-methyl AAS, a 5α-/5β-reduction with an isomer ratio depending on the individual structure of the AAS, hydrogenation of double bonds, interconversion of a 17β-hydroxy group to the 17-ketone and epimerization of 17β-hydroxy-17α-methyl AAS (Schänzer, 1996). Only in 2006, 17β-hydroxymethyl-17α-methyl-18-norandrost-13-ene derivatives

were discovered as a new group of LTM of several 17-methylated AAS including OT, which can enlarge the detection window up to ~20 days and more if used as target metabolite (Schänzer et al., 2006; Parr et al., 2010; Sobolevsky and Rodchenkov, 2012). The phase-I metabolism of OT is schematically summarized in **Figure 3.2** in comparison with the products characterized in this work.

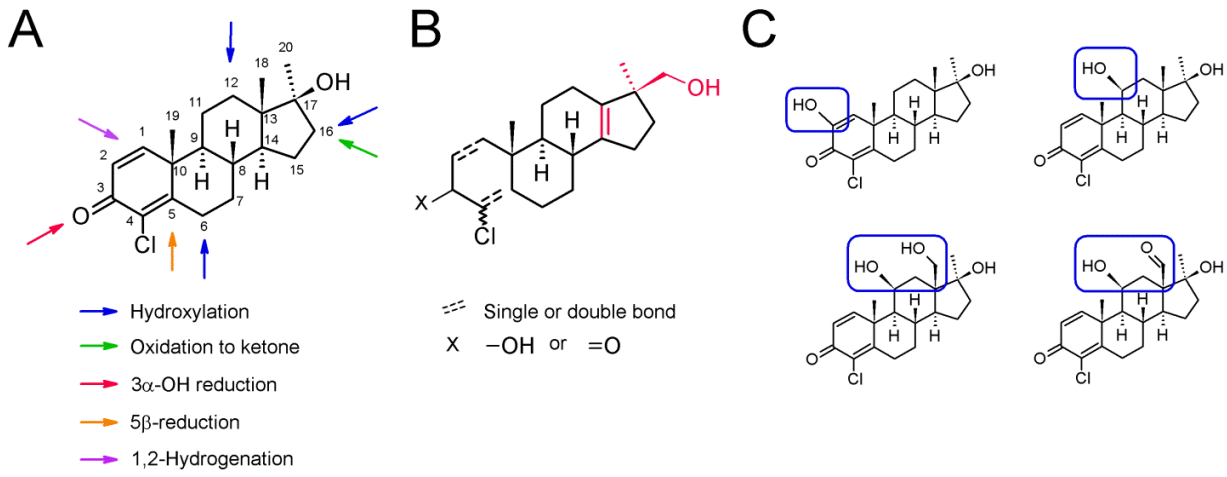


Figure 3.2: Summary of phase-I metabolism of OT described in the literature in comparison with the new derivatives from this work. (**A**) Indication of modifications identified as reviewed by (Schänzer, 1996). (**B**) General structure of novel LTMs discovered between 2006 and 2012 (Schänzer et al., 2006; Parr et al., 2010; Sobolevsky and Rodchenkov, 2012), which share a 17β-hydroxymethyl-17α-methyl-18-norandrost-13-ene structure (highlighted in red) combined with different A-ring modifications indicated in (**A**). (**C**) OT derivatives identified in this work with new modifications highlighted by blue frames.

The new OT derivatives differ from all metabolites described so far and, thus, constituted interesting candidates for an evaluation as putative target metabolites for doping controls. Therefore, urine samples of a volunteer collected 6, 24 and 96 h after oral administration of a single dose of 5 mg OT were surveyed for the presence of the new OT derivatives by LC- and GC-tandem mass spectrometry as described in 5.1.1. Experiments were carried out by the Center for Preventive Doping Research at the German Sport University Cologne. Unfortunately, none of the compounds could be detected. As the timeframe of urine collection is considered as sufficient for a detection of all metabolites, which are excreted via the kidney, it is suggested that other modifications hampering the detection are introduced prior or subsequent to the putative *in-vivo* turnover by CYP11A1, CYP11B1 and CYP11B2. These modifications could include 5β-reduction, 3α-hydroxy-reduction and additional hydroxylations, as

described above as common reactions of OT metabolism, but also an oxidation of the new 11β-hydroxyl to its ketone by an 11β-hydroxy steroid dehydrogenase (11β-HSD). Moreover, a Wagner-Meerwein rearrangement subsequent to the CYP11B2-dependent biotransformation could lead to the formation of 11β-hydroxy-17,17-dimethyl-18-nor-13-ene and 11β-hydroxy-17β-hydroxymethyl-17α-methyl-18-nor-13-ene products (Parr et al., 2012). Comparably, endogenous steroid hormones are also excessively metabolized by 11β-HSD, 3α-HSD, 5α-/5β-reductase and 20α-/20β-HSD prior to excretion and are excreted only to a minor amount in their original biologically active form (Wudy et al., 2007). Selected potential follow-up reactions are shown in **Figure 3.3** by taking 11β,18-diOH-OT as an example. The presented P450-dependent whole-cell system with *E. coli*, which enabled the transformation of OT by CYP11B2 in a preparative scale, can serve as source for the lead structures for further chemical or enzymatic modification to synthesize these alternative metabolites. Besides, combinatorial biotransformations of steroids in a single set up by all the enzymes mentioned represents a sophisticated approach to produce a set of derivatives, which are produced by the interplay of all enzymes contributing to human steroid biosynthesis and metabolism. Therefore, all enzymes of interest need to be present in the same conversion system, which can be realized by their co-synthesis in a single host cell. A recombinant strain of *Saccharomyces cerevisiae* expressing four steroidogenic P450s (CYP11A1, CYP11B1, CYP17A1 and CYP21A2), their redox partners AdR and Adx as well as a 3β-HSD was established for the synthesis of cortisol from yeast membrane sterols (Szczebara et al., 2003). This example impressively demonstrates the functionality of multi enzyme cascades in microbial whole-cell catalysis of steroids and an analog system expressing the aforementioned enzymes could deliver interesting new AAS metabolites.

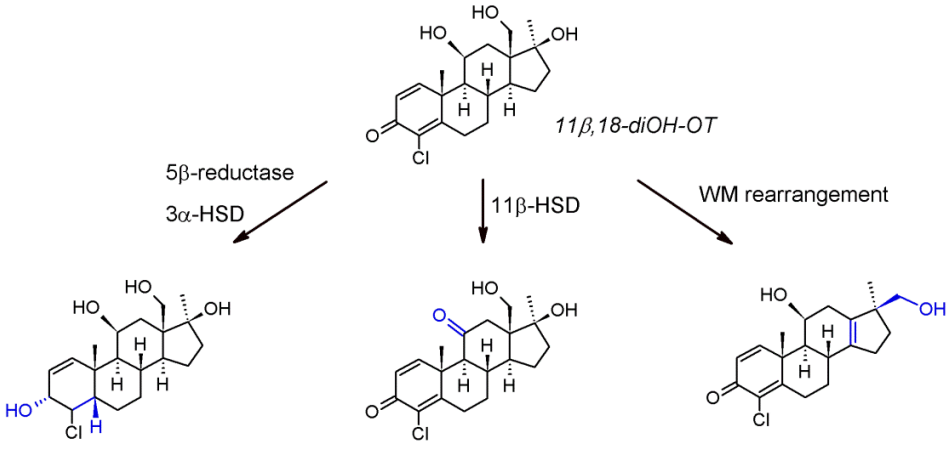


Figure 3.3: Putative enzymatic and non-enzymatic follow-up transformations of 11β,18-diOH-OT in humans. Modifications are highlighted and could be introduced by 5β-reductase and 3α-HSD (**left**), which are known to contribute to OT metabolism (Schänzer, 1996), 11β-HSD (**middle**), which

interconverts natural 11β-OH- and 11-keto- corticosteroids (Wudy et al., 2007) or by Wagner-Meerwein (WM) rearrangement to a 17β-hydroxymethyl-17α-methyl-18-norandrost-13-ene product (**right**).

However, one might also argue that the total of product formation by the CYP11 family and consecutively the excreted amounts are below the detection limit. CYP11A1 is expressed in classical steroidogenic and a few other tissues like the skin and gastrointestinal tract, brain and immune system. CYP11B1 and CYP11B2 expression is even more restricted and limited to the respective zone of the adrenal cortex and the brain, where its function is still unclear (Erdmann et al., 1996). Their putative expression in the heart is controversial (Yoshimura et al., 2002; MacKenzie et al., 2012).

In conclusion, the newly identified OT derivatives cannot directly serve as target analytes for doping conviction but are interesting intermediates for the synthesis of putative follow-up metabolites. A proof of their formation in humans remains to be established. Nevertheless, the detection of 11β-OH-metandienone and 18-OH-metandienone, which were synthesized by purified CYP11B2 and by recombinant *Schizosaccharomyces pombe*, were detected in the urine after a single administration of 25 mg metandienone (Parr et al., 2012). 18-OH metabolites were also found after administration of mesterolone, methenolone, and stenbolone (Masse and Goudreault, 1992). This makes a contribution of steroidogenic P450s to human *in-vivo* pharmacokinetics conceivable. Still, it has to be considered carefully that the formation of 11β-OH-testosterone by human liver microsomes (Choi et al., 2005) and the formation of 18-hydroxylated metabolites of C_{19} steroids by rat liver microsomes (Gustafsson and Lisboa, 1970) has been demonstrated. This hints at additional extra-adrenal sites of steroid 11β- and 18-hydroxylase activity.

3.1.3 Metabolism of pharmaceuticals by the CYP11B subfamily is limited to synthetic steroid derivatives.

Besides AAS, the mineralocorticoid receptor antagonists spironolactone and canrenone were identified as new xenobiotic substrates for CYP11B1 and CYP11B2 during the investigations outlined in manuscript 2.4. The emerging products were to date unknown, but could be structurally identified in this work. 11β-OH-spironolactone, 18-OH-spironolactone and 19-OH-spironolactone are produced from spironolactone by both CYP11B isoforms. Canrenone is transformed to 11β-OH-canrenone and 18-OH-canrenone by both isoforms and in addition to 11β,18-diOH-canrenone by only CYP11B2. Additionally, another AAS, methyltestosterone (17β-OH-17α-methyl-testosterone; MT), and the synthetic gestagen medroxyprogesterone (6α-methyl-progesterone, MP) could successfully be converted with reconstituted *in-vitro* systems of CYP11B1 and CYP11B2. HPLC chromatograms (**Figures S1 and S2** in 5.2 Supplemental data) revealed the formation of one major product for both substrates by CYP11B1 and

CYP11B2 and only minor side product formation, which is slightly higher for CYP11B2 as concluded from the peak area portion. The major products were identified as 11β-OH-MT by NMR (**Table S1** in 5.1.2) and 11β-OH-MP (also called medran) by comparison with the retention time of a commercially available standard in the HPLC measurements (**Figure S1** in 5.2). **Scheme S1** illustrates the respective biotransformations with structural formulas. OT remains the only xenobiotic steroid studied here, which was shown to undergo the complete reaction repertoire of CYP11B2 including an oxidation to the 18-aldyhyde. This strengthens the hypothesis that the 3-keto-4-chloro-Δ^4 motif of OT allows an extended retention in the active site and processive oxidations. MT and MP also induced a *high spin* shift of the heme iron and K_d values were determined by difference spectroscopy (**Figure S3** in 5.2). All values determined in this work are summarized in **Table 3.1**.

Table 3.1: Summary of dissociation constants (K_d) determined in this work by difference spectroscopy. Values were determined as described in 2.3, 2.4 and **Figure S4** by plotting the absorbance changes of the difference spectra against the ligand concentration and subsequent regression. For CYP11B2, a type-II-difference spectrum due to a *low spin* shift of the heme iron was observed upon the addition of stanozolol, while all other compounds induced a *high spin* shift as shown by the type-I-difference spectrum. No spin shift changes could be observed for CYP11B1 when titrated with stanozolol indicated by (-). n.d.: not determined. For the comparison of the 2 CYP11B isoforms, the lower K_d indicating the higher affinity of the enzyme is highlighted in blue.

