PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 75 (2000) 57–63

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Development of a simple and rapid assay for the evaluation of inhibitors of human 17α-hydroxylase-$C_{17,20}$-lyase (P450cl7) by coexpression of P450cl7 with NADPH-cytochrome-P450-reductase in *Escherichia coli*

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Received 10 March 2000; accepted 17 August 2000

Abstract

P450c17 is a microsomal enzyme catalyzing the last step in androgen biosynthesis. As inhibitors of P450c17 are promising drug candidates for the treatment of prostate cancer, it was our goal to develop a new cellular assay for the in vitro evaluation of potential inhibitors. Human P450c17 was expressed in *E. coli* and hydroxylase activity was determined using 1,2[^{3}H]-progesterone. As the activity was low (1.7 pmol/min/mg protein), due to a lack of the requisite electron transfer partner NADPH-cytochrome-P450-reductase (NADPH-P450-reductase), coexpression of both the enzymes had to be performed. For that purpose, a plasmid was constructed which encoded human P450c17 and rat NADPH-P450-reductase in a transcriptional unit. This strategy led to a 100-fold increase in P450cl7 activity (175 pmol/min/mg protein). Time, pH and temperature dependence of progesterone conversion of this new monooxygenase system was determined. The K_M of progesterone was 2.75 μM. An assay procedure for the evaluation of inhibitors was established and modified for high throughput screening using 96-well plates. Selected compounds were tested for their inhibitory activity using this whole cell assay. The data was compared to the results obtained in microsomal testicular preparations.

Keywords: P450c17; NADPH–P450 reductase; Coexpression; Inhibitor screening; 17α-hydroxylase; Whole cell assay; E. coli

1. Introduction

A modern treatment option against the hormone dependent prostate cancer is the use of inhibitors of 17α-hydroxylase-$C_{17,20}$-lyase (P450cl7). This drug target is a key enzyme for biosynthesis of androgens. Highly specific, it catalyzes the 17α-hydroxylation of progesterone and pregnenolone and the subsequent cleavage of the $C_{20,21}$ acetyl group to yield the corresponding androgens [1,2]. The presence of the NADPH-cytochrome-P450-reductase (NADPH-P450-reductase), which is a requisite electron transfer partner, is a basic requirement for the function of the enzyme. Several categories of steroidal [3,4] and nonsteroidal [5–8] inhibitors have been developed in our laboratory. The inhibitory activities of these compounds have been tested using human testicular microsomes. The availability of human tissue, however, is limited and the prepared microsomes show a high variation of the enzymatic activity. By using a microsomal assay it is not possible to identify the compounds which cannot penetrate the cell membranes. There is need for a new assay which should consist of intact cells, being capable of recombinant expression of human P450cl7. Studies by Imai et al. [9] report on the expression of human P450c17 in *E. coli*. As bacteria do not contain the NADPH-P450-reductase, the enzymatic activity in these cells is very low [10,11]. Efforts by Dong and Porter resulted in an expression plasmid, which enables coexpression of microsomal P450 monooxygenases with rat NADPH-P450-reductase [12]. Using a different plasmid, Shet et al. [13] succeeded in coexpressing

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0960-0760/$ - see front matter
PII: S0960-0760(00)00137-0

bovine P450c17 and NADPH-P450-reductase in *E. coli*. As this bovine system is not appropriate for evaluation of inhibitors of the human enzyme, in the following the development of a simple and rapid cellular assay is described by coexpressing human P450c17 with rat NADPH-P450-reductase in *E. coli*. Using selected enzyme inhibitors its suitability for drug screening is investigated.

