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Research Paper

Biotransformation of prednisone and dexamethasone by cytochrome P450 based systems – Identification of new potential drug candidates

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ABSTRACT

Prednisone and dexamethasone are synthetic glucocorticoids widely used as anti-inflammatory and immunosuppressive drugs. Since their hydroxylated derivatives could serve as novel potential drug candidates, our aim was to investigate their biotransformation by the steroid hydroxylase CYP106A2 from *Bacillus megaterium* ATCC13368. *In vitro* we were able to demonstrate highly selective 15β-hydroxylation of the steroids with a reconstituted CYP106A2 system. The reactions were thoroughly characterized, determining the kinetic parameters and the equilibrium dissociation constant. The observed lower conversion rate in the case of dexamethasone hydroxylation was clarified by quantum chemical calculations, which suggest a rearrangement of the intermediately formed radical species. To identify the obtained conversion products with NMR, CYP106A2-based *Bacillus megaterium* whole-cell systems were applied resulting in an altered product pattern for prednisone, yet no significant change for dexamethasone conversion compared to *in vitro*. Even the MS941 control strain performed a highly selective biotransformation of prednisone producing the known metabolite 20β-dihydrocortisone. The identified novel prednisone derivatives 15β, 17, 20β, 21-tetrahydroxy-preg-4-en-3,11-dione and 15β, 17, 20β, 21-tetrahydroxy-preg-1,4-dien-3,11-dione as well as the 15β-hydroxylated variants of both drugs are promising candidates for drug-design and development approaches.

1. Introduction

Cytochromes P450 (P450s) comprise one of the largest and oldest enzyme families, being present in almost every life form. Today the P450 database (http://drnelson.uthsc.edu/CytochromeP450.html; status of April 2016) lists more than 35,000 representatives, reflecting the central role of these enzymes in nature. P450s are external monooxygenases, catalyzing the scission of molecular oxygen and the following monooxygenation of an aliphatic or aromatic substrate (Ortiz de Montellano, 2010). Furthermore, P450s encompass a broad range of reactions such as hydroxylation, dehalogenation, *N*-oxidation, *N*-, *O*-, *S*-dealkylation, and C—C bond cleavage (Bernhardt, 2006; Bernhardt and Urlacher, 2014). Unusual P450 reactions including cyclopropanation via the carbene transfer (Coelho et al., 2013) and intramolecular C—H amination are also described (McIntosh et al., 2013). Besides the wide range of catalyzed reactions, their broad substrate spectrum is also remarkable. P450s perform the oxyfunctionalization of a variety of complex substrates such as alkaloids, polyketides, terpenes and steroids (Bernhardt, 2006). The appeal of P450s rests upon their unique ability to perform reactions under mild conditions, including atmospheric pressure and temperatures ranging from 20 to 37 °C (Urlacher and Girhard, 2012).

Glucocorticoids (GCs) are a class of steroid hormones that regulate the glucose metabolism via interaction with the intracellular glucocorticoid receptor. GCs are so-called stress hormones and, as such, they exhibit potent anti-inflammatory and immunosuppressive effects. Besides their fundamental anti-inflammatory activities, they display a plethora of pleiotropic effects on multiple signaling pathways (Rhen and Cidlowski, 2005). The steroid mode of action can be regulated by the introduction of a functional group, in particular, an oxyfunctionalization in specific orientation and position, attached to the steroid core. The GC activity requires a 17-hydroxylated, 21-carbon steroid with an activating

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hydroxylation at position 11β, also shown to be crucial for anti-inflammatory activity. The 11β-hydroxylation regulates the GC activity via higher affinity towards the intracellular GC receptor. When the 11-hydroxyl group is converted to a keto group, it is not able to bind and activate the GC and the mineralocorticoid (MC) receptor. The enzyme responsible for the pivotal reaction, activating glucocorticoids is the 11β-hydroxysteroid-dehydrogenase type 1 (HSD1) (Chapman et al., 2013b). The reverse reaction, inactivating glucocorticoids is catalyzed by the tissue-specific 11β-hydroxysteroid-dehydrogenase type 2 (HSD2), whose role is to convert endogenous GCs into their inactive keto forms (Chapman et al., 2013a). Both cortisone and 11-dehydrocorticosterone have a substantially reduced affinity towards the GC receptor compared to cortisol and corticosterone (Chapman et al., 2013a; Hunter and Bailey, 2015). This protective mechanism is essential in aldosterone target tissues, such as the liver, lungs, colon and kidneys, to prevent unfavourable activation of the MC receptor, and in the placenta to control foetal exposure to maternal GCs (Brown et al., 1996; Chapman et al., 2013a).

The main targets of GC drugs are inflammatory and autoimmune diseases, such as Crohn's disease, ulcerative colitis, psoriasis, rheumatic and allergic disorders, lupus and many more. However, due their long-term application and complex actions, GCs show various side effects including hypogonadism, hypertension, bone necrosis, acne, development disorders, Cushing's syndrome and changes in behaviour (Rhen and Cidlowski, 2005). In certain cases long-term GC therapy can not be avoided, consequently, synthetic GCs have been developed to overcome some of these side effects, especially those triggered by the transactivation of the MC receptor. This transactivation can be reduced by derivatization at position C-16 on the steroid core, like 16α-methylation (present in dexamethasone) and 16β-methylation (present in betamethasone). Both methylations decrease MC transactivation by selectively increasing the GC receptor activation (Diederich et al., 2004). On the contrary, a 9α-fluorination (present in dexamethasone) increases both, receptor transactivation along with a reduced oxidation via HSD2. The Δ1-dehydro derivatization, however, increases HSD2 activity, resulting in an enhanced inactivation of the GC receptor (Diederich et al., 2004).