Steroid	**K_d CYP11B1 [µM]**	**K_d CYP11B2 [µM]**
DOC	8.1 ± 0.5	1.3 ± 0.1 •
RSS	6.6 ± 0.4	n.d.
B	n.d.	115 ± 6
OT	17.7 ± 2.2	5.4 ± 0.4
Medroxyprogesterone	17.7 ± 2.2	50.2 ± 5.1
Methyltestosterone	12.4 ± 0.6	18.0 ± 0.7
Spironolactone	9.3 ± 0.7	4.1 ± 0.4
Canrenone	74.5 ± 9	5.5 ± 0.4
Stanozolol	-	5.0 ± 0.3

• (Hobler et al., 2012)

Interestingly, MT and MP are the only exogenous steroids with a higher affinity for CYP11B1 than for CYP11B2. For MT, where only a slight difference is observed, this can be explained by the small

overall size of the molecule, which does not require a high degree of flexibility of the P450 to enable an efficient binding. In case of MP, affinity is almost three times higher for CYP11B1, which hints at an enhancing effect of the 6α-methyl group for the interaction of the steroidal α-face with the hydrophobic amino acids of the active site pocket (Strushkevich et al., 2013), which is more dominant in CYP11B1. As CYP11B2 turned out to be the generally more efficient catalyst for xenobiotic oxidation, a set of marketed non-steroidal drugs was tested for conversion with CYP11B2. Structures of these drugs are shown in **Figure S4** (see 5.2). However, none of these substances was converted. In conclusion, the current experimental status suggests that the potential of the two human CYP11B subfamily members as metabolizers of xenobiotics is limited to synthetic steroid derivatives. The core structure, which is shared by all substrates, and permitted deviations are schematically represented in **Figure 3.4**.

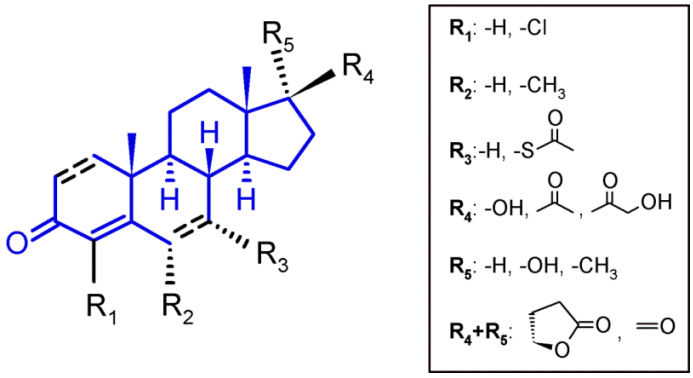


Figure 3.4: Schematic representation of the core structure of all CYP11B substrates and their variety. For the design of the scheme, the natural substrates RSS and DOC, all substrates discovered in this work, metandienone (Parr et al., 2012) as well as progesterone, testosterone and androstenedione (Strushkevich et al., 2013) were considered. 11β- and 18-hydroxylated reaction intermediates are not taken into account. The common steran scaffold shared by all substrates is highlighted in blue. Dashed bonds represent putative double bonds. Composition and stereochemistry of substituents are indicated.

3.1.4 The consideration of steroidogenic P450s as drug metabolizers can improve drug safety testing.

Synthetic derivatives of steroid hormones, which were identified in this study as substrates for selected steroidogenic P450s, are frequently applied pharmaceuticals for hormone replacement therapies and the treatment of various medicinal issues. Steroidal mineralocorticoid receptor antagonists are used as diuretics and for the treatment of hypertension and related diseases. AAS are abused for doping purposes as aforementioned, but a clinical application in burn injuries (Rojas et al., 2012) and several muscle-wasting diseases (Shahidi, 2001) is also indicated. Moreover, beneficial effects for the treatment

of postmenopausal osteoporosis (Andreopoulou and Bockman, 2015) and the rehabilitation of older people after severe surgeries (Farooqi et al., 2014) are discussed. Glucocorticoids are potent anti-inflammatory and immunosuppressive drugs. Clinically applied ones are for example the major human glucocorticoid cortisol itself and the synthetic products premedrol and medrol (methylprednisolone). Synthetic gestagens (also termed "progestines") are used as contraceptives, whereby they can be combined with estrogens (Dewick, 1999), and for the treatment of endometriosis. MP is a synthetic gestagen, which was never marketed, but is excessively formed during the metabolism of medroxyprogesterone-17α-acetate (Ishihara et al., 1976), which is included in the List of Essential Medicines of the World Health Organization (World Health Organization, 2015). This work shows the potential of CYP11B1 and CYP11B2 to catalyze the conversion of compounds from different classes of steroidal pharmaceuticals: Anabolics, aldosterone antagonists and gestagens. Due to the large variety of derivatives available for pharmaceutical applications, many more new CYP11B substrates can be expected to be found in the near future. Consequently, the "critical role" (Guengerich et al., 2005), which is demanded for the functional classification of human P450s into different substrate categories, is established. A critical role in xenobiotic metabolism can be attributed to an enzyme, if the rate of oxidation is high enough to be considered as significant in the *in-vivo* metabolism and the biotransformation has a biological effect and might be critical to health. A demonstration of the critical role is provided by this work on both levels: Firstly, relevant binding affinities and catalytic rates are reported for different synthetic substrates by characterization on enzymatic level. Secondly, altered pharmacodynamic properties of the emerging metabolites are shown for spironolactone and canrenone, whose CYP11B-derived products vary in their antagonistic properties for the MR compared to the parent compounds (see manuscript 2.4). In summary, the results strongly suggest the consideration of steroidogenic P450s as drug-metabolizers and consecutively the consideration of emerging metabolites in drug metabolite safety testing for the elucidation of putative adverse effects. Thereby, especially cross-reactivity with other steroid hormone receptors is relevant. Alterations of the effects on the MR by 11β- and 18-hydroxylation were already shown for spironolactone and canrenone (2.4). Moreover, it is of particular interest to analyze the CYP11B-derived products for cross-activation of the glucocorticoid receptor (GR), whose activation depends on the formation of a hydrogen bond with an 11β-OH group of the steroidal ligand in addition to H-bonding involving a 21-OH, a 20-keto and a 17α-OH function (Bledsoe et al., 2002; Kauppi et al., 2003). In case of both, MR and GR, the tissue-specific expression pattern overlaps with the expression patterns of CYP11B1 and CYP11B2, which are, besides in the adrenal, also expressed in the brain and putatively in the heart. MR expression is reported for the epithelial target tissues of kidney, parotid and colon, but also for the hippocampus and heart (Funder et al., 1988; Zennaro et al., 1997). GR is expressed in the brain, skeletal muscle, lung kidney, liver, heart, colon, nasal mucosa, macrophages, eosinophils, peripheral blood mononuclear cells and neutrophils

(Pujols et al., 2002). The co-localization of enzyme and receptor points towards an autocrine or paracrine influence of CYP11B metabolism and, therefore, possible tissue-specific adverse effects. For the androgen, estrogen and progesterone receptors an impairment of ligand binding by CYP11B-derived metabolites can be expected, which will reduce the efficiency of administered compounds targeting these receptors, as the ligand binding pocket is aligned by mainly hydrophobic residues (Brzozowski et al., 1997; Williams and Sigler, 1998; Sack et al., 2001). However, for the evaluation of possible effects sufficient amounts of the novel metabolites need to be made available.

3.2 Biotechnological relevance of the CYP11B1- and CYP21A2-dependent *E. coli* catalysts

3.2.1 The developed biocatalysts exhibit relevant productivities for industrial glucocorticoid production.

The application of mammalian steroidogenic P450s in biotechnology is of great interest for different purposes. Their newly established role as metabolizers of (steroidal) drugs leads to a demand of their metabolites for bioactivity/toxicity testing and as authentic analytical standards for periodical calibration as made compulsory by the instructions of *i.e.* Good Laboratory Practice and Good Manufacturing Practice. Furthermore, due to their outstanding selectivity, steroidogenic P450s are attractive biocatalysts for the production of steroidal pharmaceuticals and many efforts have been made to date to realize and optimize their application. CYP11A1 can be used for the production of pregnenolone, the precursor for further functionalizations, from cholesterol as well as plant and fungal sterols and was recently made accessible for whole-cell conversion in *Bacillus megaterium* (Gerber et al., 2015). Subsequently, especially the employment of CYP11B1, CYP17A1 and CYP21A2 is desirable, because they catalyze the hydroxylations that are essential for the activation of the glucocorticoid receptor (Bledsoe et al., 2002; Kauppi et al., 2003) and thereby the commercially relevant anti-inflammatory and immunosuppressive effects. For the application of CYP11B1 and CYP21A2, several yeast-based systems have been developed (Dumas et al., 1996; Dragan et al., 2005; Hakki et al., 2008; Zehentgruber et al., 2010) and even a combinatorial system including CYP11A1 and CYP17A1 for the synthesis of cortisol from membrane ergosterol was established (Szczebara et al., 2003). The bacterial systems presented in this work with *E. coli* significantly outcompete these eukaryotic systems with efficiencies of 0.84 g cortisol $*L^{-1}*d^{-1}$ and 0.65 g premedrol $*L^{-1}*d^{-1}$. These productivities fit into the operational window defined for P450 processes in the industry, but are still at the lower limit (Straathof et al., 2002; Julsing et al., 2008). Major reasons of the limitations are the membrane-associated nature of the applied P450s, which complicates their expression in prokaryotes, and the generally low activity of steroidogenic P450s, which has evolved along with their biosynthetic function. The highest reaction

velocity of a P450 reported to date is 363 s^{-1} for a mutant of the bacterial CYP102A1 (BM3) (Eiben et al., 2006). Human CYP11B1 and bovine CYP21A2, which were investigated in this work, exhibit respective maximal velocities of 1.6 s^{-1} for the formation of cortisol from RSS (this work, **Figure S5 in 5.2**) and in the range of 0.7 s^{-1} for the 21-hydroxylation of progesterone and 17-OH-progesterone (Pallan et al., 2015). Therefore, engineering of the biocatalysts is required to identify and overcome the bottlenecks of product formation. During the optimization of the CYP11B1- and CYP21A2-expressing whole-cell systems, presented in the scientific contributions 2.1 and 2.2, engineering on 2 levels of the P450 biocatalysts was successfully performed. First, engineering on the level of the P450 was conducted by molecular evolution and screening for enhanced product formation, wherefrom several CYP11B1 variants with enhanced activity emerged (see 2.1). Second, the electron transfer system was engineered by quantitative and qualitative approaches with the enhancement of *Adx* expression (see 2.1) and the replacement by heterologous redox partners (see 2.2). Gained insights are transferable to other P450s and, hence, of general relevance for the optimization of P450 biocatalysts. The transferability of both aspects will be discussed in the following sections.