2. Materials and methods

2.1. Chemicals and enzymes

5-Aminolevulinic acid (ALA) was obtained from Boehringer Ingelheim (Heidelberg, Germany), ethacridine lactate from Hoechst (Frankfurt, Germany), IPTG (dioxane free) and riboflavin were obtained from Roth (Karlsruhe, Germany). Ketoconazole was obtained from Synopharm (Hamburg, Germany), mercury dichloride from Merck (Darmstadt, Germany), progesterone from Caelo (Hilden, Germany) and 1,2-[^{3}H]-progesterone (40–60 Ci/mmol) was obtained from NEN (Boston, MA, USA). Restriction enzymes *Xba*I, *Hind*III, *Kpn*I and T_4-DNA ligase were purchased from Hybaid (Heidelberg, Germany), *Acl*I from New England Biolabs (Schwalbach, Germany). Calf intestine alkaline phosphatase (CIAP) was obtained from TaKaRa (Otsu, Japan) and Taq DNA polymerase (recombinant from *E. coli*) was purchased from Sigma (St. Louis, MO, USA).

2.2. Bacterial strains and plasmids

E. coli XL1 was obtained from Stratagene (La Jolla, CA, USA) and *E. coli* Nova Blue Singles from Novagen (Madison, WI, USA). The plasmid pJL2E1/OR was a generous gift of Dr T.D. Porter (University of Kentucky, Lexington, KY, USA). The plasmid pCWH17mod(His)$_4$ was kindly provided by Dr M.R. Waterman (Vanderbilt University, Nashville, TN, USA).Vector pSTBluel was bought from Novagen (Madison, WI, USA).

2.3. PCR-primer, DNA amplification and preparation of the PCR products

Oligonucleotides PE001 (5′-GCT CTA GAC CAT GGC TCT GTT ATT AGC-3′) and PE002 (5′-GCT CTA GAG GTG CTA CCC TCA GCC TGG GC-3′) were synthesized by MWG-Biotech (Ebersberg, Germany). The primer pair PE00l/PE002 was used to amplify complete coding sequence of human P450c17 with pCWH17mod(His)$_4$ as template. Amplification conditions included 30 cycles of denaturation at 95°C for 90 s, annealing at 55°C for 90 s and extension at 72°C for 90 s. The PCR product was inserted into the EcoRV site of the vector pSTBlue1 and transformed into *E. coli* Nova Blue Singles using the Perfectly Blunt Cloning Kit (Novagen, Madison, WI, USA). The plasmids were isolated from transformants and partially digested with *Xba*I. The DNA was separated by agarose gel electrophoresis and a 1.5-kb fragment, encoding the entire human P450c17, was purified by the use of the QIAquick Gel Extraction Kit (Quiagen, Hilden, Germany).

2.4. Construction of P450c17/NADPH-P450-reductase coexpression plasmid pJL17/OR

By digesting the plasmid pJL2E1/OR (Fig. 1.C) using *Xba*I, a 5.1-kb DNA fragment was obtained that contained the vector backbone as well as the coding sequence of the rat NADPH-P450-reductase (Fig. 1.D). The 5′ends were dephosphorylated using calf intestine alkaline phosphatase. The 1.5-kb fragment (see above), encoding the human P450c17 (Fig. 1.B) was ligated into the *Xba*I site of the 5.1-kb plasmid. This resulted in the new construct pJL17/OR (Fig. 1.E) which was transformed into *E. coli* XL1. The plasmid DNA was isolated from transformants and the correct orientation of the 1.5-kb insert was checked by restriction digestion using *Acl*I, *Hind*III, and *Kpn*I.

2.5. Expression of pJL17/OR and pCWH17mod(His)$_4$ in E. coli

E. coli XL1 was transformed with pJL17/OR or pCWH17mod(His)$_4$, respectively, by the method of Inoue et al. [14]. Ampicillin-resistant colonies were streaked on a fresh LuriaBertani (LB) agar plate (containing 100 μg of ampicillin per ml). A single colony was selected and inoculated into 10 ml of LB (containing 100 μg of ampicillin per ml) for growth overnight at 37°C with vigorous shaking. 2 ml of the overnight culture was transferred into 200 ml of terrific broth (TB) [15] (containing 50 μg of ampicillin per ml). Bacteria were grown at 37°C with vigorous shaking up to an OD_{578} of 0.4–0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce enzyme expression. 5-Aminolevulinic acid (ALA) and riboflavin were added to a final concentration of 1 mM. The temperature was then reduced to 29°C and the shaking rate was decreased to 120 rpm. The time of expression was extended to 40 h.

