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Inhibition and stimulation of activity of purified recombinant CYP11A1 by therapeutic agents

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ABSTRACT

In vertebrates, the biosynthesis of steroid hormones is initiated by cytochrome P450 CYP11A1 which converts cholesterol to pregnenolone. We investigated whether some of the experimental and FDA-approved therapeutic agents alter the activity of CYP11A1 in the reconstituted system *in vitro*. We found that under the experimental conditions used and when phospholipids are included, ketoconazole, posaconazole, carbenoxolone, and selegiline inhibit CYP11A1-mediated production of pregnenolone by at least 67%. Conversely, pemirolast, clobenpropit, desogestrel, dexmedetomidine, and tizanidine stimulate the enzyme activity by up to 70%. We then evaluated the identified inhibitors and activators for spectral binding to CYP11A1 and their effect on enzyme activity in the absence of phospholipids. The data obtained provide insight into how different drugs interact with CYP11A1 and demonstrate that P450 association with the lipid bilayer determines, in many cases, a drug's effect on enzyme activity.

1. Introduction

Cytochrome P450 11A1 (CYP11A1) or cholesterol side chain cleavage enzyme, is a mitochondrial monooxygenase catalyzing the conversion of cholesterol to pregnenolone, the precursor of all steroid hormones. CYP11A1 has been intensively studied during the last 40 years (reviewed in Guengerich (2005), Pikuleva (2006), and Miller et al. (2011)), yet little is currently known about the effect of marketed drugs on activity of CYP11A1, both *in vivo* and *in vitro*. Our knowledge in this area is mainly limited to earlier studies in the field showing that CYP11A1, as well as some other steroidogenic P450s, are inhibited by the antifungal agent ketoconazole and anticonvulsant aminoglutethimide (withdrawn from the US market in 1986), whose administration leads to adrenocortical dysfunction (Bossche, 1992; Harvey et al., 2003). Recently, we determined the crystal structure of CYP11A1 (Mast et al., 2011) and compared it to the structure of CYP46A1, another P450 which utilizes cholesterol as the endogenous substrate. CYP46A1, or cholesterol 24-hydroxylase, is a microsomal enzyme expressed predominantly in the brain whether it initiates the major pathway of cholesterol removal (Lutjohann et al., 1996; Russell et al., 2009). We found that in CYPs 11A1 and 46A1, enzymes that share <25% of amino acid sequence identity, the shape of the active site is similar, a long curved tube, as is the positioning of cholesterol (Mast et al., 2011). The major difference is that the active site in CYP11A1 is longer and more narrow, providing an explanation for the more strict substrate specificity of CYP11A1 as compared to that of CYP46A1 which may bind compounds structurally unrelated to cholesterol (Mast et al., 2003, 2008, 2010). Similar architecture of the active sites in CYP11A1 and CYP46A1 gave impetus to the present work in which we investigated whether the pharmaceuticals that modulate CYP46A1 activity *in vitro*, also affect the activity of purified recombinant CYP11A1. We identified several strong CYP11A1 inhibitors, and unexpectedly found that CYP11A1 activity could also be stimulated. The latter is a novel finding which enhances our understanding of CYP11A1 and opens new directions in studies of this enzyme as a target for therapeutic agents.

2. Materials and methods

2.1. Materials

Pharmaceuticals for screening were purchased from one of the following sources: Sigma–Aldrich Co (St. Louis, MO), Cayman Chemical Company (Ann Arbor, MI), Alfa Aesar (Ward Hill, MA), Waterstone Technology LLC (Carmel, IN), and Toronto Research Chemicals Inc. (North York, Ontario, Canada). Cholesterol and [^{3}H]cholesterol were from Steraloids Inc (Newport, RI) and American Radiolabeled Chemicals, Inc (St. Louis, MO), respectively. All

Abbreviations: Adx, adrenodoxin; Adr, adrenodoxin reductase; DLPC, dilauroylglycerol-3-phosphatidylcholine; HPCD, 2-hydroxypropyl-β-cyclodextrin; KP_i, potassium phosphate; PL, phospholipid.