3.2.2 Selected CYP11B1 mutations can set a basis for the rational design of other P450 catalysts.

Selected expression or activity improving mutations discovered in the CYP11B1 library and described in 2.1 can be used for the optimization of the other biotechnologically interesting steroidogenic P450s by SDM as concluded from the primary sequence alignments shown in **Figure 3.5 A**. A replacement, analogous to the N-terminal G to R replacement in CYP11B1 (G23R), which was demonstrated to significantly increase the expression level in *E.coli*, is promising to comparably enhance the expression of CYP17A1 and CYP21A2, whose wildtype sequences encode non-charged amino acids in the respective positions. Respective exchanges are S33R for CYP17A1 and G36R for CYP21A2. This idea is supported by the fact that modifications of the N-terminus including single amino acid exchanges, sequence replacement and truncations of hydrophobic parts appear as the key to the expression of eukaryotic P450s in bacteria (Gillam, 2008). The occurrence of the other mutations (except for L271M) in the center of helices of the conserved P450 folding (E, I and J, see **Figure 3.5 A**) makes them more likely to have general beneficial effects on P450 function than only CYP11B1-specific effects. Especially the transferability to CYP11A1, which has a common evolutionary ancestor with the CYP11B subfamily (Nelson et al., 2013), becomes evident from the sequence alignment. CYP11A1 encodes the same amino acids as CYP11B1 in the respective positions aligned to H171 and L271 of CYP11B1. The same exchanges (H→L and L→M) as observed to be beneficial for CYP11B1 activity are thus interesting candidates for CYP11A1 SDM. The threonine residue of CYP11A1 aligned to position 286 of CYP11B1 represents another interesting mutagenesis spot. **Figure 3.5 B** shows in detail the localization of this position in the CYP11B1 model in the I-helix. Its proximity to the conserved

acid/alcohol-pair (D294/T295), which is essential for proton transfer and progression of the catalytic cycle (**Figure 1.2 A**), suggests that the selected mutation contributes to these processes and thereby promotes substrate hydroxylation or prevents of ROS formation. Saturation mutagenesis of position 286 and the neighboring ones can help to further elucidate this aspect. As there is very limited information in the literature about mutational strategies to improve P450s activity, which are not primarily motivated by an improvement of the selectivity, the identification of crucial residues is of considerable value to develop strategies for rational enzyme engineering.

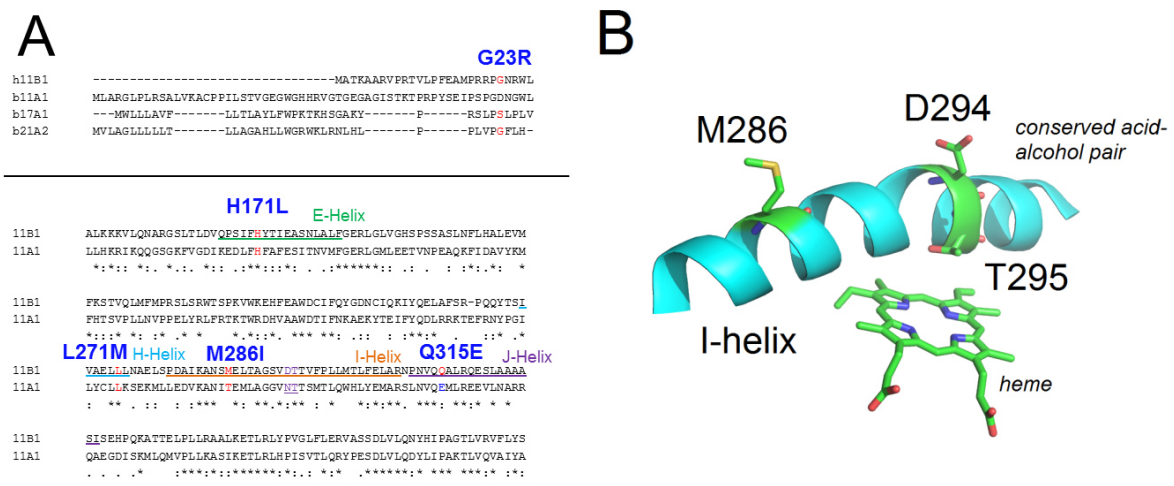


Figure 3.5: Localization of CYP11B1 activity enhancing mutations and alignment with other P450s. (**A**) – Alignment of selected sequence parts of CYP11B1 with other steroidogenic P450s. Sequences were extracted from uniprot.org (bovine CYP11A1: P00189; bovine CYP17A1: P05185; bovine CYP21A2: P00191). The CYP11B1 sequence is shown as modified for expression in *E. coli* (Zöllner et al., 2008). The alignment was done with the Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). Relevant helical structures are indicated as in the latest CYP11B1 homology model (Hobler, 2013). (**B**) Extract of the CYP11B1 homology model showing M286 and the conserved acid-alcohol pair in element color-coded stick representation in the I-helix (cartoon representation) above the heme.

3.2.3 A combination of Arh1 and adjusted Adx expression leads to a versatile redox system for P450 whole-cell application.

The efforts made in publication 2.1 to improve the activity of the CYP11B1 biocatalyst revealed *Adx* expression as a limiting factor, which could successfully be overcome by the introduction of additional cDNA copies of *Adx* into the polycistronic expression plasmid. The presented strategy to improve P450 activity by enhancing *Adx* expression in the whole-cell catalyst is as well transferable to other P450

systems, as Adx can also transfer electrons to bacterial and *class II* P450s (Ewen et al., 2012). Because of its soluble nature and small size of ~14 kDa it is easy to be synthesized in high levels by *E. coli* by simply enhancing its mRNA level. A dependency of P450 activity on Adx concentration is generally accepted, as the electron transfer from Adx to the P450 is known as a rate-limiting step (Cao and Bernhardt, 1999; Guengerich, 2002). Enhancing its expression is thus a very straight-forward strategy and can be advantageous over the artificial construction of self-sufficient P450-redox partner fusions, which are likely to facilitate the final electron transport step, but whose design and expression are challenging (Munro et al., 2007). The presented unique polycistronic approach still enables the expression of all constituents of the redox system from one plasmid and requires only a single antibiotic. It is, therefore, well suited for up-scaling of the reaction volume towards industrial standards in contrast to approaches, which regulate the expression ratio of different genes by the employment of different plasmids, which need additional costly antibiotics to be stably maintained. The replacements of the autologous redox system of CYP21A2 by heterologous ones for the optimization of the whole-cell activity impressively demonstrate the possibility to support one P450 by versatile combinations of redox proteins from different organisms and classes (see publication 2.2). Arh1 from *Schizosaccharomyces pombe* was identified as the most efficient reductase due to its soluble character and capability to accept NADH in addition to NADPH. The use of *Arh1* in a plasmid with several copies of *Adx* would be the next step of engineering the redox system to combine the qualitative and quantitative optimizations. This mixed system can efficiently supply a large variety of P450s in whole-cell systems. Its application for the screening of orphan P450s with unknown redox partners for novel reactions is, therefore, recommended. Current DNA sequencing technologies deliver genomic sequence information at a steadily increasing rate and their bioinformatic evaluation leads to the identification of new P450s. A screening of substrate libraries with the new P450s in combination with the redox system constituted of Arh1 and Adx at the adjusted expression level can reveal novel reactions with biotechnological relevance.

4. Outlook

This work demonstrates the capability of steroidogenic P450s and among them the CYP11B subfamily in particular to convert xenobiotic steroids from different classes with relevant catalytic rates. This raises the question, how far their substrate spectrum can be extended and motivates a systematic search for additional substrates. For the screening of substrate libraries, a whole-cell approach with the CYP11B1- or CYP11B2-expressing *E. coli* cells employed in this work is well-suited when it is down-scaled to a microtiter plate format as it enables a time- and cost-efficient analysis. For both CYP11B isoforms expression systems in 1-mL scale are available and were shown to mimic the full catalytic potential of the purified enzymes. Therefore, these systems are promising tools for the identification of new substrates. CYP11B1 and CYP11B2 have been ascertained to be highly polymorphic (Bernhardt and Waterman, 2007; Schiffer et al., 2015). Several polymorphisms have to date been cloned and demonstrated to exhibit altered activities *in-vitro* (reviewed by (Schiffer et al., 2015)). It is, therefore, of interest to study the metabolism of the new pharmaceutical substrates by P450s displaying different polymorphisms and consider the results in combination with the adverse effects that might emerge from the products, *i.e.* the reduction of the MR antagonist properties of spironolactone by CYP11B1- and CYP11B2-dependent conversion. Within the model of *personalized medicine*, these insights into the relation between polymorphisms and the emergence of adverse effects can help to improve drug usage and dosage for the benefit of the patient. However, to study putative adverse effects of metabolites formed by CYP11B1 and CYP11B2, sufficient amounts need to be supplied. The recombinant *E. coli* system, which was used in this study under non-optimized conditions, is a promising basis for the optimization of metabolite production and the major products were already successfully produced for purification in low mg amounts. Nevertheless, the production of minor metabolites in appropriate amounts can be problematic. Therefore, the selectivity of the CYP11B isoforms can be modulated by rational design. Combinatorial randomization of the active site residues interacting with the steroid α-face (W116, F130 and F487 in CYP11B2) with a reduced amino acid alphabet that maintains the aromatic nature (F, W, Y) could be used to alter substrate binding in a way, which favors 18- and 19-hydroxlation. To target C_{19}, SDM following a structural alignment with CYP19A1, which catalyzes successive hydroxylations at C_{19} position for its substrates, is an interesting approach. For an enhancement of 18-hydroxylase activity, a sequence comparison with the CYP11B3 isoform from rat, which has a preference for 18- over 11β-hydroxylase activity (Zhou et al., 1995), could give helpful information for possible SDM.

Regarding the engineering of CYP11B1 for the biotechnological production of glucocorticoids by 11β-hydroxylation, detailed *in-vitro* studies of the effect of the selected mutations on parameters of

CYP11B1 activity should be performed (*i.e.* the determination of kinetic parameters k_{cat} and K_M in dependency on substrate and Adx concentration, substrate binding, thermostability, solvent tolerance) to understand the underlying structure-function relationship and to establish a rational basis for a second mutant generation.

The discovery of medroxyprogesterone as new substrate for CYP11B1, which is selectively 11β-hydroxylated (**Figure S1 in 5.2, D**) in *E. coli*, opens up a new synthetic path for the direct production of premedrol from medroxyprogesterone with a single biocatalyst expressing CYP11B1 and CYP21A2. As both P450s accept Adx as redox partners, a single electron transfer chain constituted of Arh1 and Adx at a suitable level, which can be regulated as aforementioned, can supply both enzymes. This potential *E. coli* biocatalyst is illustrated in **Figure 3.6**. However, it has to be mentioned that the balanced co-expression of two P450s and their redox partners will be a challenging task. Alternatively, the desired cascade reaction could be realized by co-cultivation of two strains expressing either P450.

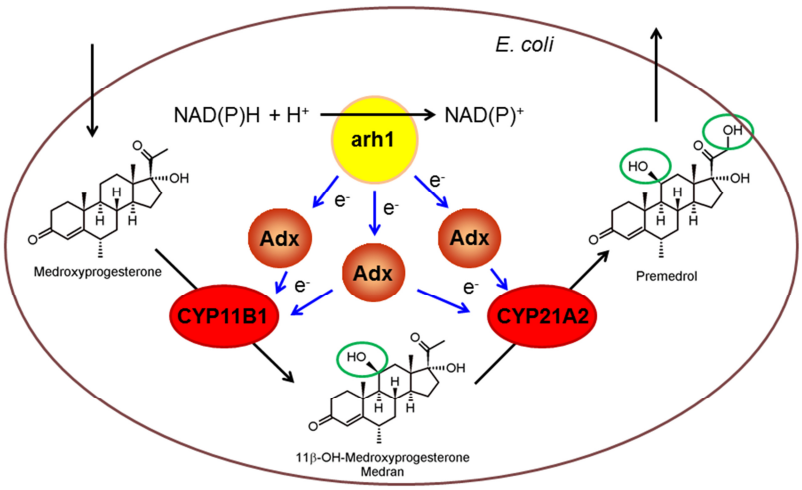


Figure 3.6: Schematic representation of an *E. coli*-based biocatalyst for the one-pot-synthesis of premedrol from medroxyprogesterone by CYP11B1 and CYP21A2. Electron transfer can be ensured by co-expression of *Arh1*, which uses NADH and NADPH as electron source, and Adx, whose expression level can be increased to its optimum by adjusting its cDNA copy number.