2.6. Measurement of protein concentration

The amount of protein in cell extracts of recombinant bacteria was measured by the method of Honn and Chavin [16]. Cell extracts were obtained by sonication (sonicator Bandelin Sonopuls HD 60, Bandelin electronic; Berlin, Germany).

2.7. Storage of recombinant bacteria for testing

The bacteria was harvested by centrifugation at 5000 × g for 15 min at a temperature of 4°C. After washing with 0.1 M sodium phosphate, pH 7.4, the cells were resuspended resulting in an OD_{578} of 15.0 in 0.1 M sodium phosphate, pH 7.4 (containing 20% glycerol). For testing in 96-well plates, OD_{578} was adjusted to 5.0. The bacteria was then frozen in an ethanol/dry ice bath and stored at −70°C.

2.8. Test assay with intact bacterial cells coexpressing human P450c17 and rat NADPH-P450-reductase

To test the inhibitory effect of compounds on P450cl7, 10 μl of 1.25 mM progesterone (containing 600 nCi of [^{3}H]progesterone) and 10 μl of an appropriate dilution of the inhibitor (both dissolved in 96% ethanol) was transferred to 1.5 ml tubes and evaporated to dryness. 400 μl of 0.1 M sodium phosphate, pH 7.4 (containing 12.5 mM glucose) was added, mixed for 5 min and incubated at 37°C for 10 min. The test was

