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DETERMINATION OF 17α-HYDROXYLASE-$C_{17,20}$-LYASE ($P450_{17α}$) ENZYME ACTIVITIES AND THEIR INHIBITION BY SELECTED STEROIDAL PICOLYL AND PICOLINYLIDENE COMPOUNDS

NIKOLETTA SZABÓ,[1] JOVANA J. AJDUKOVIĆ,[2] EVGENIJA A. DJURENDIĆ,[2] MARIJA N. SAKAČ,[2] IMRE IGNÁTH,[1] JÁNOS GARDI,[1] GÁBOR MAHMOUD,[1] OLIVERA R. KLISURIĆ,[3] SUZANA JOVANOVIĆ-ŠANTA,[2] KATARINA M. PENOV GAŠI[2] and MIHÁLY SZÉCSI[1]*

[1]1st Department of Medicine, University of Szeged, Korányi fasor 8–10, H-6720 Szeged, Hungary
[2]Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, 3 Trg D. Obradovića, 21000 Novi Sad, Serbia
[3]Department of Physics, Faculty of Sciences, University of Novi Sad, 4 Trg D. Obradovića, 21000 Novi Sad, Serbia

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17α-hydroxylase-$C_{17,20}$-lyase ($P450_{17α}$) is a key regulator enzyme of the steroid hormone biosynthesis in both the adrenals and the testes. Inhibition of this enzyme can block androgen synthesis in an early step, and may thereby be useful in the treatment of several androgen-dependent diseases. We developed radiosubstrate *in vitro* incubation methods for the determination of the distinct 17α-hydroxylase and $C_{17,20}$-lyase activities of the enzyme using rat testicular homogenate as enzyme source. With this method we have studied the inhibiting activity of selected steroidal picolyl and picolinylidene compounds. Tests revealed a substantial inhibitory action of the 17-picolinyliden-androst-4-en-3-one compound.

Keywords: EC 1.14.99.9 / EC 4.1.2.30 – 17α-hydroxylase-$C_{17,20}$-lyase ($P450_{17α}$) inhibitors – *exo*-heterocyclic steroids – rat testis – abiraterone

INTRODUCTION

17α-hydroxylase-$C_{17,20}$-lyase ($P450_{17α}$) plays an important role in the pathways of steroid hormone biosynthesis [26, 28]. This is a cytochrome P450 enzyme located on the endoplasmic reticulum of the cells. It is expressed and active in the adrenal glands, testes, ovaries and placenta, primarily. The $P450_{17α}$ is capable of catalysing two reactions engaged on a single active site and is active both in the Δ^5 and Δ^4 pathways. The enzyme converts C_{21} precursors pregnenolone and progesterone (Prog) first to their 17α-hydroxy derivatives 17-hydroxypregnenolone and 17-hydroxy-progesterone (17OHProg). A subsequent side chain cleavage process forms C_{19} steroids, dehydroepiandrosterone (DHEA) and androstenedione (4-en-dione) (Fig. 1). Prog is a precursor of mineralocorticoids, whereas its hydroxylated derivative 17OHProg may result in glucocorticoids. C_{19} steroids are androgen prehormones: in steroido-

*Corresponding author; e-mail: szecsi.mihaly@med.u-szeged.hu

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genic tissues and target organs they can be further metabolized to more potent androgens such as testosterone and dihydrotestosterone. Inhibition of $P450_{17\alpha}$ can block androgen synthesis, and may be competent in pharmacological treatment of hormone dependent disorders [4, 16, 18]. This enzyme is also a potential target for a drug designed to treat androgen dependent prostatic carcinoma [12, 27]. Inhibitory action was found to be related to lone electron pair coordination to the heme iron atom at the active site of the enzyme and many steroidal compounds bearing different heteroaromatic moieties on ring D of the sterane skeleton were analyzed against $P450_{17\alpha}$. Pyridyl analogues, for example, exhibited highly effective inhibition of the enzyme, and abirateron [17-(3-pyridyl)androsta-5,16-dien-3β-ol] is recently introduced into medical application [2, 32, 36].

We report here radiosubstrate *in vitro* incubation methods for the specific determination of 17α-hydroxylase and $C_{17,20}$-lyase activities. Using these methods we have studied inhibitory action of selected steroidal 17-picolyl and 17-picolinylidene derivatives exerted towards the rat $P450_{17\alpha}$. Earlier publications describe synthesis methods and chemical properties of these compounds [7, 14, 25].

