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A fission yeast-based test system for the determination of IC_{50} values of anti-prostate tumor drugs acting on CYP21

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Abstract
Human steroid 21-hydroxylase (CYP21) and steroid 17α-hydroxylase/17,20-lyase (CYP17) are two closely related cytochrome P450 enzymes involved in the steroidogenesis of glucocorticoids, mineralocorticoids, and sex hormones, respectively. Compounds that inhibit CYP17 activity are of pharmacological interest as they could be used for the treatment of prostate cancer. However, in many cases little is known about a possible co-inhibition of CYP21 activity by CYP17 inhibitors, which would greatly reduce their pharmacological value. We have previously shown that fission yeast strains expressing mammalian cytochrome P450 steroid hydroxylases are suitable systems for whole-cell conversion of steroids and may be used for biotechnological applications or for screening of inhibitors. In this study, we developed a very simple and fast method for the determination of enzyme inhibition using *Schizosaccharomyces pombe* strains that functionally express either human CYP17 or CYP21. Using this system we tested several compounds of different structural classes with known CYP17 inhibitory potency (i.e. Sa 40, YZ5ay, BW33, and ketoconazole) and determined IC_{50} values that were about one order of magnitude higher in comparison to data previously reported using human testes microsomes. One compound, YZ5ay, was found to be a moderate CYP21 inhibitor with an IC_{50} value of 15 μM, which is about eight-fold higher than the value determined for CYP17 inhibition (1.8 μM) in fission yeast. We conclude that, in principle, co-inhibition of CYP21 by CYP17 inhibitors cannot be ruled out.

Keywords: *CYP17, CYP21, fission yeast, prostate cancer, Schizosaccharomyces pombe*, inhibition, 17α-hydroxylase/17,20-lyase, steroid 21-hydroxylase

Introduction

Prostate cancer is the second leading cause of death from cancer and the most prevalent cancer amongst men in the western world. Since approximately 80% of human prostatic tumors are androgen dependent, inhibitors of enzymes involved in the key steps of androgen synthesis are of potential pharmacological importance. The cytochrome P450 dependent steroid 17α-hydroxylase/17,20-lyase (CYP17) is one of these key target enzymes [1,2] as it catalyzes the 17α-hydroxylation of pregnenolone (Preg) and progesterone (Prog) and the subsequent cleavage of the C20,21-acetyl group to yield dehydroepiandrosterone (DHEA) and androstenedione (AD), respectively [3]. As CYP17 is expressed in the adrenals and testes [4], its inhibition should decrease the production of both testicular and adrenal androgens. Ketoconazole, an antimycotic and unspecific inhibitor of several CYP enzymes that also inhibits CYP17, has been used clinically in the treatment of advanced prostate cancer [5–7]. Although this compound had shown anti-tumor activity, it was withdrawn from clinical use because of its short half-life and its non-selective side effects. Consequently, several research groups have aimed for new steroidal and non-steroidal compounds with CYP17 inhibitory potency [1,8–13]. However, little information has been available so far about a possible co-inhibition of steroid 21-hydroxylase (CYP21) by these compounds. Human CYP21 is a

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Table I. Parental strain and derived strains used in this work.

Strain	Genotype	Expressed P450	Reference
MB175	*h- ade6.M210 leu1.32 ura4.dl18 his3.Δ1*	none	[24]
CAD8	*h- ade6.M210 leu1.32 ura4.dl18 his3.Δ1 / pNMT1-hCYP17*	CYP17	this work
CAD18	*h- ade6.M210 leu1.32 ura4.dl18 his3.Δ1 / pNMT1-hCYP21*	CYP21	[23]

microsomal cytochrome P450 enzyme that is closely related to CYP17 and converts Prog to 11-deoxycorticosterone (DOC) and 17α-hydroxyprogesterone (17Prog) to 11-deoxycortisol (RSS). The activity of this enzyme is essential for the formation of gluco- and mineralocorticoids, and its impairment causes congenital adrenal hyperplasia (CAH) [14,15], the most frequent inherited disorder of steroid metabolism. In these patients, adrenocorticotropic hormone (ACTH) levels increase because of defective cortisol synthesis, which results in overproduction and accumulation of cortisol precursors, particularly 17Prog proximal to the block. This in turn causes excessive production of androgens and results in virilization [16]. Human CYP17 and CYP21 differ by only 14 amino acids in length, share 29% amino acid identity, and hydroxylate their steroidal substrates at two carbon atoms that lie a mere 0.26 nm apart. Moreover, the CYP17 and CYP21B genes have identical intron/exon organization [17,18], and are very closely related from an evolutionary point of view [19]. But while the only activities that have been demonstrated for CYP21 are the two 21-hydroxylation reactions mentioned above, CYP17 is not only a 17α-hydroxylase and a 17,20-lyase, but can also display 16α-hydroxylase and Δ^{16}-ene synthase activities [20]. Both enzymes share the common substrate Prog, and at least some compounds (e.g. the enantiomer of Prog [21]) competitively inhibit progesterone metabolism of both enzymes. Thus, it cannot *a priori* be ruled out that CYP17 inhibitors significantly inhibit CYP21, which would greatly reduce their pharmacological value. The aim of this study was to develop a rapid and convenient test system that identifies compounds with inhibitory potency towards CYP17 and CYP21. For this purpose we made use of recombinant fission yeast strains that strongly express either human CYP17 or CYP21, respectively, and display high steroid hydroxylation activity. Three CYP17 inhibitors that belong to different structural classes were tested with these strains and the resulting data were compared to the effect of ketoconazole.