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other chemicals were from Sigma–Aldrich unless otherwise specified. Bovine recombinant CYP11A1 was purified as previously described (Mast et al., 2011), except that 22R-hydroxycholesterol was omitted from all the buffers, and detergent (CHAPS) was removed after the last purification step by repeated dilutions and concentrations of the enzyme preparation with detergent-free buffer, 50 mM potassium phosphate (KP_i), pH 7.2, containing 1 mM EDTA and 20% glycerol. Bovine recombinant adrenodoxin reductase (Adr) and adrenodoxin (Adx) were purified as previously described (Sagara et al., 1992, 1993).

2.2. Enzyme assay

Incubations were carried out as previously described (Heo et al., 2012). The reaction volume was 1 ml and the buffer was 40 mM KP_i, pH 7.2, containing 1 mM EDTA. Liposomes (20 μg, prepared from dilauroylglycerol-3-phosphatidylcholine (DLPC)) were added to the buffer first followed by addition of cold cholesterol (1 μM), [^{3}H]cholesterol (250,000 cpm), proteins (0.1 μM CYP11A1, 1.6 μM Adx and 0.4 μM Adr), and a drug (10 μM). The components were reconstituted for 20 min at room temperature and then for 2 min at 30 °C followed by addition of 1 mM NADPH to initiate the enzymatic reaction. The enzymatic reaction was carried out for 2 min and terminated by the addition of CH_2Cl_2 (two 2 ml extractions). Organic layer was isolated, and evaporated, and the extract was dissolved in CH_3CN and analyzed by HPLC (Pikuleva et al., 1997). The utilized concentration of cholesterol (added from 1 mM stock in 4.5% aqueous 2-hydroxypropyl-β-cyclodextrin (HPCD)) was equal to 0.5 K_m of CYP11A1 for cholesterol. The concentration of test drug was equal to 5 K_m for cholesterol. These conditions were selected based on our previous studies of CYP46A1 showing that if, under such a cholesterol concentration and drug-to-substrate ratio, a test drug inhibits P450-mediated cholesterol hydroxylation by $\geqslant$50%, this is usually an indication that the drug in question has nanomolar to low micromolar affinity for the P450 (Mast et al., 2008). The drugs were dissolved in solvents in which they had maximal solubility; methanol, DMSO, water or HPCD (Supplementary Table 1).

2.3. Spectral binding assay

Binding affinities of different drugs for CYP11A1 were estimated as described previously (Pikuleva et al., 1995), except the buffer (40 mM KP_i, pH, 7.2, 1 mM EDTA) did not contain any detergent. The temperature was 18 °C. When titrations were carried out in the presence of phospholipids (PLs), liposomes were added to the buffer first, at a final concentration of 40 μg/ml, followed by the addition of 0.4 μM CYP11A1. To test drug binding to cholesterol-bound CYP11A1, the reagents were placed in a tube in the following order: buffer, liposomes (40 μg/ml), cholesterol (4 μM), and CYP11A1 (0.4 μM). Apparent binding constants (K_d) were calculated using either the $\Delta A = (\Delta A_{max}[L])/(K_d + [L])$ or $\Delta A = 0.5\Delta A_{max}(K_d + [E] + [L] - \sqrt{K_d + [E] + [L])^2 - 4[E][L]})$ equations, in which ΔA is the spectral response at different ligand (drug) concentrations [L], and ΔA_{max} is the maximal amplitude of the spectral response.