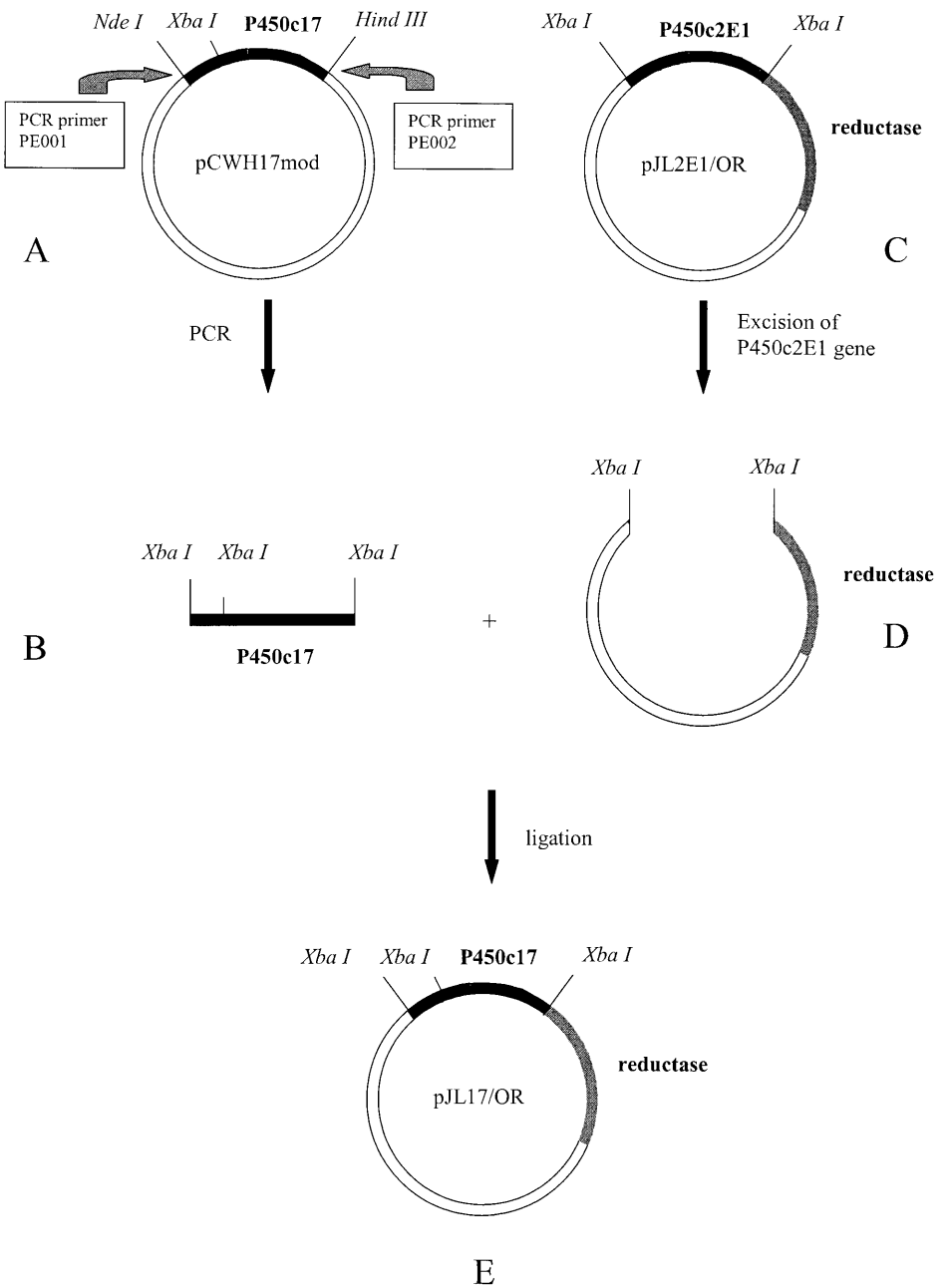


Fig. 1. Construction of the human P450c17 and rat NADPH-P450-reductase coexpression plasmid pJL17/OR.

started by the addition of 100 μl of thawed bacterial suspension (prepared as described in the previous paragraph). The tubes were horizontally shaken (200 rpm) at 37°C for 45 min. The reaction was stopped by heating the tubes at 95°C for 5 min. Steroids were extracted with 600 μl of cold ethylacetate for 5 min. After centrifugation at 3000 × g for 5 min at a temperature of 4°C, 400 μl of the supernatant were removed, evaporated to dryness, dissolved in 50 μl of methanol and analyzed by HPLC. As a control, the incubation was performed without inhibitor using the same conditions.

2.9. Test assay for inhibitors of P450c17 in 96-well plates

For screening of high numbers of compounds, a test assay was established using 96-well polypropylene plates with polystyrol mats (Corning Costar; Bodenheim, Germany).Then, 10 μl of 0.125 mM progesterone with 600 nCi of [^{3}H]progesterone (dissolved in 96% ethanol) was added to each well and evaporated to dryness. 15 μl of 0.13 M sodium phosphate pH 7.4 (containing 33.3 mM glucose) and 5 μl of an appropriate solution of the inhibitor (dissolved in DMSO) was added and incubated at 37°C for 10 min with shaking. The test was started by the addition of 30 μl of thawed bacterial suspension (prepared as described above). The plate was covered by a polystyrol mat and incubated at 37°C for 45 min with vigorous shaking (250 rpm). The reaction was stopped by the addition of 5 μl stop-mix (containing 5 mg ethacridine lactate and 6 mg of mercury dichloride per ml of water). The steroids were extracted with 95 μl of cold ethylacetate in the covered 96-well plate for 15 min. Further, after 15 min without shaking for separation of phases, 40 μl of the supernatant was removed, transferred into a 1.5 ml tube, evaporated to dryness, dissolved in 50 μl of methanol and analyzed by HPLC.

2.10. HPLC (and determination of IC_{50} values)

HPLC was performed by the use of the autosampler system 851 -AS (Jasco; Tokyo, Japan), high pressure solvent pump M 6000 A (Waters; Milford, USA) and radioactive flow detector LB 506C (Berthold; Wildbad, Germany). For analysis HLABE 1.6.5 software (Berthold; Wildbad, Germany) was used. Using the solvent methanol, water (1:1) with a flow rate of 0.4 ml/min, the steroids were separated on a Nukleosil RP 8 (125 × 2 × mm) column (Macherey–Nagel; Düren, Germany). Time of retention for 16α-hydroxyprogesterone (formed as a byproduct by P450c17 [17]) was 8 min, for 17α-hydroxyprogesterone 14 min and for progesterone 29 min.