Materials and methods

Chemicals

Radioactive [^{14}C]progesterone was obtained from NEN (Boston, MA), non-radioactive steroids and ketoconazole were from Sigma (Deisenhofen, Germany). The CYP17 inhibitors Sa 40 [12], YA5ay [11] and BW33 [22] have been described before.

Fission yeast strains and culture

Fission yeast strain CAD18 (all genotypes are listed in Table I) has been described previously [23]. Briefly, it is a derivative of parental strain MB175 [24] and contains the human P450 gene cloned into plasmid pNMT-TOPO® (Invitrogen; Carlsbad, CA) that allows strong expression under the control of the *nmt1* promoter [25]. Using pNMT-TOPO® the proteins of interest are expressed with two C-terminal tags, a hexahistidine tag and a Pk tag, respectively; the latter allows convenient immunological detection of the proteins [26]. Media and genetic methods for studying fission yeast have been described in detail [27,28]. Generally, strains were cultivated at 30°C in Edinburgh Minimal Medium (EMM) with supplements (final concentration 0.1 g·L^{-1}) of adenine, leucine, histidine, and uracil, respectively, as required. Thiamine was used at a concentration of 5 μM throughout. General DNA manipulation methods were performed using standard techniques [29].

Construction of a fission yeast strain expressing human CYP17

The cDNA of human CYP17 was PCR-amplified using *Pyrococcus furiosus* (Pfu) DNA polymerase (Promega; Madison, WI) and cloned into the fission yeast expression vector pNMT1-TOPO® (Invitrogen) to give pNMT1-hCYP17. Fission yeast strain MB175 was the transformed with this plasmid by the lithium acetate method [28] to yield strain CAD8 (all genotypes are listed in Table I). Transformed cells were plated on EMM with 0.1 g·L^{-1} adenine, histidine, uracil and 5 μM thiamine and incubated at 30°C. Transformants were checked for plasmid incorporation by colony PCR.

Protein detection

Early stationary phase cultures were used for denaturing protein extraction, where a total amount of approximately $2.5 \cdot 10^8$ cells were processed as previously described [23]. Protein preparation, SDS-PAGE and Western blot analysis were performed using standard techniques [29]. An α-Pk antibody (MCA1360, Serotec; Oxford, England) and a secondary peroxidase coupled α-rabbit antibody (DakoCytomation; Glostrup, Denmark) were used

for immunologic detection. Visualization was done using 2.0 mL of 2 mg·mL^{-1} chloronaphthol in 98% EtOH mixed with 25 mL PBS and 10 μl H_2O_2 (30%).

Medium scale steroid hydroxylation assays

For steroid bioconversion, cells were grown to early stationary phase in EMM with supplementes but without thiamine, centrifuged, washed and resuspended in 10 mL of the same media to a cell density of approximately 5·10^7 cells·mL^{-1}. The cell suspension was then transferred to a 250 mL Erlenmeyer flask, steroid substrate was added to a final concentration of 1.0 mM, and the culture was shaken for 72 h at 30°C and 300 rpm. Samples were taken at 0 h, 24 h, 48 h, and 72 h, respectively. Steroids were extracted with an equal volume of chloroform and analyzed on a HPLC device (Jasco; Tokyo, Japan) composed of an autosampler AS-950, pump PU-980, gradient mixer LG-980-02 and an UV-detector UV-975 equipped with a reversed phase Nova-Pak® C18 column (Waters; Milford, MA). The mobile phase was methanol:water (60:40) yielding retention times around 45 min for Prog, 20 min for 17Prog, 16.5 min for DOC and 10 min for RSS. Absorption was recorded at 240 nm and peak detection was done using the algorithm of the analysis software Borwin™ v1.50 (Jasco). Dilutions of respective pure steroids were used as references and as internal standard references as well as for calibrations. In the case of Preg conversion by CAD8, steroids had to be converted to the 4-ene-3-one species by cholesterol oxidase (Serva; Heidelberg, Germany) in order to be detectable at 240 nm. As the activity of cholesterol oxidase is significantly decreased at the low pH values [30] that are typical for fission yeast cultures, Preg conversion was only qualitatively described but not used for quantitative analysis of steroid hydroxylation activity or inhibitor assays.