5. Appendix

5.1 Supplemental methods

5.1.1 OT administration study and screening for CYP11B-derived metabolites

The OT administration study and post-administration analysis of urine were performed by the Center for Preventive Doping Research, Prof. Dr. Mario Thevis, German Sport University, 50933 Cologne, Germany. A single dose of 5 mg OT was orally administered by a male subject without further medication, urine was periodically sampled and stored at -20 °C until analysis (Tretzel et al., 2015). Urine analysis and screening for 11β-OH-OT, 11β,18-diOH-OT and 11β-OH-OT-18-al were performed by liquid chromatography-tandem mass spectrometry, including triple quadrupole mass spectrometry and high-resolution/high-accuracy mass spectrometry, and gas chromatography-tandem mass spectrometry as described previously (Guddat et al., 2013).

5.1.2 NMR spectroscopy of 11β-OH-methyltestosterone

After whole-cell biotransformation of methyltestosterone with CYP11B2 as described in 2.3 and 2.4 the major product was purified by RP-HPLC and analyzed by NMR spectroscopy. NMR measurements were performed by Dr. Josef Zapp (Department of Pharmaceutical Biology) and the results were evaluated by Dipl.-Chem. Martin Litzenburger (Institute of Biochemistry). ^{1}H and ^{13}C NMR were recorded on a Bruker (Rheinstetten, Germany) 500 NMR spectrometer. Two-dimensional NMR spectra were recorded as gs-HHCOSY, gs-HSQC, and gs-HMBC. All chemical shifts are relative to $CDCl_3$ (δ = 77.00 for ^{13}C-NMR; δ = 7.24 for ^{1}H-NMR) using the standard δ notion in parts per million. The spectra showed signals for an additional secondary hydroxyl group (δ_H 4.41 m; δ_C 68.30), which could be located at C-11. Its proton showed correlations to H-9 (δ 0.94, dd) and H-12a (δ 1.53, dd) and H-12b (δ 1.65, dd). Thereby, H-11 must be in equatorial position due to its small coupling constants to H-9 (J= 3.5 Hz) and H-12 (J= 3.5) as well as coupling with both H-1 in the 2D-NMR measurements and is therefore in α-orientation. Consecutively, the hydroxyl at C-11 is β-orientated. The following **Table S1** summarizes all chemical shifts in parts per million and coupling constants assigned to the respective carbon atoms numbered according to standard steran scaffold numbering.

Carbon number	^{1}H NMR	coupling constants [Hz]	^{13}C NMR
1	1,80-1,87 m (1H) + 2,15-2,21 m (1H)		34,94
2	2,30-2,37 m (1H) + 2,42-2,50 m(1H)		33,83
3	-		199,53
4	5,65 d (1H)	1,7	122,32
5	-		172,16
6	2,19-2,24 m(1H) + 2,42-2,50 m(1H)		32,05
7	0,98-1,05 m (1H) + 1,95-2,02 m(1H)		32,39
8	1,97-2,04 m(1H)		32,48
9	0,94 dd (1H)	11,1; 3,5	56,32
10	-		39,28
11	4,41 m (1H)		68,30
12	1,53 dd (1H) + 1,65 dd (1H)	3,5; 13,7	41,07
13	-		44,52
14	1,12-1,18 m (1H)		51,70
15	1,37 dd (1H) + 1,59-1,65 m (1H)		23,28
16	1,69-1,77 m (1H) + 1,80-1,87 m (1H)		38,79
17	-		81,85
18	1,14 s (3H)		16,53
19	1,44 s (3H)		20,87
20	1,16 s (3H)		25,89
NOESY crosspeaks	4,41 m (1H)	1,83; 2,17	

5.2 Supplemental data

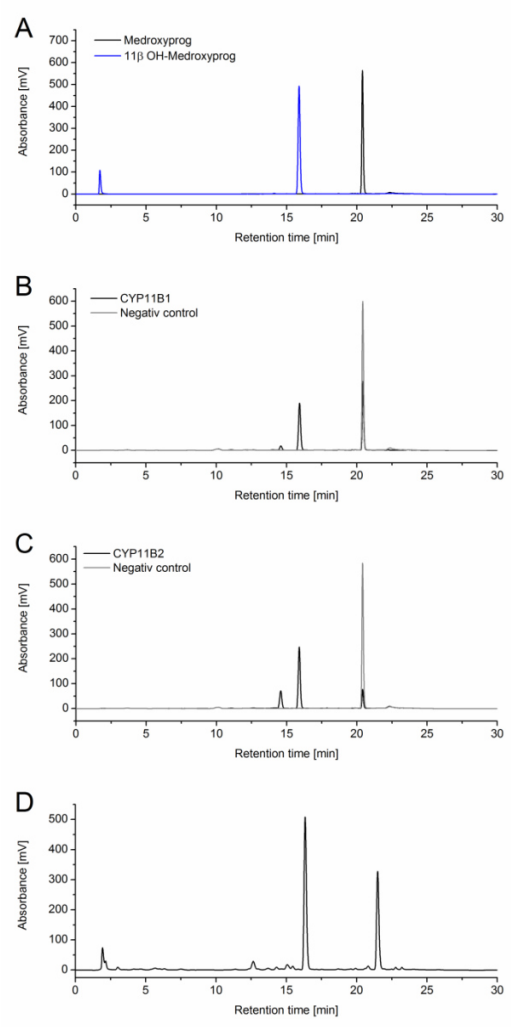


Fig. S1: HPLC chromatograms of CYP11B1- and CYP11B2-dependent conversion of medroxyprogesterone. Steroids were separated on a reverse phase column with an acetonitrile/water gradient and monitored at 240 nm, whereby standards of medroxyprogesterone (black line) and 11β-OH-medroxyprogesterone (blue line) showed retention times of 20.4 min and 15.9 min, respectively (**A**). Conversions of medroxyprogesterone by CYP11B1 (**B**) and CYP11B2 (**C**) were conducted *in-vitro* with purified enzymes as described in 2.3 (Schiffer *et al.* 2015b) and yielded in the formation of 11β-OH-medroxyprogesterone and a minor product (RT 14.6), which is formed to a greater extent by CYP11B2. The negative control (grey line) was incubated without NADPH. *E. coli* based whole-cell conversion of 250 µM medroxyprogesterone with CYP11B1 as described in 2.1 (Schiffer *et al.* 2015a) enabled the production of 11β-OH-medroxyprogesterone without significant formation of minor products with a volumetric productivity of 54 $mg*L^{-1}*d^{-1}$ as calculated from peak area portions in the HPLC chromatogram after 24h of conversion (**D**).

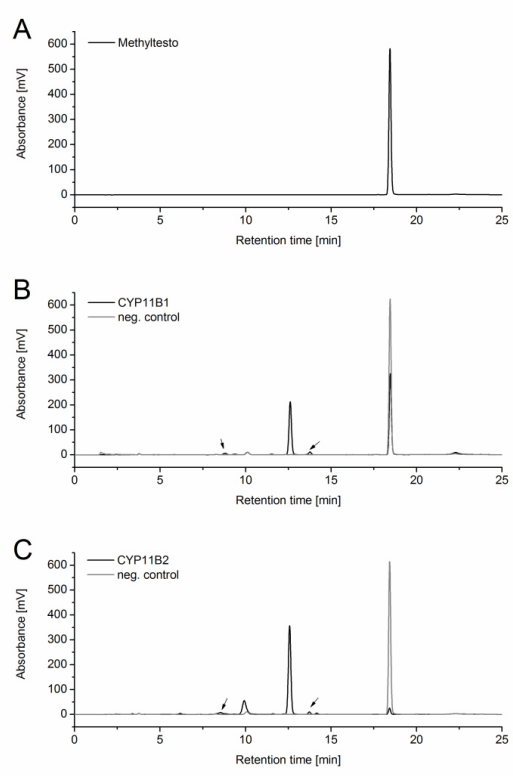
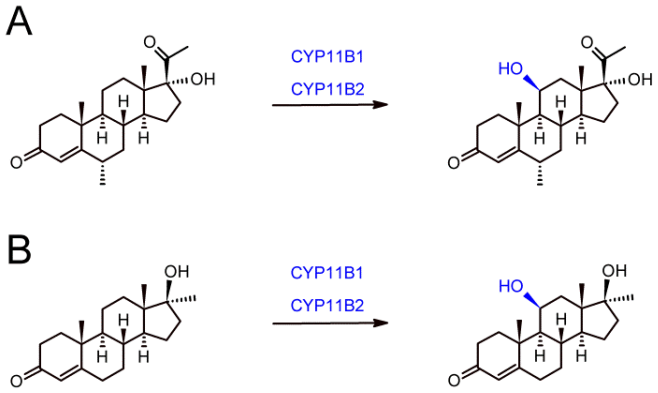


Fig. S2: HPLC chromatograms of CYP11B1- and CYP11B2-dependent conversion of methyltestosterone. Steroids were separated on a reverse phase column with an acetonitrile/water gradient and monitored at 240 nm, whereby methyltestosterone showed a retention time of 18.4 min (**A**). Conversions of methyltestosterone by CYP11B1 (**B**) and CYP11B2 (**C**) were conducted *in-vitro* with purified enzymes as described under 2.3 (Schiffer *et al.* 2015b) and lead to the formation of the major product 11β-OH-methyltestosterone (RT 12.6 min; see **Table S1** for structural identification by NMR) and a minor product (RT 9.9 min) in case of CYP11B2. Additional putative minor products are marked by arrows. The negative control (grey line) was incubated without NADPH.



Scheme S1: Structural formula of the CYP11B1- and CYP11B2-dependent conversion of medroxyprogesterone (A) and methlytestosterone (B). The 11β-hydroxylation of medroxyprogesterone was identified by HPLC analysis and comparison of the retention time with a commercially available standard. 11β-OH-methyltestosterone was identified by NMR spectroscopy as described in **Table S1**.