In this work, we aimed to convert the synthetic GCs prednisone and dexamethasone in order to produce novel hydroxylated derivatives of both drugs. The opportunity to selectively introduce hydroxyl groups into prednisone and dexamethasone opens up the possibility to observe new effects of these substances. In addition, such hydroxysteroids could have the potential to be used in subsequent studies developing them into newly functionalized drugs with altered specificity and pharmacokinetics. Our primary goal included the efficient hydroxylation of both steroids using a P450 based biotransformation system. To achieve this, we used CYP106A2 from *Bacillus megaterium* (*B. megaterium*) ATCC13368 as biocatalyst. This enzyme has been extensively studied in our group, and researchers have been investigating its steroid hydroxylating capacity since the late 1970s (Berg et al., 1976; Berg and Rafter, 1981; Rauschenbach et al., 1993; Lisurek et al., 2004; Virus et al., 2006; Zehentgruber et al., 2010; Kiss et al., 2015a). The conversion of prednisone and dexamethasone has been proved in our previous studies, however, the respective hydroxylation positions have not yet been determined (Schmitz et al., 2014; Kiss et al., 2015a). With the goal of detailed characterization of these CYP106A2-based reactions, substrate binding as well as reconstituted *in vitro* assays were performed. To understand the binding and kinetic behaviour of the P450 with the substrates, computational methods were applied. The conversion of both glucocorticoids was further investigated *in vivo* using different CYP106A2-based *B. megaterium* whole-cell systems. These preparative scale conversions enabled us to obtain sufficient amounts of reaction products for structural elucidation via NMR. Interestingly, the main product of all dexamethasone conversions was identified as 15β-hydroxydexamethasone whereas prednisone was converted with *B. megaterium* strains ATCC13368 and MS941 into different and novel hydroxyprednisone derivatives besides 15β-hydroxyprednisone. The former strain gave a product with rehydrated C-1 and subsequent loss of the Δ1-dehydro moiety, the latter one with a reduced keto group at position 20β.

2. Materials and methods

2.1. Chemicals and solvents

Prednisone and dexamethasone were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 17, 20β, 21-trihydroxy-preg-4-en-3,11-dione (20β-dihydrocortisone, Q3960-000) was purchased from Steraloids (Newport, RI, USA). All solvents and other chemicals were of highest purity available and were obtained from standard sources.

2.2. Protein expression and purification

CYP106A2 and its bovine redox partners, the truncated form of adrenodoxine ($Adx_{4\text{-}108}$) and adrenodoxine reductase (AdR) were expressed and purified as described before (Uhlmann et al., 1992; Sagara et al., 1993; Simgen et al., 2000; Lisurek et al., 2004).

2.3. Spectroscopic substrate binding assay

The binding behaviour of dexamethasone and prednisone to CYP106A2 was investigated by difference spectroscopy, using tandem quartz cuvettes as described before. (Jefcoate, 1978; Schenkman and Jansson, 1998). The titration of the CYP106A2 enzyme solution with the corresponding steroid substrate dissolved in DMSO was performed according to Schmitz et al. (Schmitz et al., 2014). The spectrum was recorded from 350 to 500 nm during each titration step, encompassing a concentration range of 0–500 μM for dexamethasone and 700 μM for prednisone. The equilibrium dissociation constant (K_d) was calculated by plotting the peak-to-through differences against the total substrate concentration. The mean values of three independent titration sets were fitted with OriginPro 9.0G software, using hyperbolic regression (OriginLab, Massachusetts, USA).

2.4. In vitro *turnover and catalytic activity*

The *in vitro* substrate turnover was performed with a reconstituted system, containing CYP106A2, bovine $Adx_{4\text{-}108}$ and AdR, in a molar ratio of 1:20:2. For the continuous electron supply, an NADPH regeneration system was used [glucose-6-phosphate-dehydrogenase (1 U), glucose-6-phosphate (5 mM) and $MgCl_2$ (1 mM)]. All reactions were carried out in 250 μL total volume at 30 °C in 50 mM potassium phosphate buffer (with 10% glycerol, pH 7.4).

The kinetic parameters were estimated with a substrate concentration range from 0 to 500 μM using 0.5 μM of P450, 10 μM of Adx and 1 μM of AdR. The reactions were started by adding 0.2 mM NADPH and quenched after 2 min for prednisone and after 10 min for dexamethasone by adding one reaction volume of ethyl acetate. Steroids were extracted twice with ethyl acetate. The organic phases were combined, evaporated to dryness and resuspended in acetonitrile/water (20:80) for high performance liquid chromatography (HPLC) analysis. The product amounts were determined from the conversion ratio using area under-peak of product and substrate from the HPLC chromatograms. The kinetic constants were calculated by plotting the product formation rates against the corresponding substrate concentrations and fitting the data using