Screening procedure and determination of IC_{50} values

Early stationary phase fission yeast cultures were used for miniaturized steroid hydroxylation assays. For each sample, 500 μL of cell suspension was transferred to 1.5 mL Eppendorf tubes and preincubated for 15 min at 30°C and 1400 rpm in a benchtop shaker with different inhibitor concentrations as indicated. After substrate addition, cells were incubated for further a 15 min. Inhibitor concentrations ranged from 100 nM to 20 μM while progesterone concentration was always 100 nM and included [^{14}C]progesterone for radiocative detection. The reaction was stopped by chloroform extraction of steroids using whole cultures. The organic phase was dried under vacuum, steroids were dissolved in 10 μL of chloroform and spotted on to glass-backed silica-coated HPTLC plates (Kieselgel 60 F_{254}, Merck; Darmstadt, Germany). In addition, small amounts of nonradioactive steroids were spotted as references. The HPTLC was developed twice in chloroform/methanol/water (300:20:1), and steroids were identified after exposure to Fuji imaging plates. Quantification was done using a phosphoimager (BAS-2500, Fuji; Stamford, CT) and the software TINA v2.10 g. The ratio R of the respective products was calculated as follows:

$$R_{17\mathrm{Prog}} = \frac{I_{17\mathrm{Prog}}}{I_{17\mathrm{Prog}} + I_{16\mathrm{Prog}} + I_{\mathrm{Bp}} + I_{\mathrm{Prog}}}, \quad (1)$$

$$R_{\mathrm{Doc}} = \frac{I_{\mathrm{DOC}}}{I_{\mathrm{DOC}} + I_{\mathrm{Prog}}}, \quad (2)$$

where I is the intensity of the respective steroid as measured by the phosphoimager and the subscript Bp denotes the byproduct (see below). Multiplication of the ratios by the initial concentration of substrate steroid gave the concentration of each steroid at every time point.

Results

Expression of human CYP17 in fission yeast strain CAD8

Fission yeast strain MB175 was transformed using pNMT1-hCYP17 as described above. After three days, the presence of the CYP17 cDNA in colonies grown on selective media was confirmed by colony PCR. The resulting strain was named CAD8. For immunologic detection of the human CYP17 protein, yeasts were grown in the absence of thiamine to induce the strong *nmt1* promoter. Protein lysates were prepared from CAD8 as well as from parental strain MB175 and examined by Western blot analysis using an α-Pk antibody. As expected, the presence of CYP17 could be detected in lysates from CAD8 but not in the parental strain (Figure 1A), and the antibody showed no cross-reaction with other fission yeast proteins.

Steroid bioconversion by strain CAD8

The functionality of the human CYP17 enzyme expressed in *S. pombe* was confirmed by steroid hydroxylation assays monitoring the conversion of Prog and Preg as described above. Both substrates were successfully converted to the respective 17α-hydroxylated products after 72 hours (Figures 1B to 1E). During the first 24 hours, 176 ± 13 μM 17Prog and 83 ± 9 μM 16Prog were produced (Figures 1F and 1G). The concentration-time course is pseudofirst-order for $t < 48$ hours. CYP17 is a microsomal enzyme that in mammalian cells receives electrons from NADPH via the NADPH-cytochrome P450 reductase (CPR), and our results demonstrate that the heterologously