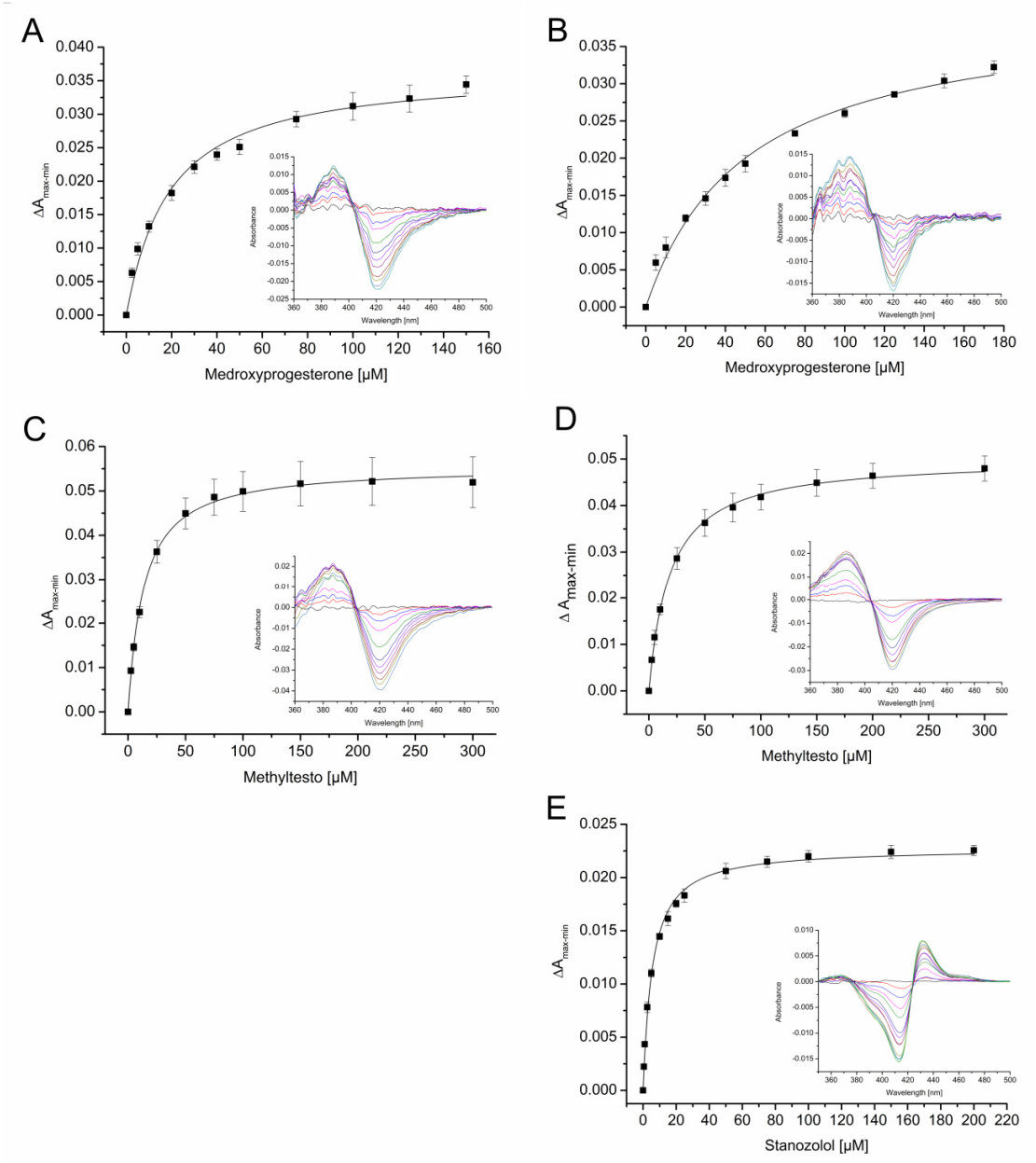


Fig. S3: Determination of dissociation constants for CYP11B1 and CYP11B2 by difference spectroscopy. Difference spectroscopy was performed as described in 2.3 (Schiffer *et al.* 2015b) (**A**) CYP11B1 with medroxyprogesterone, (**B**) CYP11B2 with medroxyprogesterone, (**C**) CYP11B1 with methyltestosterone, (**D**) CYP11B2 with methyltestosterone, (**E**) CYP11B2 with stanozolol. No effect on the spin state equilibrium was observed for CYP11B1 upon titration with stanozolol up to 200 µM. Peak-to-trough ($\Delta A_{max\text{-}min}$) were plotted against the ligand concentration and dissociation constants were determined by hyperbolic regression for B (R^2 0.990) or regression by tight binding equation for A (R^2

0.985), C (R^2 0.998), D (R^2 0.999) and E (R^2 0.995). Values represent the mean of three titrations with standard deviation. Resulting constants are summarized in **Table 3.1**.

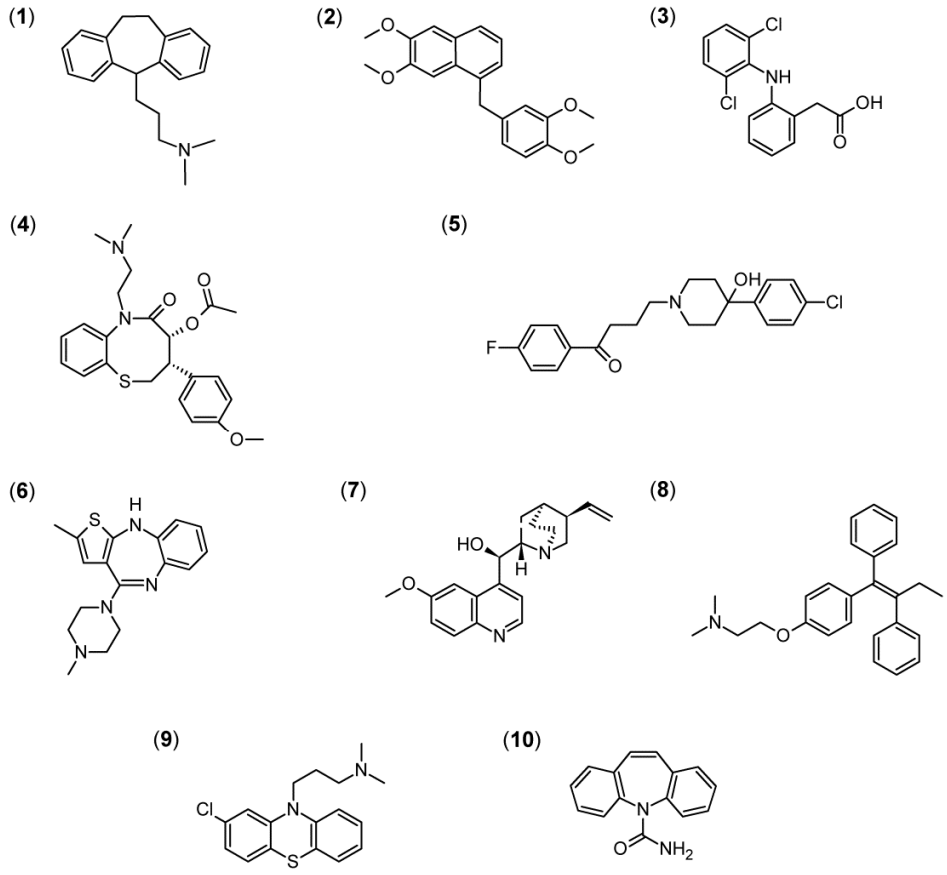


Fig. S4: Structural formula of drugs assayed for metabolism by CYP11B2. (**1**) amitriptyline – antidepressant; (**2**) papaverine- antispasmodic drug; (**3**) diclofenac – analgesic; (**4**) diltiazem – calcium antagonist; (**5**) haloperidol – neuroleptic; (**6**) olanzapine – neuroleptic; (**7**) quinine – antipyretic, analgesic, anti-malaria; (**8**) tamoxifen – selective estrogen receptor modulator; (**9**) chlorpromazine – neuroleptic; (**10**) carbamazepine - anticonvulsive. 200 µM of each compounds were incubated with a reconstituted CYP11B2 system as described in 2.3 (Schiffer *et al.* 2015b) for 3-4 h. Extraction and HPLC analysis were performed with compound specific protocols and detection wavelengths. For none of the drugs a conversion by CYP11B2 was observed.

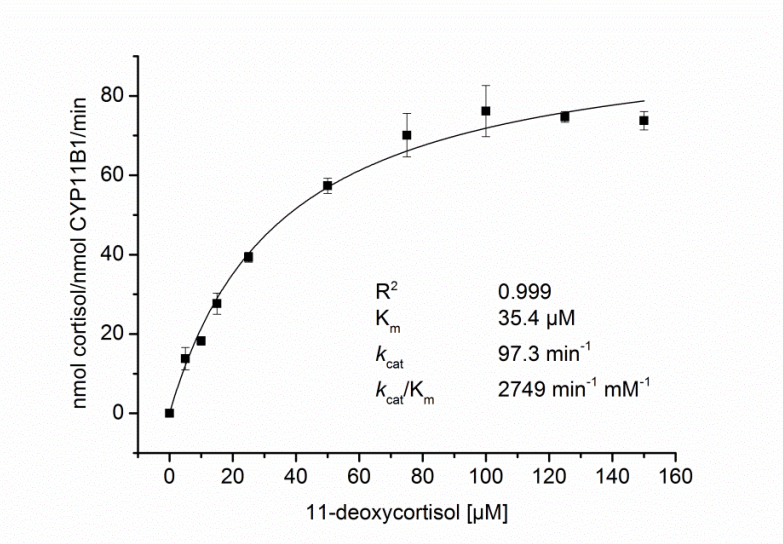


Fig. S5: Steady-state kinetics of CYP11B1-dependent conversion of 11-deoxycortisol to cortisol. Reactions were performed in a reconstituted *in-vitro* system with purified enzymes in the presence of an NADPH regeneration system as described in 2.3 (Schiffer *et al*. 2015b). Enzyme concentrations were 0.25 µM for CYP11B1 and human AdR and 5 µM for human Adx. Reactions took place at 37 °C and 750 rpm and were stopped after 1-4 minutes under steady-state conditions. Progesterone was added as internal standard and samples were extracted with chloroform. Cortisol formation was quantified by HPLC analysis with an acetonitrol/water gradient and detection at 240 nm with a standard curve. Reaction velocity was plotted against the substrate concentration and kinetic parameters were determined by hyperbolic regression according to Michealis-Menten.

5.3 Abbreviations and units

AAS	Anabolic androgenic steroids
Andro	Androstenedione
AdR	Adrenodoxin reductase
Adx	Adrenodoxin
Aldo	Aldosterone
AR	Androgen receptor
Arh1	Adrenodoxin reductase homologue 1
B	Corticosterone
CPR	Cytochrome P450 reductase
d	Day
DHEA	Dehydroepiandrosterone
DOC	11-deoxycorticosterone
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FDA	Food and drug administration
FdR	Ferredoxin reductase
Fdx	Ferredoxin
FMN	Flavin mononucleotide
g	Gram
GR	Glucocorticoid receptor
h	Hour
HPLC	High performance liquid chromatography
HSD	Hydroxy steroid dehydrogenase
K_d	Dissociation constant
L	Liter
LC	Liquid chromatography
LTM	Long-term metabolite
m	Meter
ME	Molecular evolution
M	Molar
MP	Medroxyprogesterone

MR	Mineralocorticoid receptor
MRA	Mineralocorticoid receptor antagonist
MS	Mass spectrometry
MT	17α-Methyltestosterone
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
n.d.	Not determined
OT	Oral-turinabol
P450	Cytochrome P45
Preg	Pregnenolone
Prog	Progesterone
ROS	Reactive oxygen species
RSS	Reichstein's substance S, 11-deoxycortisol
s	Second
SDM	Site directed mutagenesis
Testo	Testosterone
UV	Ultra violett
WADA	World Anti-Doping Agency
WM	Wagner Meerwein

Abbreviations for amino acids

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartate	P	Proline
E	Glutamate	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophane
L	Leucine	Y	Tyrosine

6. References

Acevedo-Rocha CG, Reetz MT, and Nov Y (2015) Economical analysis of saturation mutagenesis experiments. *Scientific Reports* **5:**10654.

Akhtar M, Calder MR, Corina DL, and Wright JN (1982) Mechanistic studies on C-19 demethylation in oestrogen biosynthesis. *Biochemical Journal* **201:**569-580.

Akhtar M and Skinner SJ (1968) The intermediary role of a 19-oxoandrogen in the biosynthesis of oestrogen. *Biochemical Journal* **109:**318-321.

Anastas P and Warner J (1998) *Green Chemistry: Theory and Practice*. Oxford Univ.Press.

Andreopoulou P and Bockman RS (2015) Management of Postmenopausal Osteoporosis. *Annual Review of Medicine* **66:**329-342.

Auchus RJ and Miller WL (1999) Molecular Modeling of Human P450c17 (17α-Hydroxylase/ 17,20-Lyase): Insights into Reaction Mechanisms and Effects of Mutations. *Molecular Endocrinology* **13:**1169-1182.

Bernhardt R (2006) Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology* **124:**128-145.

Bernhardt R and Urlacher VB (2014) Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. *Appl Microbiol Biotechnol* **98:**6185-6203.

Bernhardt R and Waterman MR (2007) Cytochrome P450 and Steroid Hormone Biosynthesis, in: *The Ubiquitous Roles of Cytochrome P450 Proteins*, pp 361-396, John Wiley & Sons, Ltd.

Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, and Xu HE (2002) Crystal Structure of the Glucocorticoid Receptor Ligand Binding Domain Reveals a Novel Mode of Receptor Dimerization and Coactivator Recognition. *Cell* **110:**93-105.

Bloom JD, Meyer MM, Meinhold P, Otey CR, MacMillan D, and Arnold FH (2005) Evolving strategies for enzyme engineering. *Current Opinion in Structural Biology* **15:**447-452.

Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC, and Robins K (2012) Engineering the third wave of biocatalysis. *Nature* **485:**185-194.

Boyd GS, Brownie AC, Jefcoate CR, and Simpson ER (1971) Cholesterol hydroxylation in the adrenal cortex and liver. *Biochemical Journal* **125:**1P-2P.

Braselton WE, Engel LL, and Orr JC (1974) The Flux of Intermediates and Products in Aromatization of C19 Steroids by Human Placental Microsomes. *European Journal of Biochemistry* **48:**35-43.

Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson J-A, and Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389:**753-758.

Cao P-r and Bernhardt R (1999) Interaction of CYP11B1 (cytochrome P-45011β) with CYP11A1 (cytochrome P-450scc) in COS-1 cells. *European Journal of Biochemistry* **262:**720-726.

Choi MH, Skipper PL, Wishnok JS, and Tannenbaum SR (2005) Characterization of testosterone 11β-hydroxylation catalyzed by human liver microsomal cytochromes P450. *Drug Metabolism and Disposition* **33:**714-718.

Chung BC, Matteson KJ, Voutilainen R, Mohandas TK, and Miller WL (1986) Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta. *Proceedings of the National Academy of Sciences of the United States of America* **83:**8962-8966.

Denisov IG, Makris TM, Sligar SG, and Schlichting I (2005) Structure and Chemistry of Cytochrome P450. *Chemical Reviews* **105:**2253-2278.

Denner K, Doehmer J, and Bernhardt R (1995) Cloning of CYP11B1 and CYP11B2 from normal human adrenal and their functional expression in COS-7 and V79 chinese hamster cells. *Endocrine Research* **21:**443-448.

Dewick PM (1999) Pharmaceutical Steroids and their Production for Hormone Replacement Therapy. *British Menopause Society Journal* **5:**12-22.

Donova MV and Egorova OV (2012) Microbial steroid transformations: current state and prospects. *Appl Microbiol Biotechnol* **94:**1423-1447.

Dragan CA, Zearo S, Hannemann F, Bernhardt R, and Bureik M (2005) Efficient conversion of 11-deoxycortisol to cortisol (hydrocortisone) by recombinant fission yeast Schizosaccharomyces pombe. *FEMS Yeast Res* **5:**621-625.

Dumas B, Cauet G, Lacour T, Degryse E, Laruelle L, Ledoux C, Spagnoli R, and Achstetter T (1996) 11 beta-hydroxylase activity in recombinant yeast mitochondria. In vivo conversion of 11-deoxycortisol to hydrocortisone. *Eur J Biochem* **238:**495-504.

Eiben S, Kaysser L, Maurer S, Kühnel K, Urlacher VB, and Schmid RD (2006) Preparative use of isolated CYP102 monooxygenases—A critical appraisal. *Journal of Biotechnology* **124:**662-669.

Erdmann B, Gerst H, Lippoldt A, Bülow H, Ganten D, Fuxe K, and Bernhard R (1996) Expression of cytochrome P45011B1 mRNA in the brain of normal and hypertensive transgenic rats. *Brain Research* **733:**73-82.

Estabrook RW, Cooper DY, and Rosenthal O (1963) The Light Reversible Carbon Monoxide Inhibition of the Steroid C21-Hydroxylase System of the Adrenal Cortex. *Biochem Z* **338:**741-755.

Ewen KM, Ringle M, and Bernhardt R (2012) Adrenodoxin--a versatile ferredoxin. *IUBMB Life* **64:**506-512.

Farooqi V, van den Berg ME, Cameron ID, and Crotty M (2014) Anabolic steroids for rehabilitation after hip fracture in older people. *Cochrane Database Syst Rev* **6**.

Fishman J, Guzik H, and Dixon D (1969) Stereochemistry of estrogen biosynthesis. *Biochemistry* **8:**4304-4309.

Food and Drug Administration (2008) Guidance for Industry Safety Testing of Drug Metabolites

Pharmacokinetic Subcommittee of the Pharmacology and Toxicology Coordinating Committee (PTCC) in the Center for Drug Evaluation and Research (CDER) US Department of Health and Human Services Food and Drug Administration.

Fragkaki AG, Angelis YS, Koupparis M, Tsantili-Kakoulidou A, Kokotos G, and Georgakopoulos C (2009) Structural characteristics of anabolic androgenic steroids contributing to binding to the androgen receptor and to their anabolic and androgenic activities: Applied modifications in the steroidal structure. *Steroids* **74:**172-197.

Freel EM, Shakerdi LA, Friel EC, Wallace AM, Davies E, Fraser R, and Connell JMC (2004) Studies on the Origin of Circulating 18-Hydroxycortisol and 18-Oxocortisol in Normal Human Subjects. *The Journal of Clinical Endocrinology & Metabolism* **89:**4628-4633.

Funder J, Pearce P, Smith R, and Smith A (1988) Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242:**583-585.

Gell JS, Carr BR, Sasano H, Atkins B, Margraf L, Mason JI, and Rainey WE (1998) Adrenarche Results from Development of a 3β-Hydroxysteroid Dehydrogenase-Deficient Adrenal Reticularis. *The Journal of Clinical Endocrinology & Metabolism* **83:**3695-3701.

Gerber A, Kleser M, Biedendieck R, Bernhardt R, and Hannemann F (2015) Functionalized PHB granules provide the basis for the efficient side-chain cleavage of cholesterol and analogs in recombinant Bacillus megaterium. *Microb Cell Fact* **14:**015-0300.

Geyer H, Schänzer W, and Thevis M (2014) Anabolic agents: recent strategies for their detection and protection from inadvertent doping. *British Journal of Sports Medicine* **48:**820-826.

Ghosh D, Griswold J, Erman M, and Pangborn W (2009) Structural basis for androgen specificity and oestrogen synthesis in human aromatase. *Nature* **457:**219-223.

Gibson GG and Tamburini PP (1984) Cytochrome P-450 spin state: Inorganic biochemistry of haem iron ligation and functional significance. *Xenobiotica* **14:**27-47.

Gillam EMJ (2007) Extending the capabilities of nature's most versatile catalysts: Directed evolution of mammalian xenobiotic-metabolizing P450s. *Archives of Biochemistry and Biophysics* **464:**176-186.

Gillam EMJ (2008) Engineering Cytochrome P450 Enzymes. *Chemical Research in Toxicology* **21:**220-231.

Girhard M, Klaus T, Khatri Y, Bernhardt R, and Urlacher VB (2010) Characterization of the versatile monooxygenase CYP109B1 from Bacillus subtilis. *Appl Microbiol Biotechnol* **87:**595-607.

Gotoh O (1992) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *Journal of Biological Chemistry* **267:**83-90.

Gregory MC, Denisov IG, Grinkova YV, Khatri Y, and Sligar SG (2013) Kinetic Solvent Isotope Effect in Human P450 CYP17A1-Mediated Androgen Formation: Evidence for a Reactive Peroxoanion Intermediate. *Journal of the American Chemical Society* **135:**16245-16247.

Gricman Ł, Vogel C, and Pleiss J (2014) Conservation analysis of class-specific positions in cytochrome P450 monooxygenases: Functional and structural relevance. *Proteins: Structure, Function, and Bioinformatics* **82:**491-504.

Groenhof AR (2007) Theoretical Investigation of the Hydroxylation Mechanism by Cytochromes P450, University of Amsterdam, Amsterdam.

Guddat S, Fußhöller G, Beuck S, Thomas A, Geyer H, Rydevik A, Bondesson U, Hedeland M, Lagojda A, Schänzer W, and Thevis M (2013) Synthesis, characterization, and detection of new oxandrolone metabolites as long-term markers in sports drug testing. *Anal Bioanal Chem* **405:**8285-8294.

Guengerich FP (2002) Rate-Limiting Steps in Cytochrome P450 Catalysis, in: *Biological Chemistry*, pp 1553.

Guengerich FP (2008) Cytochrome P450 and Chemical Toxicology. *Chemical Research in Toxicology* **21:**70-83.

Guengerich FP and Cheng Q (2011) Orphans in the Human Cytochrome P450 Superfamily: Approaches to Discovering Functions and Relevance in Pharmacology. *Pharmacological Reviews* **63:**684-699.

Guengerich FP, Liebler DC, and Reed DL (1985) Enzymatic activation of chemicals to toxic metabolites. *CRC Critical Reviews in Toxicology* **14:**259-307.

Guengerich FP and Munro AW (2013) Unusual Cytochrome P450 Enzymes and Reactions. *Journal of Biological Chemistry* **288:**17065-17073.

Guengerich FP, Wu Z-L, and Bartleson CJ (2005) Function of human cytochrome P450s: Characterization of the orphans. *Biochemical and Biophysical Research Communications* **338:**465-469.

Guo IC, Hu MC, and Chung BC (2003) Transcriptional Regulation of CYP11A1. *Journal of Biomedical Science* **10:**593-598.

Gustafsson JÅ and Lisboa BP (1970) Studies on the metabolism of C19 steroids in rat liver. 7. 18-hydroxylation of 17-oxo-C19 steroids in rat liver microsomes. *Steroids* **15:**723-735.

Hakki T, Zearo S, Drăgan C-A, Bureik M, and Bernhardt R (2008) Coexpression of redox partners increases the hydrocortisone (cortisol) production efficiency in CYP11B1 expressing fission yeast Schizosaccharomyces pombe. *Journal of Biotechnology* **133:**351-359.

Hanlon SP, Friedberg T, Wolf CR, Ghisalba O, and Kittelmann M (2007) Recombinant Yeast and Bacteria that Express Human P450s: Bioreactors for Drug Discovery, Development, and Biotechnology, in: *Modern Biooxidation*, pp 233-252, Wiley-VCH Verlag GmbH & Co. KGaA.

Hannemann F, Bichet A, Ewen KM, and Bernhardt R (2007) Cytochrome P450 systems—biological variations of electron transport chains. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1770:**330-344.

Hobler A (2013) Enzymatische Charakterisierung der humanen Aldosteronsynthase (CYP11B2) durch in vitro Untersuchungen sowie Homologiemodellierung und Dockingstudien, in: *Biochemistry*, Saarland University, Saarbruecken.

Hobler A, Kagawa N, Hutter MC, Hartmann MF, Wudy SA, Hannemann F, and Bernhardt R (2012) Human aldosterone synthase: Recombinant expression in E. coli and purification enables a detailed biochemical analysis of the protein on the molecular level. *The Journal of Steroid Biochemistry and Molecular Biology* **132:**57-65.

Hofmann F, Schwab M, and Eichelbaum M (2013) Kapitel 1 - Allgemeine Pharmakologie und Toxikologie, in: *Allgemeine und spezielle Pharmakologie und Toxikologie (11, Überarbeitete Auflage)* (Aktories K, Förstermann U, Hofmann F, and Starke K eds), pp 1-93, Urban & Fischer, Munich.

Imai M, Shimada H, Watanabe Y, Matsushima-Hibiya Y, Makino R, Koga H, Horiuchi T, and Ishimura Y (1989) Uncoupling of the cytochrome P-450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine: possible role of the hydroxy amino acid in oxygen activation. *Proceedings of the National Academy of Sciences of the United States of America* **86:**7823-7827.

Ishihara M, Kirdani RY, Osawa Y, and Sandberg AA (1976) The metabolic fate of medroxyprogesterone acetate in the baboon. *Journal of Steroid Biochemistry* **7:**65-70.

Julsing MK, Cornelissen S, Bühler B, and Schmid A (2008) Heme-iron oxygenases: powerful industrial biocatalysts? *Current Opinion in Chemical Biology* **12:**177-186.

Kahn RA and Durst F (2000) Chapter Six Function and evolution of plant cytochrome P450, in: *Recent Advances in Phytochemistry* (John T. Romeo RILV and Vincenzo De L eds), pp 151-189, Elsevier.

Katagiri M, Kagawa N, and Waterman MR (1995) The Role of Cytochrome b5 in the Biosynthesis of Androgens by Human P450c17. *Archives of Biochemistry and Biophysics* **317:**343-347.

Katagiri M, Takemori S, Itagaki E, Suhara K, and Gomi T (1976) Characterization of purified cytochrome P-450scc and P-450(11)beta from bovine adrenocortical mitochondria. *Adv Exp Med Biol* **74:**281-289.