3. Results

3.1. Effect of CYP46A1 inhibitors on activity of CYP11A1

Only compounds inhibiting CYP46A1 activity by more than 45% in our previous studies (Mast et al., 2012) were tested in this CYP11A1 enzyme assay. More than half of these pharmaceuticals did not significantly affect CYP11A1 activity under the experimental conditions used (Fig. 1, Supplementary Table 1). Yet, ketoconazole, posaconazole, carbenoxolone and selegeline decreased pregnenolone production by >65%, whereas clobenpropit and dexmedetomidine increased CYP11A1-mediated cholesterol metabolism by up to 170%. Pharmacologic stimulation of CYP11A1 activity prompted us to test desogestrel, pemirolast and tizanidine. In studies of CYP46A1, desogestrel had no effect, and pemirolast and tizanidine had a slight stimulatory effect on enzyme activity. These compounds increased CYP11A1 activity by 161%, 125%, and 170%, respectively.

3.2. Spectral changes in CYP11A1 elicited by inhibitors and activators

The identified strong CYP11A1 inhibitors and all of the enzyme activators were then evaluated in the spectral binding assay. Of them, only 4 elicited significant spectral shifts in CYP11A1 (Figs. 2–4). These were two inhibitors (ketoconazole and posaconazole) and two activators (clobenpropit and dexmedetomidine). At saturating concentrations, ketoconazole and posaconazole shifted λ_{max} in the CYP11A1 absolute spectrum from 417 nm to 422 nm (Fig. 2A and C), the same wavelength as observed in previous studies with amine-containing steroids that bind to the CYP11A1 active site and serve as the enzyme inhibitors by coordinating the P450 heme iron with their nitrogen atom (Sheets et al., 1982, 1983). Clobenpropit and dexmedetomidine also red-shifted the λ_{max} of CYP11A1 (Figs. 3A and 4A), but the position of the Soret peak was at a shorter wavelength (420–421 nm). The difference spectra of CYP11A1 in the presence of ketoconazole, posaconazole, clobenpropit and dexmedetom idine were all similar and of type II (Remmer et al., 1966; Schenkman et al., 1967) with the troughs at 412 nm and the peaks at 433–434 nm (Figs. 2B and D and 3B and 4B). Equilibrium binding constants were determined from the difference spectra (Table 1). The spectral K_d values were 1.0 μM and 1.5 μM for the inhibitors, and 7.0 μM and 18 μM for the activators.

3.3. Effect of phospholipids on drug modulation of CYP11A1 activity

CYP11A1 is a membrane-bound enzyme residing in the inner membrane of the mitochondrion. To gain insight into whether association with the membrane contributes to pharmacologic modulation of CYP11A1 activity, a set of drugs investigated in Section 3.1 was tested in the *in vitro* enzyme assay again, except that PLs were omitted from the reaction mixture. Lack of PLs affected CYP11A1 activity in a drug-dependent manner (Fig. 5). The extent of inhibition remained the same, as in the presence of PLs, in the case ketoconoazole and was increased in the case of posaconazole, selegeline, ravuconazole, clotrimazole and hexaconazole. In contrast, inhibitory effect of carbenoxolone was abolished as is the stimulatory effect of pemirolast, clobenpropit, desogestrel, dexmedetomidine and tizanidine.

3.4. Effect of cholesterol and PLs on activator-induced spectral changes in CYP11A1

Since PLs seem to be essential for a stimulatory effect of drugs on CYP11A1, we carried out spectral titrations of this P450 under the conditions modeling those in the enzyme assay: 10:1 (M/M) ratio of cholesterol to CYP11A1 but a 4-fold increased substrate and enzyme concentrations to obtain good quality spectra. Consistent with previous studies (Takikawa et al., 1978), cholesterol-induced spectral shifts in CYP11A1 were of type I (Fig. 3A and C) occurring when compound in question binds to the P450 active site and displaces water molecule coordinating the heme iron in substrate-free enzyme. Subsequent titration of cholesterol-bound CYP11A1 with either pemirolast, clobenpropit, desogestrel, dexmedetomidine or tizanidine revealed that, as in experiments with substrate-free CYP11A1, only clobenpropit and dexmedetomidine elicited spectral changes in substrate-bound CYP11A (Figs. 3A and D and 4A and D). At subsaturating clobenpropit or dexmedetomidine concentrations (we