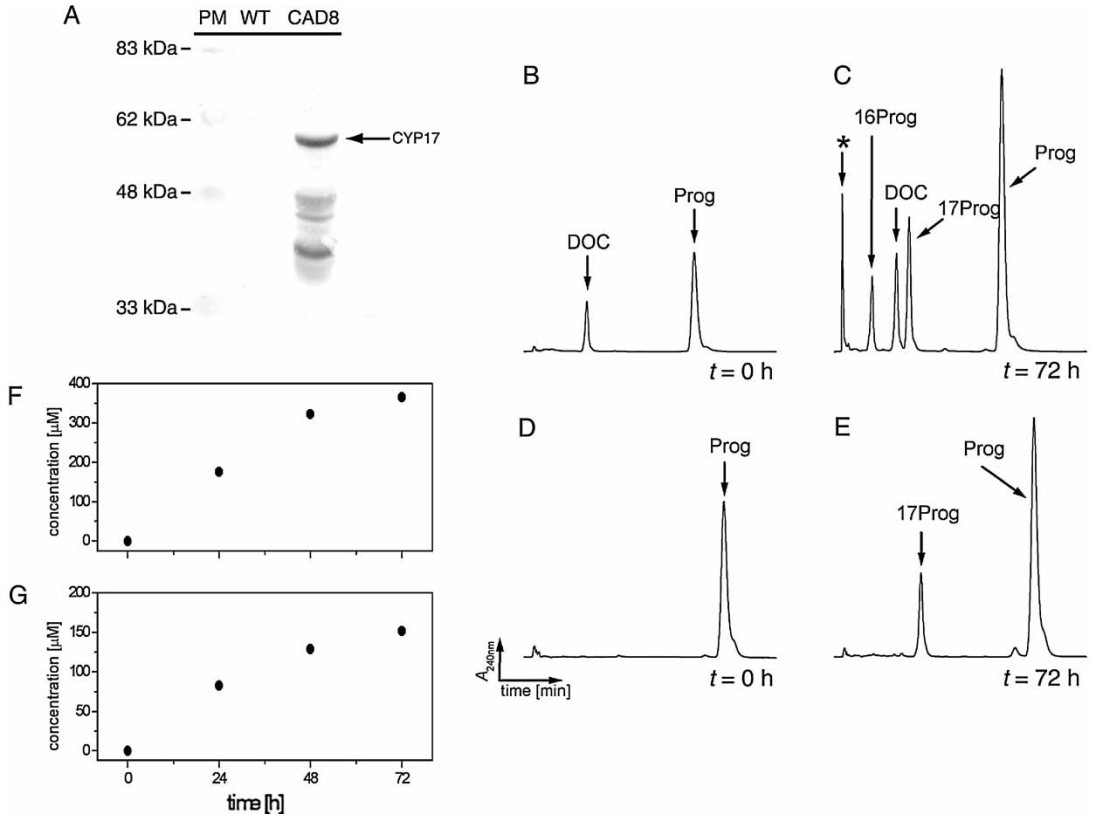

Figure 1. Fission yeast strain CAD8 expressing the human CYP17 can convert progesterone to 17α(hydroxyprogesterone. A: Immunologic detection of CYP17 heterologously expressed in strain CAD8. Protein extraction and detection were done as described above. The indicated band is in good agreement with the calculated mass of 59 kDa. PM: protein marker; WT: parental strain (MB175); CAD8: CYP17 expressing strain. **B, C, D and E:** HPLC chromatograms of steroid hydroxylation assays using strain CAD8 as described above. Prog: progesterone, 17Prog: 17α(hydroxyprogesterone), 16Prog: 16α(hydroxyprogesterone), DOC: 11(deoxycorticosterone (internal standard)), ⋆: an unidentified compound that was also detected in the absence of steroid substrates (data not shown). **B and C** Conversion of 1.0 mM progesterone by strain CAD8 for 72 h. **D and E** Conversion of 1.0 mM pregnenolone by strain CAD8 for 72 h. Prior to steroid extraction with chloroform, the cell suspension was incubated with cholesterol oxidase (see above) in order to convert the Δ_5−steroids into Δ_4−species. **F and G:** Concentration increase of 17α(hydroxyprogesterone) **(F)** and 16α(hydroxyprogesterone) **(G)** measured during the bioconversion assay with CAD8 incubated with 1.0 mM progesterone. All values were calculated from extraction loss corrected peak areas using calibration with pure steroids and represent mean ± standard error of mean from three independent experiments.

expressed human enzyme is also efficiently reduced by *ccr1*, the fission yeast CPR homologue [31]. During all experiments, formation of AD or of DHEA (resulting from AD after cholesterol oxidase treatment) was never detected, which indicates that human CYP17 expressed in fission yeast does not catalyze the steroid 17,20-lyase reaction under these conditions.

Establishment of a cellular inhibitor assay using fission yeast that expresses either CYP17 or CYP21 and validation of CYP17 inhibition

The functional expression of human CYP21 in *S. pombe* was previously shown by us [23], and the functionality of human CYP17 in this yeast is demonstrated in this study. As a first step towards the set-up of an inhibitor testing procedure, we monitored the conversion of progesterone (Prog) by CYP17 to 17α-hydroxyprogesterone (17Prog) and 16α-hydroxyprogesterone (16Prog), and the CYP21-dependent hydroxylation of Prog to 11-deoxycorticosterone (DOC), respectively, in miniaturized steroid hydroxylation assays as described above. Under these conditions that employ lower substrate concentrations (100 nM), strain CAD18 (expressing CYP21) converted Prog to DOC virtually to completion within 3 hours without the detectable formation of byproducts (Figure 2). Strain CAD8 (expressing CYP17) converted Prog to 17Prog, 16Prog and a byproduct that appears to be more polar than 16Prog and 17Prog (data not shown). No substrate conversion was observed when using the parental strain MB175 (data not shown). The apparent rate constant of substrate consumption could be determined by fitting the decay function,

$$c(t) = c_0 \cdot \exp(-k_{app} t) \qquad (3)$$

with $c(t)$ being the concentration as a time dependent function and k_{app} the apparent substrate consumption rate constant. Data analysis yielded $k_{app} = 2.7 \pm 0.1\,h^{-1}$ for strain CAD8 (correlation coefficient $r^2 = 0.996$) and $k_{app} = 2.5 \pm 0.1\,h^{-1}$ for CAD18 ($r^2 = 0.991$).