3. Results

3.1. Hydroxylation of progesterone by E. coli cells, expressing P450cl7

For expression of human P450c17, *E. coli* XL1 was transformed with pCWH17mod(His)$_4$ [9] and grown as described under Materials and Methods. The enzyme was active showing a progesterone hydroxylation rate of approximately 1.7 pmol/min/mg protein. As described for COS cells expressing human P450cl7 [17], formation of 16α-hydroxyprogesterone was observed. The ratio of 17α and 16α-hydroxylation was approximately 4:1.

3.2. Cloning and construction of coexpression plasmid

The bacterial plasmid pCWHl7mod(His)$_4$ contains the sequence of human P450c17. In comparison with the native sequence, the cDNA shows modifications within the aminoterminal eight codons, which are favorable for expression in *E. coli*, as well as codons for four histidine residues at the carboxyl terminus. The PCR primers PE001 and PE002 were used to amplify the sequence (Fig. 1.A). Thereby the restriction sites were changed (Fig. 1.B) and the (His)$_4$ encoding region was removed. The coding region for P450c2E1 (Fig. 1.C) in the plasmid pJL2El/OR [12] was replaced by the modified sequence of P450cl7. The resulting 6.6-kb plasmid was named pJL17/OR (Fig. 1.E). It contains the human P450c17 and rat NADPH-P450-reductase cDNAs, which can be transcribed and translated sequentially from a single RNA.

3.3. Hydroxylation of progesterone by E. coli cells, coexpressing human P450c17 and rat NADPH-P450-reductase

For coexpression of human P450cl7 and NADPH-P450-reductase, *E. coli* was transformed with pJL17/OR, bacteria was grown and the expression was induced as described. Thus, a functional monooxygenase system was generated. Progesterone was hydroxylated at much higher rates than in the experiments using *E. coli* expressing P450c17 only. The ratio of 17α and 16α product was as described above. Using radiolabeled progesterone, the K_M value was determined to be 2.75 μM. Bacterial cells incubated with 25μM progesterone showed a rapid disappearance of this substrate (Fig. 2). The maximum activity was seen at a pH of 7.4 (Fig. 3) and a temperature of 37°C (Fig. 4). The specific hydroxylase activity was determined to be 175 pmol/min/mg protein. Bacterial suspensions (prepared as described above) could be stored at −70°C with no decrease in enzyme activity after 5 months.

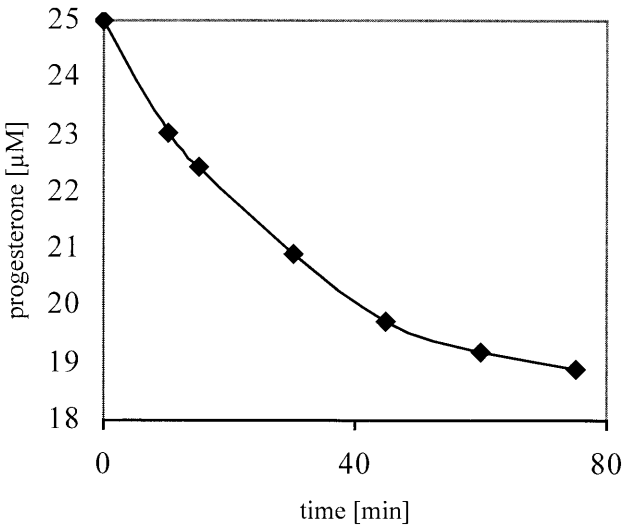


Fig. 2. Progesterone conversion by *E. coli* XL1 pJL17/OR. 500 µl of bacterial suspension ($OD_{578} = 3.0$) in 0.1 M sodium phosphate, pH 7.4, 10 mM glucose and 25 µM of progesterone was incubated at 37°C in 1.5 ml cups. The shaking rate of the horizontal positioned cups was 200 rpm. The reaction was stopped at indicated times by heating the cups at 95°C for 5 min. The loss of substrate was quantified by HPLC.