Kauppi B, Jakob C, Färnegårdh M, Yang J, Ahola H, Alarcon M, Calles K, Engström O, Harlan J, Muchmore S, Ramqvist A-K, Thorell S, Öhman L, Greer J, Gustafsson J-Å, Carlstedt-Duke J, and Carlquist M (2003) The Three-dimensional Structures of Antagonistic and Agonistic Forms of the Glucocorticoid Receptor Ligand-binding Domain: RU-486 INDUCES A TRANSCONFORMATION THAT LEADS TO ACTIVE ANTAGONISM. *Journal of Biological Chemistry* **278:**22748-22754.

Kawamoto T, Mitsuuchi Y, Ohnishi T, Ichikawa Y, Yokoyama Y, Sumimoto H, Toda K, Miyahara K, Kuribayashi I, Nakao K, Hosoda K, Yamamoto Y, Imura H, and Shizuta Y (1990a) Cloning and expression of a cDNA for human cytochrome P-450aldo as related to primary aldosteronism. *Biochemical and Biophysical Research Communications* **173:**309-316.

Kawamoto T, Mitsuuchi Y, Toda K, Miyahara K, Yokoyama Y, Nakao K, Hosoda K, Yamamoto Y, Imura H, and Shizuta Y (1990b) Cloning of cDNA and genomic DNA for human cytochrome P-45011β. *FEBS Letters* **269:**345-349.

Khatri Y, Gregory MC, Grinkova YV, Denisov IG, and Sligar SG (2014) Active site proton delivery and the lyase activity of human CYP17A1. *Biochemical and Biophysical Research Communications* **443:**179-184.

Khatri Y, Hannemann F, Girhard M, Kappl R, Même A, Ringle M, Janocha S, Leize-Wagner E, Urlacher VB, and Bernhardt R (2013) Novel family members of CYP109 from Sorangium cellulosum So ce56 exhibit characteristic biochemical and biophysical properties. *Biotechnology and Applied Biochemistry* **60:**18-29.

Kimata Y, Shimada H, Hirose T, and Ishimura Y (1995) Role of THR-252 in Cytochrome P450cam: A Study with Unnatural Amino Acid Mutagenesis. *Biochemical and Biophysical Research Communications* **208:**96-102.

Kiss FM, Khatri Y, Zapp J, and Bernhardt R (2015a) Identification of new substrates for the CYP106A1-mediated 11-oxidation and investigation of the reaction mechanism. *FEBS Letters* **589:**2320-2326.

Kiss FM, Schmitz D, Zapp J, Dier TKF, Volmer DA, and Bernhardt R (2015b) Comparison of CYP106A1 and CYP106A2 from Bacillus megaterium – identification of a novel 11-oxidase activity. *Appl Microbiol Biotechnol* **99:**8495-8514.

Kuchner O and Arnold FH (1997) Directed evolution of enzyme catalysts. *Trends in Biotechnology* **15:**523-530.

Labinger JA (2004) Selective alkane oxidation: hot and cold approaches to a hot problem. *Journal of Molecular Catalysis A: Chemical* **220:**27-35.

Lamb DC, Lei L, Warrilow AGS, Lepesheva GI, Mullins JGL, Waterman MR, and Kelly SL (2009) The First Virally Encoded Cytochrome P450. *Journal of Virology* **83:**8266-8269.

Locuson CW, Hutzler JM, and Tracy TS (2007) Visible Spectra of Type II Cytochrome P450-Drug Complexes: Evidence that "Incomplete" Heme Coordination Is Common. *Drug Metabolism and Disposition* **35:**614-622.

Loughlin WA (2000) Biotransformations in organic synthesis. *Bioresource Technology* **74:**49-62.

Lund B-O and Lund J (1995) Novel Involvement of a Mitochondrial Steroid Hydroxylase (P450c11) in Xenobiotic Metabolism. *Journal of Biological Chemistry* **270:**20895-20897.

MacKenzie SM, Connell JMC, and Davies E (2012) Non-adrenal synthesis of aldosterone: A reality check. *Molecular and Cellular Endocrinology* **350:**163-167.

Martinis SA, Atkins WM, Stayton PS, and Sligar SG (1989) A conserved residue of cytochrome P-450 is involved in heme-oxygen stability and activation. *Journal of the American Chemical Society* **111:**9252-9253.

Masse R and Goudreault D (1992) Studies on anabolic steroids—11. 18-hydroxylated metabolites of mesterolone, methenolone and stenbolone: New steroids isolated from human urine. *The Journal of Steroid Biochemistry and Molecular Biology* **42:**399-410.

Mestres J (2005) Structure conservation in cytochromes P450. *Proteins* **58:**596-609.

Miller WL and Auchus RJ (2011) The Molecular Biology, Biochemistry, and Physiology of Human Steroidogenesis and Its Disorders. *Endocrine Reviews* **32:**81-151.

Mizutani M (2012) Impacts of Diversification of Cytochrome P450 on Plant Metabolism. *Biological and Pharmaceutical Bulletin* **35:**824-832.

Morant M, Bak S, Møller BL, and Werck-Reichhart D (2003) Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. *Current Opinion in Biotechnology* **14:**151-162.

Mosa A, Neunzig J, Gerber A, Zapp J, Hannemann F, Pilak P, and Bernhardt R (2015) 2β- and 16β-hydroxylase activity of CYP11A1 and direct stimulatory effect of estrogens on pregnenolone formation. *The Journal of Steroid Biochemistry and Molecular Biology* **150:**1-10.

Mulatero P, Curnow KM, Aupetit-Faisant B, Foekling M, Gomez-Sanchez C, Veglio F, Jeunemaitre X, Corvol P, and Pascoe L (1998) Recombinant CYP11B Genes Encode Enzymes that Can Catalyze Conversion of 11-Deoxycortisol to Cortisol, 18-Hydroxycortisol, and 18-Oxocortisol. *The Journal of Clinical Endocrinology & Metabolism* **83:**3996-4001.

Munro AW, Girvan HM, and McLean KJ (2007) Cytochrome P450–redox partner fusion enzymes. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1770:**345-359.

Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato RYO, and Waterman MR (1987) The P450 Gene Superfamily: Recommended Nomenclature. *DNA* **6:**1-11.

Nelson D (2009) The Cytochrome P450 Homepage. *Human Genomics* **4:**59 - 65.

Nelson DR (2013) A world of cytochrome P450s. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **368:**20120430.

Nelson DR, Goldstone JV, and Stegeman JJ (2013) The cytochrome P450 genesis locus: the origin and evolution of animal cytochrome P450s. *Philos Trans R Soc Lond B Biol Sci* **368:**19.

Nelson SD (1982) Metabolic activation and drug toxicity. *Journal of Medicinal Chemistry* **25:**753-765.

Neunzig J, Sánchez-Guijo A, Mosa A, Hartmann MF, Geyer J, Wudy SA, and Bernhardt R (2014) A steroidogenic pathway for sulfonated steroids: The metabolism of pregnenolone sulfate. *The Journal of Steroid Biochemistry and Molecular Biology* **144, Part B:**324-333.

Norman AW and Litwack G (1997a) Chapter 2 - Steroid Hormones: Chemistry, Biosynthesis, and Metabolism, in: *Hormones (Second Edition)* (Litwack AWN ed), pp 49-86, Academic Press, San Diego.

Norman AW and Litwack G (1997b) Chapter 10 - Adrenal Corticoids, in: *Hormones (Second Edition)* (Litwack AWN ed), pp 281-318, Academic Press, San Diego.

Norman AW and Litwack G (1997c) Chapter 12 - Androgens, in: *Hormones (Second Edition)* (Litwack AWN ed), pp 341-360, Academic Press, San Diego.

Norman AW and Litwack G (1997d) Chapter 13 - Estrogens and Progestins, in: *Hormones (Second Edition)* (Litwack AWN ed), pp 361-386, Academic Press, San Diego.

Norman AW and Litwack G (1997e) Chapter 14 - Hormones of Pregnancy and Lactation, in: *Hormones (Second Edition)* (Litwack AWN ed), pp 387-411, Academic Press, San Diego.

O'Reilly E, Kohler V, Flitsch SL, and Turner NJ (2011) Cytochromes P450 as useful biocatalysts: addressing the limitations. *Chemical Communications* **47:**2490-2501.

Omura T and Sato R (1962) A New Cytochrome in Liver Microsomes. *Journal of Biological Chemistry* **237:**1375-1376.

Omura T and Sato R (1964a) The Carbon Monoxide-binding Pigment of Liver Microsomes: I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry* **239:**2370-2378.

Omura T and Sato R (1964b) The Carbon Monoxide-binding Pigment of Liver Microsomes: II. Solubilization, Purification and Properties. *Journal of Biological Chemistry* **239:**2379-2385.

Ortiz de Montellano PR (2010) Hydrocarbon Hydroxylation by Cytochrome P450 Enzymes. *Chemical Reviews* **110:**932-948.

Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Jiang H, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, and Newman JD (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* **496:**528-532.

Pallan PS, Wang C, Lei L, Yoshimoto FK, Auchus RJ, Waterman MR, Guengerich FP, and Egli M (2015) Human Cytochrome P450 21A2, the Major Steroid 21-Hydroxylase: Structure of the Enzyme-Progesterone Substrate Complex and Rate-Limiting C-H Bond Cleavage. *Journal of Biological Chemistry*.

Pandey AV and Miller WL (2005) Regulation of 17,20 Lyase Activity by Cytochrome b5 and by Serine Phosphorylation of P450c17. *Journal of Biological Chemistry* **280:**13265-13271.

Parr MK, Fusshöller G, Gütschow M, Hess C, and Schänzer W (2010) GC-MS(/MS) investigations on long-term metabolites of 17-methyl steroids, in: *Recent Advances in Doping Analysis* (Schänzer W, Geyer H, Gotzmann A, and Mareck U eds), pp 64-73, SPORTVERLAG Strauss, Cologne, Cologne.

Parr MK, Zöllner A, Fußhöller G, Opfermann G, Schlörer N, Zorio M, Bureik M, and Schänzer W (2012) Unexpected contribution of cytochrome P450 enzymes CYP11B2 and CYP21, as well as CYP3A4 in xenobiotic androgen elimination – Insights from metandienone metabolism. *Toxicology Letters* **213:**381-391.

Pechurskaya TA, Harnastai IN, Grabovec IP, Gilep AA, and Usanov SA (2007) Adrenodoxin supports reactions catalyzed by microsomal steroidogenic cytochrome P450s. *Biochemical and Biophysical Research Communications* **353:**598-604.

Peterson JA and Graham SE (1998) A close family resemblance: the importance of structure in understanding cytochromes P450. *Structure* **6:**1079-1085.

Petrunak EM, DeVore NM, Porubsky PR, and Scott EE (2014) Structures of Human Steroidogenic Cytochrome P450 17A1 with Substrates. *Journal of Biological Chemistry* **289:**32952-32964.

Petzoldt K, Annen K, Laurent H, and Wiechert R (1982) Process for the preparation of 11-beta-hydroxy steroids (Germany) SAB ed, US patent.

Pujols L, Mullol J, Roca-Ferrer J, Torrego A, Xaubet A, Cidlowski JA, and Picado C (2002) Expression of glucocorticoid receptor α- and β-isoforms in human cells and tissues. *American Journal of Physiology - Cell Physiology* **283:**C1324-C1331.

Rainey WE (1999) Adrenal zonation: clues from 11β-hydroxylase and aldosterone synthase. *Molecular and Cellular Endocrinology* **151:**151-160.

Renault H, Bassard J-E, Hamberger B, and Werck-Reichhart D (2014) Cytochrome P450-mediated metabolic engineering: current progress and future challenges. *Current Opinion in Plant Biology* **19:**27-34.

Rendic S, Nolteernsting E, and Schänzer W (1999) Metabolism of anabolic steroids by recombinant human cytochrome P450 enzymes: Gas chromatographic–mass spectrometric determination of metabolites. *Journal of Chromatography B: Biomedical Sciences and Applications* **735:**73-83.