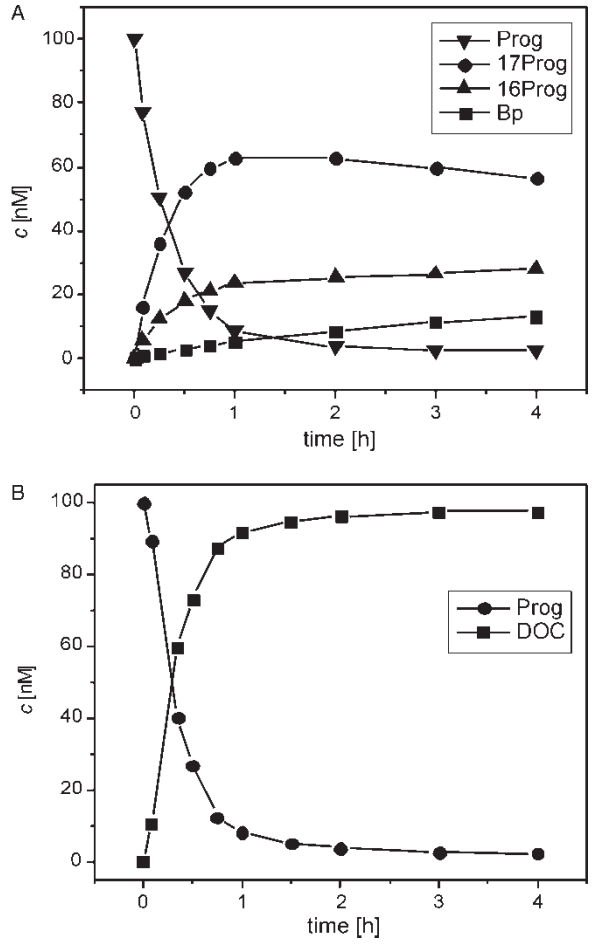

Figure 2. Time course of product formation of fission yeast strains expressing human CYP17 and CYP21. Miniaturized steroid conversion assays were carried out as described in "Materials and methods" with 100 nM progesterone. Samples were taken at the indicated time points, separated by HPTLC and analyzed using a PhosphoImager. Ratios were calculated using Equations (1) and (2) (for the byproducts of the CAD8 assay, Equation (1) was rearranged). Prog: progesterone; DOC: 11-deoxycorticosterone; 17Prog: 17α-hydroxyprogesterone; 16Prog: 16α-hydroxy progesterone; Bp: byproduct.

As before, no products of 17,20-lyase activity of CYP17 were detected. Due to these findings, the assay period could conveniently be set to 15 min, where both strains caused between 25 and 40% substrate conversion. Next, we used strain CAD8 to test for the inhibitory action of the broad range P450 inhibitor ketoconazole [32] and of the specific CYP17 inhibitors Sa 40 [12], YZ5ay [11], and BW33 [22] (all structures are shown in Figure 3). Since 17,20-lyase activity was not observed, inhibitory action refers only to the 17α-/16α-hydroxylase activity of CYP17. At a concentration of 100 nM, none of the tested compounds displayed a strong inhibition, while at 500 nM, Sa 40 acted as the most potent inhibitor; at 20 μM, all three specific inhibitors but not ketoconazole strongly reduced CYP17 activity (Table II). IC_{50} values calculated from these data are presented in Table III. These results indicate that fission yeasts expressing human CYP17 are suitable for rapid inhibitor screening, although higher inhibitor concentrations were required than in previous assays with human testes microsomes (see Discussion).

Determination of inhibitory action of CYP17 inhibitors on human CYP21

Next, fission yeast strain CAD18 was used for the determination of IC_{50} values as described in above. An representative experiment is shown in Figure 4, showing the inhibition of CYP21 activity by increasing concentrations of compound YZ5ay. Data calculated from six independent experiments for each compound are shown in Figure 5, and the percent inhibition values for the highest inhibitor concentration used (20 μM) are given in Table II. In general, all tested compounds inhibited CYP21 less efficiently than CYP17, with YZ5ay being the only compound to cause more than 50% inhibition at a concentration of 20 μM. Inhibition data points of YZ5ay for the four highest inhibitor concentrations were used to calculate an IC_{50} of 15 μM (Table III). Compounds Sa 40 and BW33 were not accessible to reliable IC_{50} determinations as no inhibition values of more than 50% could be measured. Additionally, we observed an unexpected activation of CAD18-mediated progesterone conversion by ketoconazole of 7% at 2 μM and 15% at 5 μM (Figure 5). This behavior reversed into inhibition at concentrations of more than 10 μM. To eliminate the possibility of a slower diffusion of ketoconazole into the cells and, therefore, of limited access of this compound to CYP21, the time dependence of the product formation ratio on the length of ketoconazole incubation was examined (Figure 6). Cells of strain CAD18 were pre-incubated with 5 μM ketoconazole for different time periods, while a 60 minute incubation with solvent alone served as control. Prog was then added to all samples, and the steroid conversion assay was performed for 15 minutes as above. This experiment surprisingly showed that CYP21 activity significantly increases with a longer pre-incubation with ketoconazole, which excludes the notion that delayed diffusion of ketoconazole into the cells could account for the weak inhibitory action of this compound in this test system.

Discussion

Heterologous expression of functional human CYP17 in fission yeast

Fission yeast cells strongly expressing human CYP17 did not show an altered microscopic phenotype, grew within one day to early stationary phase under induced conditions (i.e., in the absence of thiamine) and could be directly used for each of the described methods. Western blot analysis of the expressed CYP17 protein revealed a strong band in the expected size range and some additional bands (Figure 1A). Multiple bands in SDS/PAGE were also detected when either

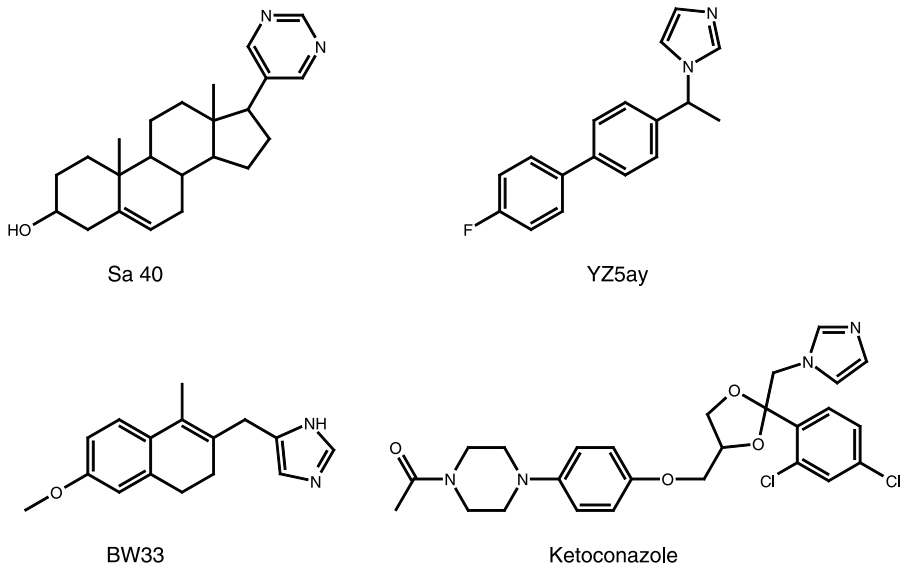

Figure 3. Structures of compounds used in this study.