3.4. Evaluation of inhibitors of P450c17

To test the inhibitory effect of the compounds on P450cl7, cells were incubated with 25 µM of radiolabeled progesterone at 37°C, pH 7.4 for 45 min. As shown in Table 1, six compounds were tested. The steroidal compounds are strong inhibitors of the human enzyme expressed in bacterial cells, especially compound 9(Z), which shows about ten times higher effects than the reference drug ketoconazole. There also exists a correlation between the IC_{50} measured in the cell assay and the assay with human microsomes. Though

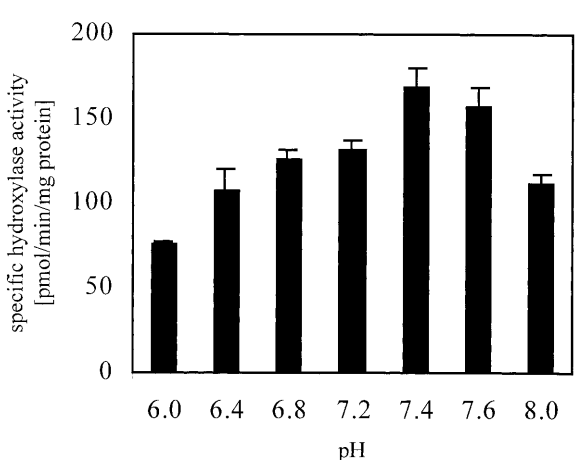


Fig. 3. pH dependence of specific P450cl7 activity in *E. coli* XL1 pJL17/OR. The incubation was performed as described in Fig. 2. over the indicated pH range. Incubation time was 45 min.

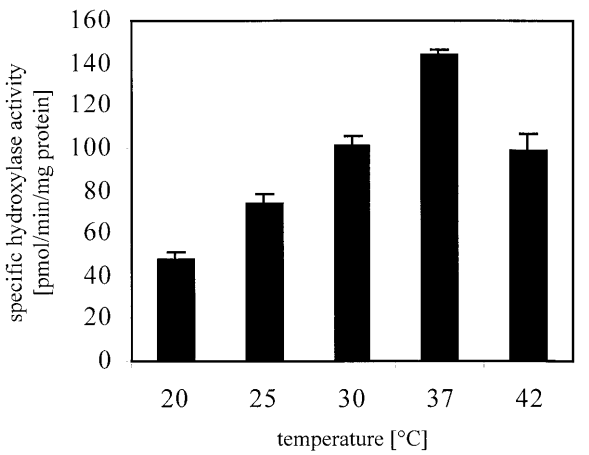


Fig. 4. Temperature dependence of specific P450c17 activity in *E. coli* XL1 pJL17/OR. The incubation was performed as described in Fig. 2. but at the indicated temperatures, incubation time was 45 min.

the IC_{50} values in the cell assay are higher, the order of decreasing potencies is similar in both assays. In the course of the evaluation of the possible drug candidates the compounds were tested on rats for their effect on reduction of the plasma testosterone concentration. Only compounds which also show a strong effect on the rat P450c17 in vitro are appropriate for the in vivo test. As can be seen from Table 1, great differences exist between the inhibitory effects of the compounds against the human and the rat enzyme. The most active compounds are 3 and 9(Z), while 10 and 11 are nearly inactive. Therefore only 3 and 9(Z) were tested in vivo. Compounds 3 and 9(Z) decrease the testosterone level after i.p. application of 0.019 mmol/kg body weight for 81 and 57%, respectively. Using this concentration, the reference compound ketoconazole had no significant effect.