Ringle M, Khatri Y, Zapp J, Hannemann F, and Bernhardt R (2013) Application of a new versatile electron transfer system for cytochrome P450-based Escherichia coli whole-cell bioconversions. *Appl Microbiol Biotechnol* **97:**7741-7754.

Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MCY, Withers ST, Shiba Y, Sarpong R, and Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440:**940-943.

Rojas Y, Finnerty CC, Radhakrishnan RS, and Herndon DN (2012) Burns: an update on current pharmacotherapy. *Expert opinion on pharmacotherapy* **13:**2485-2494.

Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR, Weinmann R, and Einspahr HM (2001) Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. *Proceedings of the National Academy of Sciences* **98:**4904-4909.

Sadeghi SJ and Gilardi G (2013) Chimeric P450 enzymes: Activity of artificial redox fusions driven by different reductases for biotechnological applications. *Biotechnology and Applied Biochemistry* **60:**102-110.

Schänzer W (1996) Metabolism of anabolic androgenic steroids. *Clinical Chemistry* **42:**1001-1020.

Schänzer W and Donike M (1993) Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Analytica Chimica Acta* **275:**23-48.

Schänzer W, Geyer H, Fußhöller G, Halatcheva N, Kohler M, Parr M-K, Guddat S, Thomas A, and Thevis M (2006) Mass spectrometric identification and characterization of a new long-term metabolite of metandienone in human urine. *Rapid Communications in Mass Spectrometry* **20:**2252-2258.

Schänzer W, Horning S, and Donike M (1995) Metabolism of anabolic steroids in humans: Synthesis of 6β-hydroxy metabolites of 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone, and metandienone. *Steroids* **60:**353-366.

Schänzer W, Horning S, Opfermann G, and Donike M (1996) Gas chromatography/mass spectrometry identification of long-term excreted metabolites of the anabolic steroid 4-chloro-1,2-dehydro-17α-methyltestosterone in humans. *The Journal of Steroid Biochemistry and Molecular Biology* **57:**363-376.

Schänzer W and Thevis M (2015) Human sports drug testing by mass spectrometry. *Mass Spectrometry Reviews***:**n/a-n/a.

Schenkman JB (1970) Studies on the nature of the type I and type II spectral changes in liver microsomes. *Biochemistry* **9:**2081-2091.

Schenkman JB, Cinti DL, Orrenius S, Moldeus P, and Kraschnitz R (1972) The nature of the reverse type I (modified type II) spectral change in liver microsomes. *Biochemistry* **11:**4243-4251.

Schenkman JB and Jansson I (2006) Spectral Analyses of Cytochromes P450, in: *Cytochrome P450 Protocols* (Phillips IR and Shephard EA eds), pp 11-18, Humana Press.

Schenkman JB, Remmer H, and Estabrook RW (1967) Spectral Studies of Drug Interaction with Hepatic Microsomal Cytochrome. *Molecular Pharmacology* **3:**113-123.

Schenkman JB, Sligar SG, and Cinti DL (1981) Substrate interaction with cytochrome P-450. *Pharmacology & Therapeutics* **12:**43-71.

Schiffer L, Anderko S, Hannemann F, Eiden-Plach A, and Bernhardt R (2015) The CYP11B subfamily. *The Journal of Steroid Biochemistry and Molecular Biology* **151:**38-51.

Schiffler B, Kiefer M, Wilken A, Hannemann F, Adolph HW, and Bernhardt R (2001) The Interaction of Bovine Adrenodoxin with CYP11A1 (Cytochrome P450scc) and CYP11B1 (Cytochrome P45011β): Acceleration of reduction and substrate conversion by site-directed mutagenesis of adrenodoxin. *Journal of Biological Chemistry* **276:**36225-36232.

Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, and Witholt B (2001) Industrial biocatalysis today and tomorrow. *Nature* **409:**258-268.

Schmitz D, Zapp J, and Bernhardt R (2012) Hydroxylation of the triterpenoid dipterocarpol with CYP106A2 from Bacillus megaterium. *FEBS Journal* **279:**1663-1674.

Schmitz D, Zapp J, and Bernhardt R (2014) Steroid conversion with CYP106A2 - production of pharmaceutically interesting DHEA metabolites. *Microbial Cell Factories* **13:**81.

Shahidi NT (2001) A review of the chemistry, biological action, and clinical applications of anabolic-androgenic steroids. *Clinical Therapeutics* **23:**1355-1390.

Simpson ER and Davis SR (2001) Minireview: Aromatase and the Regulation of Estrogen Biosynthesis—Some New Perspectives. *Endocrinology* **142:**4589-4594.

Sirim D, Widmann M, Wagner F, and Pleiss J (2010) Prediction and analysis of the modular structure of cytochrome P450 monooxygenases. *BMC Structural Biology* **10:**34.

Sligar SG (1976) Coupling of spin, substrate, and redox equilibriums in cytochrome P450. *Biochemistry* **15:**5399-5406.

Slominski AT, Li W, Kim T-K, Semak I, Wang J, Zjawiony JK, and Tuckey RC (2015) Novel activities of CYP11A1 and their potential physiological significance. *The Journal of Steroid Biochemistry and Molecular Biology* **151:**25-37.

Sobolevsky T and Rodchenkov G (2012) Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine. *The Journal of Steroid Biochemistry and Molecular Biology* **128:**121-127.

Sono M, Roach MP, Coulter ED, and Dawson JH (1996) Heme-Containing Oxygenases. *Chemical Reviews* **96:**2841-2888.

Straathof AJJ, Panke S, and Schmid A (2002) The production of fine chemicals by biotransformations. *Current Opinion in Biotechnology* **13:**548-556.

Strushkevich N, Gilep AA, Shen L, Arrowsmith CH, Edwards AM, Usanov SA, and Park H-W (2013) Structural Insights into Aldosterone Synthase Substrate Specificity and Targeted Inhibition. *Molecular Endocrinology* **27:**315-324.

Strushkevich N, MacKenzie F, Cherkesova T, Grabovec I, Usanov S, and Park H-W (2011) Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. *Proceedings of the National Academy of Sciences of the United States of America* **108:**10139-10143.

Swart AC, Schloms L, Storbeck K-H, Bloem LM, Toit Td, Quanson JL, Rainey WE, and Swart P (2013) 11β-Hydroxyandrostenedione, the product of androstenedione metabolism in the adrenal, is metabolized in LNCaP cells by 5α-reductase yielding 11β-hydroxy-5α-androstanedione. *The Journal of Steroid Biochemistry and Molecular Biology* **138:**132-142.

Szczebara FM, Chandelier C, Villeret C, Masurel A, Bourot S, Duport C, Blanchard S, Groisillier A, Testet E, Costaglioli P, Cauet G, Degryse E, Balbuena D, Winter J, Achstetter T, Spagnoli R, Pompon D, and Dumas B (2003) Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat Biotech* **21:**143-149.

Tapolcsányi P, Wélfling J, Mernyák E, and Schneider G (2004) The Mitsunobu Inversion Reaction of Sterically Hindered 17-Hydroxy Steroids. *Monatshefte für Chemie* **135:**1129-1136.

Thompson EA and Siiteri PK (1974a) The Involvement of Human Placental Microsomal Cytochrome P-450 in Aromatization. *Journal of Biological Chemistry* **249:**5373-5378.

Thompson EA and Siiteri PK (1974b) Utilization of Oxygen and Reduced Nicotinamide Adenine Dinucleotide Phosphate by Human Placental Microsomes during Aromatization of Androstenedione. *Journal of Biological Chemistry* **249:**5364-5372.

Townsley JD and Brodie HJ (1968) Mechanism of estrogenbiosynthesis. III. Stereochemistry of aromatization of C19 and C18 steroids. *Biochemistry* **7:**33-40.

Tretzel L, Thomas A, Geyer H, Pop V, Schanzer W, and Thevis M (2015) Dried blood spots (DBS) in doping controls: a complementary matrix for improved in- and out-of-competition sports drug testing strategies. *Analytical Methods* **7:**7596-7605.

Tsubaki M, Matsumoto N, Tomita S, Ichikawa Y, and Hori H (1998) 20β-hydroxy-C21-steroid 20β-oxidase activity of cytochrome P450c21 purified from bovine adrenocortical microsomes. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1390:**197-206.

Urlacher VB and Eiben S (2006) Cytochrome P450 monooxygenases: perspectives for synthetic application. *Trends in Biotechnology* **24:**324-330.

Vail RB, Homann MJ, Hanna I, and Zaks A (2005) Preparative synthesis of drug metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450 reductase expressed in Escherichia coli. *J IND MICROBIOL BIOTECHNOL* **32:**67-74.

Vidakovic M, Sligar SG, Li H, and Poulos TL (1998) Understanding the Role of the Essential Asp251 in Cytochrome P450cam Using Site-Directed Mutagenesis, Crystallography, and Kinetic Solvent Isotope Effect. *Biochemistry* **37:**9211-9219.

WADA (2015) 2014 Anti-Doping Testing Figures Report. *World Anti-Doping Agency*.

Williams SP and Sigler PB (1998) Atomic structure of progesterone complexed with its receptor. *Nature* **393:**392-396.

World Health Organization (2015) WHO Model List of Essential Medicines (19th list), World Health Organization.

Wudy SA, Hartmann MF, and Remer T (2007) Sexual dimorphism in cortisol secretion starts after age 10 in healthy children: urinary cortisol metabolite excretion rates during growth. *American Journal of Physiology - Endocrinology and Metabolism* **293:**E970-E976.

Yoshimura M, Nakamura S, Ito T, Nakayama M, Harada E, Mizuno Y, Sakamoto T, Yamamuro M, Saito Y, Nakao K, Yasue H, and Ogawa H (2002) Expression of Aldosterone Synthase Gene in Failing Human Heart: Quantitative Analysis Using Modified Real-Time Polymerase Chain Reaction. *The Journal of Clinical Endocrinology & Metabolism* **87:**3936-3940.

Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & Therapeutics* **138:**103-141.

Zehentgruber D, Drăgan C-A, Bureik M, and Lütz S (2010) Challenges of steroid biotransformation with human cytochrome P450 monooxygenase CYP21 using resting cells of recombinant Schizosaccharomyces pombe. *Journal of Biotechnology* **146:**179-185.

Zennaro M-C, Farman N, Bonvalet J-P, and Lombès M (1997) Tissue-Specific Expression of α and β Messenger Ribonucleic Acid Isoforms of the Human Mineralocorticoid Receptor in Normal and Pathological States. *The Journal of Clinical Endocrinology & Metabolism* **82:**1345-1352.

Zhang K, Shafer BM, Demars MD, Stern HA, and Fasan R (2012) Controlled Oxidation of Remote sp3 C–H Bonds in Artemisinin via P450 Catalysts with Fine-Tuned Regio- and Stereoselectivity. *Journal of the American Chemical Society* **134:**18695-18704.

Zhou M-Y, Gomez-Sanchez EP, Foecking MF, and Gomez-Sanchez CE (1995) Cloning and expression of the rat adrenal cytochrome P-450 11B3 (CYP11B3) enzyme cDNA: Preferential 18-hydroxylation over 11β-hydroxylation of DOC. *Molecular and Cellular Endocrinology* **114:**137-145.

Zöllner A, Buchheit D, Meyer MR, Maurer HH, Peters FT, and Bureik M (2010a) Production of human phase 1 and 2 metabolites by whole-cell biotransformation with recombinant microbes. *Bioanalysis* **2:**1277-1290.

Zöllner A, Kagawa N, Waterman MR, Nonaka Y, Takio K, Shiro Y, Hannemann F, and Bernhardt R (2008) Purification and functional characterization of human 11β hydroxylase expressed in Escherichia coli. *FEBS Journal* **275:**799-810.

Zöllner A, Parr MK, Drăgan C-A, Dräs S, Schlörer N, Peters FT, Maurer HH, Schänzer W, and Bureik M (2010b) CYP21-catalyzed production of the long-term urinary metandienone metabolite 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one: a contribution to the fight against doping. *Biological Chemistry* **391:**119-127.

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