Escherichia coli [33] or *Saccharomyces cerevisiae* [34,35] were used as a host for the expression of human CYP17. Even in the case of COS-1 (African green monkey kidney) cells expressing bovine CYP17, two bands of different intensity were detected [36]. In all studies including this one, the distance between the two highest protein bands indicates a mass difference of roughly 10 kDa, which is by far too large to be accounted for by the loss of the localization signal in the mature protein as compared to the preprotein. Therefore, we assume that overexpressed CYP17 is readily degraded by specific proteolysis, which seems to invariably occur in different hosts including mammalian cells.

The functionality of the human CYP17 enzyme expressed in fission yeast was demonstrated by *in vivo* conversion assays using the natural substrates Preg and Prog (Figure 1). We observed a distinct 17α- and 16α-hydroxylase activity of CAD8 towards Preg and Prog, but no detectable 17,20-lyase activity. This suggests that the human P450 can successfully couple to the fission yeast NADPH P450 oxidoreductase (CPR) *ccr1*.

It has been described that the 17,20-lyase activity of human CYP17 is not only dependent on electron delivery from CPR but can be augmented by the presence of cytochrome b_5, even when the latter is not involved in electron transfer itself [34]. It is intriguing that *S. pombe* lacks this function, although its own cytochrome b_5 shares 33% identity and 55% similarity with the human homologue. But *Saccharomyces cerevisiae* strains expressing bovine CYP17 also exhibited poor 17,20-lyase activity towards 17α-hydroxypregnenolone and nearly none towards 17Prog [37–39]; however, lyase activity was enhanced after co-expression of human cytochrome b_5 [34]. Furthermore, human CYP17 expressed in *E. coli* and reconstituted with rat CPR showed no detectable lyase activity for 17Prog [33]. Recently, a classification system for CYP17 enzymes from different species was suggested, in which the human and the bovine enzyme are part of the group B CYP17s, which have no or insignificant 17,20-lyase activities in relation to 17Prog [40].

As shown in Figure 1, we detected a byproduct in the 100 nM substrate conversion assays that appeared to be formed only after CYP17 expression (strain CAD8) and not in strains MB175 or CAD18. This strongly points towards a reaction that takes place downstream of the Prog → 17Prog/16Prog reaction, whereby the precursor of the byproduct (i.e., either 16Prog or 17Prog) remains to be identified. In order to answer this question by using the time course data obtained in this study, two potential mechanisms can be postulated with rate constants (k) indicated above and below the arrows. In Model A, the byproduct (Bp) is made from 17Prog in a reversible reaction:

$$\begin{array}{ccc} \mathrm{Prog} & \xrightarrow{k1} & 17\mathrm{Prog} \underset{k4}{\overset{k3}{\rightleftarrows}} \mathrm{Bp} \\ & \downarrow^{k2} & \\ & 16\mathrm{Prog} & \end{array} \qquad (4)$$

By contrast, Model B assumes that Bp is made from 16Prog in a reversible reaction:

$$\begin{array}{ccc} \mathrm{Prog} & \xrightarrow{k1} & 17\mathrm{Prog} \\ & \downarrow^{k2} & \\ & 16\mathrm{Prog} \underset{k4}{\overset{k3}{\rightleftarrows}} & \mathrm{Bp} \end{array} \qquad (5)$$

Table II. Percent inhibition of selected CYP17 inhibitors for the 17α-hydroxylase activity of heterologously expressed human CYP17 (CAD8) and the 21-hydroxylase activity of heterologously expressed human CYP21 (CAD18) in fission yeast at different inhibitor concentrations.

Compound	Percentage inhibition of CYP17 $c_{inh} = 100$ nM	Percentage inhibition of CYP17 $c_{inh} = 500$ nM	Percentage inhibition of CYP17 $c_{inh} = 20$ μM	Percentage inhibition of CYP21 $c_{inh} = 20$ μM
Sa 40	8.0 ± 2.0	57.0 ± 3.0	97.3 ± 0.1	10.0 ± 1.0
YZ5ay	8.0 ± 1.0	15.4 ± 0.4	95.3 ± 0.1	57.0 ± 7.0
BW33	7.0 ± 2.0	0	90.2 ± 0.2	9.0 ± 3.0
Ketoconazole	16.0 ± 2.0	21.9 ± 0.9	20.7 ± 2.6	6.0 ± 3.0

Values represent mean ± standard error of mean of three independent experiments for the human CYP17 while five different experiments were used for the human CYP21. The concentration of Prog was 100 nM throughout.