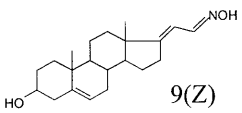
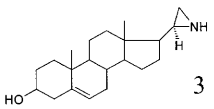
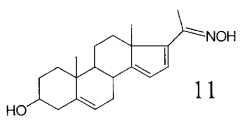
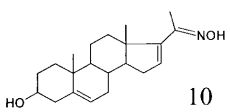
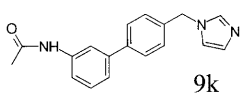
3.5. Modification of the assay for high throughput screening of potential inhibitors

The whole cell assay was modified for high throughput screening. Some parameters were changed and the reaction volume was reduced by 90% to make it applicable to 96-well plates. The IC_{50} values obtained for the compounds 9(Z), 3, 11, 10, 9k were identical to those shown above (Table 1).

4. Discussion

The present paper shows that the recombinant expression of human P450c17 in *E. coli* results in cells showing a poor enzyme activity, as described by Grigoryev et al. [18]. Therefore, this cellular system is not ideal as a screening assay for inhibitor evaluation. Isolation of the enzyme expressed in *E. coli* followed by

Table 1
Effect of P450cl7 inhibitors on different P450cl7 assays and on testosterone plasma concentration in rats

Compound [§]	IC_{50} [μM] human microsomes [a]	IC_{50}[μM] cellular assay [a]	IC_{50}[μM] rat microsomes [a]	in vivo effect (rat)
 9(Z)	0.077	0.229	0.52	-57 % [+]
 3	0.54	0.335	0.21	-81 % [+]
 11	0.2	0.415	> 100	ND *
 10	0.17	0.523	> 100	ND *
 9k	0.23	1.520	3.0	ND *
ketoconazole	0.74	2.780	67.0	+6% [+]

[§] 9(Z): 21-Hydroxyimino-pregna-5,17(20)-diene-3β-ol [4], 3: 20(S),21-Aziridinylpregn-5-en-3β-ol [3], 11: 20-Hydroxyimino-pregna-5,14,16-triene-3β-ol [4], 10: 20-hydroxyimino-pregna-5,16-diene-3β-ol [4], 9k: 3′Acetamido-4-(imidazol-1yl-methyl)-biphenyl [7]. [+] reduction of plasma testosterone two hours after i.p. application of 0.019 mmol/kg body weight. * not determined. [a] substrate concentration: 25 μM progesterone.

reconstitution using phospholipids, NADPH and NADPH-P450-reductase increases enzyme activity (data not shown), but is complicated and labor intensive. By coexpression of human P450c17 and rat NADPH-P450-reductase in *E. coli*, however, an elegant method was developed leading to an enhancement of enzyme activity by a factor of 100 compared to the sole expression of P450cl7. For this purpose the cDNA of human P450c17 was cloned into the backbone of plasmid pJL2E1/OR, which contained cDNA of rat NADPH-P450-reductase [12]. Using this monooxygenase system in *E. coli*, an assay was established for inhibitor evaluation. Compared to the standard procedure using microsomes from human testes, the enzyme activity is higher (175 pmol/min/mg protein and 9–18 pmol/min/mg protein, respectively) and above all it is constant. The fact that the assay can be performed in 96-well plates enables high numbers of potential inhibitors to be tested efficiently. The whole cell assay is closer to the in vivo situation compared to the microsomal procedure. Thus, using microsomes compound 3 is less active than 9(Z), in the whole cell assay it shows similar inhibitory activity and in rat it is superior to 9(Z). The increase in vivo activity can also be explained by the fact that 3 shows higher activity towards the rat P450c17 compared to 9(Z). As a consequence, compounds showing strong effects in microsomes but not in the whole cellular assay should not be tested in vivo (9k). In conclusion the assay described in this paper should be a valuable tool for inhibitor screening. In addition it might be used for mechanistic studies implying site directed mutagenesis. On the other hand, the procedure described here can be applied to other microsomal P450 enzymes.

Acknowledgements

We would like to thank Dr Todd D. Porter for providing the plasmid pJL2EI/OR and Dr Michael R. Waterman for the construct pCWHI7mod$(HiS)_4$. This investigation was supported by the Fonds der Chemischen Industrie.

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