Unfortunately the solution set of the dynamic linear equation system is infinite for both models. However, for times $t \leq 0.5$ h we can assume a slow backward reaction of Bp due to relatively low byproduct concentrations bringing about an error of approximately 15% to the solution. Consequently, within the first 30 minutes we further assume the reactions of 17Prog and 16Prog to be described by $c(t) = c_0$ (1(exp((*k*$_i$*t*)), where k_i ($i = 1,2$) is the apparent substrate consumption rate constant for either 17Prog or 16Prog. Data fitting yielded $k_1 = 1.61 \pm 0.1\,h^{-1}$ and $k_2 = 0.43 \pm 0.04\,h^{-1}$. The rate constant for the byproduct reaction was found to be $k_3 = 0.07 \pm 0.01\,h^{-1}$. The differential equation system was solved numerically using MATLAB's ode45 solver (Natick, Massachusetts, USA) yielding the data presented in Figure 7. Comparison of the simulated versus the experimentally gained data strongly suggests that Bp is made from 17Prog. In case model B would hold, there should be a decrease in 16Prog over time and no significant built up of Bp. Furthermore, there should be no decrease in 17Prog. The decreasing slope of the concentration time course of Bp (Figure 2A) indicates that the back reaction has a rate greater than the Bp production, which was roughly estimated to be about 2 to 4 times higher than k_3 ($k_4 = 3k_3$ in simulation). Remarkably, at low substrate concentrations the steroid conversion rates for CYP17 and CYP21 are roughly equal although there are great differences at 1.0 mM progesterone, where, in agreement with the literature [23], the apparent production rate is ten times lower for DOC than for 17Prog. In bakers yeast expressing CYP17, an endogenous 20α-hydroxysteroid dehydrogenase (20α-HSD) was shown to convert 17Prog to 17α,20α-dihydroxypregn-4-ene-3-on [39], and *S. pombe* was also reported to exhibit 20α-HSD activity towards progesterone [41]; in this study, we observed a weak progesterone conversion by wild type fission yeast only at higher substrate concentrations (data not shown). Taken together, it can be assumed that the unidentified byproduct is 17α,20α-dihydroxypregn-4-ene-3-one.

IC_{50} determination with fission yeast expressing human microsomal P450 enzymes

In this study, we report the creation of a fission yeast based test system suited for the determination of IC_{50} values of inhibitory compounds acting on human CYP17 and CYP21 with an assay duration time of 15 min. The validity of this system was shown by testing three known CYP17 inhibitors with high potency (Sa 40 [12], YZ5ay [11], and BW33 [42]) and ketoconazole [32]. A comparison of IC_{50} values previously determined in assays using testes microsomal preparations of human CYP17 with data obtained in this study shows that higher inhibitor

Table III. Comparison of IC_{50} values of selected CYP17 inhibitors determined either in human testes microsomes or in fission yeast strains CAD8 or CAD18, respectively.

Compound	IC_{50} CYP17 in microsomes (μM)	IC_{50} CYP17 in fission yeast (μM)	IC_{50} CYP21 in fission yeast (μM)
Sa 40	0.024 [12]	0.8	n.d.
YZ5ay	0.24 [11]	1.8	15
BW33	0.11 [42]	2.8	n.d.

n.d.: Not determined.

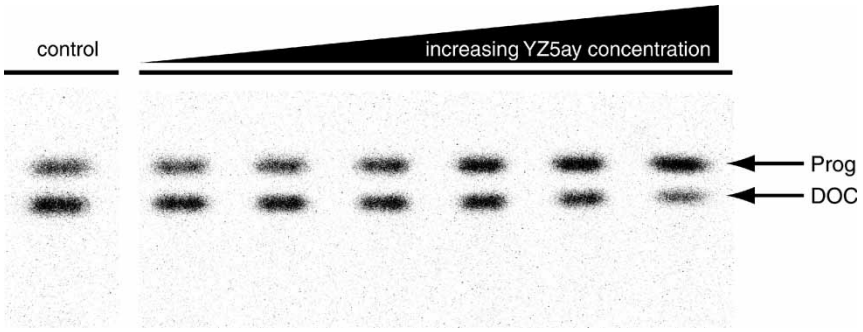

Figure 4. Autoradiographic detection of steroid hydroxylation activity. Cells of strain CAD18 were incubated with increasing concentrations of the CYP17 inhibitor YZ5ay as described in "Materials and methods". Steroids were extracted with chloroform, separated by HPTLC and analyzed using a PhosphoImager. CO: control reaction of strain CAD18 cells (solvent only). Subsequent six lanes contain CAD18 incubations with increasing concentrations of YZ5ay from the left to the right. Prog: progesterone (substrate); DOC: 11-deoxycorticosterone (product)

concentrations are needed in the fission yeast test system to reduce CYP17 activity (Table III). In human testes microsomes, the IC_{50} value of ketoconazole for the inhibition of CYP17 was found to be 740 nM [11], while this compound inhibited CYP21 only weakly [7,43]. This is also reflected by our fission yeast results, where ketoconazole was found to be an ineffective inhibitor of CYP17 or CYP21 even at a concentration of 20 μM (Table II). Of the specific CYP17 inhibitors tested here, only YZ5ay displayed significant inhibitory potency towards CYP21, while Sa 40 and BW33 showed a strong selectivity towards CYP17. Even in the case of YZ5ay, the selectivity of this compound is about eight-fold higher towards CYP17 than towards CYP21. Still, these findings corroborate our initial apprehension that CYP17 inhibitors may also co-inhibit CYP21 and stress the necessity to test drug candidates for this co-inhibitory effect.

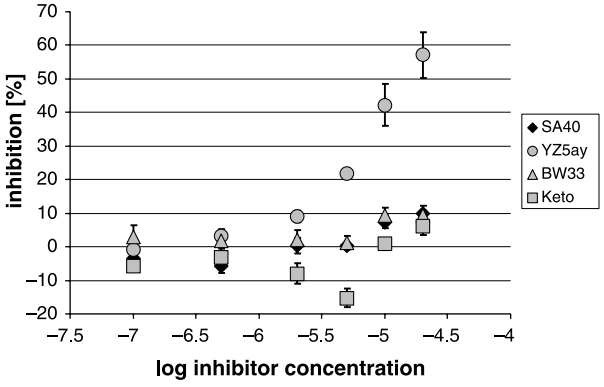

Figure 5. Log inhibitor concentration(CYP21 inhibition plot for all tested compounds. CAD18 cells were incubated with 100 nM progesterone and increasing concentrations of the different compounds as described in "Materials and methods". Ratios of DOC were normalized to the control reaction and plotted on a half logarithmic scale.

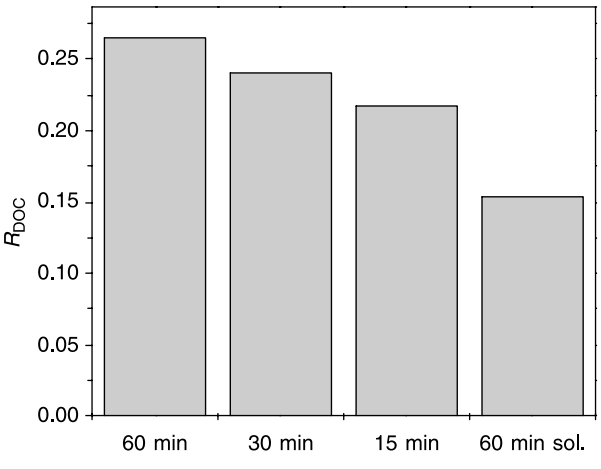

Figure 6. Activating effect of ketoconazole on human CYP21 expressed in fission yeast. Cells of strain CAD18 were incubated with 100 nM progesterone and 5.0 μM ketoconazole for different time periods as indicated. The ratio of DOC at the end of the incubation period was measured as described in "Materials and methods". 60 min sol.: Sample incubated for 60 minutes with solvent only (control).

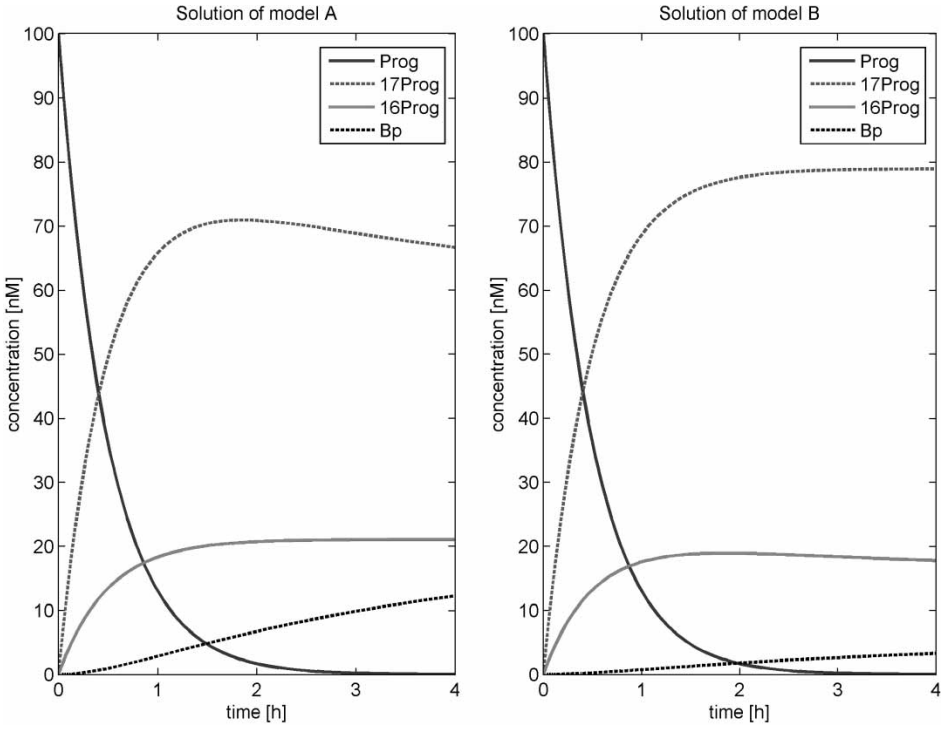

Figure 7. Simulation of the concentration time course in miniaturized progesterone bioconversion assays with fission yeast strain CAD8. The initial progesterone concentration was 100 nM; the rate constants k_1, k_2, k_3 and k_4 were set to 1.61, 0.43 0.07 and 0.21, respectively. The equation systems derived from models A and B (see discussion) were numerically solved using the MATLAB ode45 algorithm under default conditions.

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