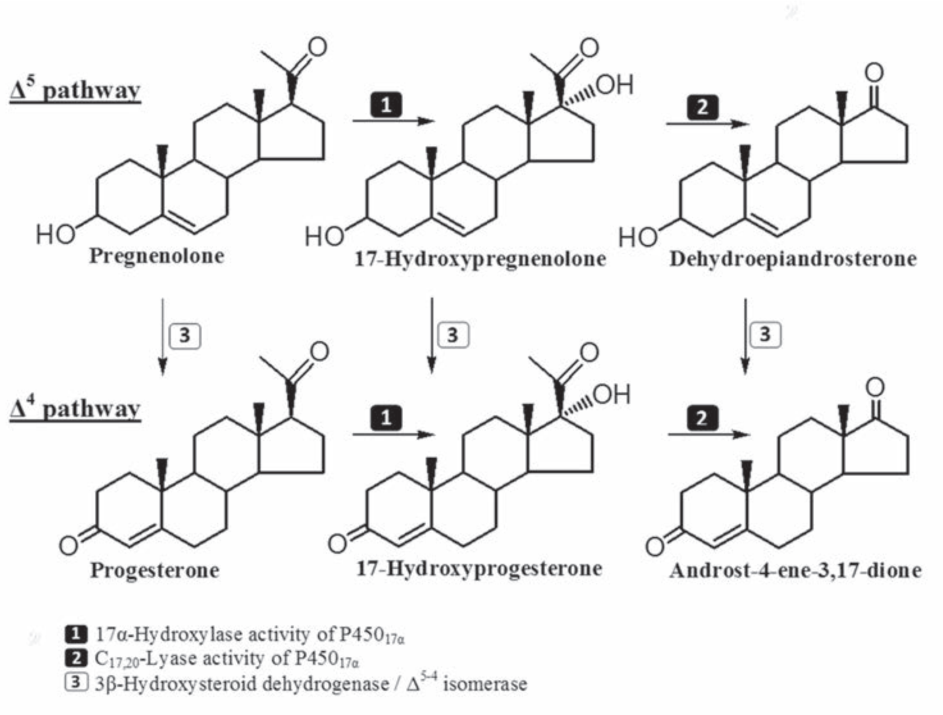

Fig. 1. Steroid biotransformations by 17α-hydroxylase-$C_{17,20}$-lyase ($P450_{17\alpha}$)

MATERIALS AND METHODS

Steroid standards and other chemicals

Radioactive [4-^{14}C]Prog with specific activity (S.A.) of 46 mCi/mmol and [1,2,6,7-^{3}H]17OH-Prog, S. A. = 50 Ci/mmol was purchased from the Radiochemical Centre, Amersham International (Amersham, UK) and American Radiolabeled Chemicals (St. Louis, MO, USA), respectively. Non-radioactive Prog and 17OH-Prog and 4-en-dione standards and reference inhibitor ketoconazole were obtained from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland). Abiraterone was prepared with the synthesis method of Potter et al. [33] and was provided by Professor Gyula Schneider and Professor János Wölfling (Department of Organic Chemistry, University of Szeged). Other chemicals and solvents with purity of analytical grade were purchased from Sigma (St. Louis, MO, USA).

Preparation of rat testicular homogenates

Testicular tissue was obtained from male rats (12-week old, 200–250 g body mass) via surgeries under ether anaesthesia. Tissue specimens were washed with an isotonic solution of NaCl. 1.0 g pieces of the testicles of five animals were mixed and the tissue sample was homogenized with an Ultra-Turrax in 20.0 ml 0.1 M HEPES buffer (pH = 7.3) containing 1 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged at 1500 *g* for 10 min at 4 °C. The supernatant was divided into portions for storage at –70 °C.

In vitro *incubations*

17α-hydroxylase: Testis homogenate aliquots containing suitable amount of enzyme (equivalent to 10 mg of testicular tissue) were incubated with 1 μM [^{14}C]Prog (25,000 dpm) in the presence of 1 mM NADPH in total volume of 200 μl HEPES buffer incubation medium (pH = 7.3). [^{14}C]Prog was added to the incubate in 20 μl 30 v/v% propylene glycol/HEPES buffer solution. Incubation was carried out at 37 °C for 20 min in air. The enzymatic reaction was stopped by the addition of ethyl acetate and freezing. After extraction, unlabeled carriers of Prog, 17OH-Prog and 4-en-dione were added to the samples to aid visualization of steroid spots on the chromatography plate by ultraviolet light. The three steroids were separated by thin layer chromatography (TLC) on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick) with the solvent system dichloromethane/diisopropyl ether/ethyl acetate (75:15:10 v/v). Ultraviolet light was used to trace the separated steroids. Prog, 17OH-Prog and 4-en-dione were found at retention factors 0.67, 0.24 and 0.48, respectively. Spots were cut out and the radioactivity of the 17OH-Prog and 4-en-

dione formed, and the Prog remaining was measured by liquid scintillation counting (Packard Tri-Carb 2200CA). The 17α-hydroxylase activity was calculated from the conversions corrected with recoveries of the 17OH-Prog and 4-en-dione formed and expressed in picomoles.

$C_{17,20}$-lyase: Testis homogenate aliquots containing suitable amount of the enzyme (equivalent to 30 mg of testicular tissue) were incubated with 1 μM [^{3}H]17OH-Prog (300,000 dpm) in the presence of 1 mM NADPH in HEPES buffer incubation medium for 20 min at 37 °C. The substrate, in this procedure, was prepared with mix of the tritiated tracer and the non-labelled 17OH-Prog, and was added to the incubate also in 20 μl 30 v/v% propylene glycol/HEPES buffer solution. Work-up procedures of the incubates and isolation of the 4-en-dione product and the substrate 17OH-Prog rest were performed as described above. The $C_{17,20}$-lyase activity was calculated from the conversion corrected with recovery of 4-ene-dione formed.

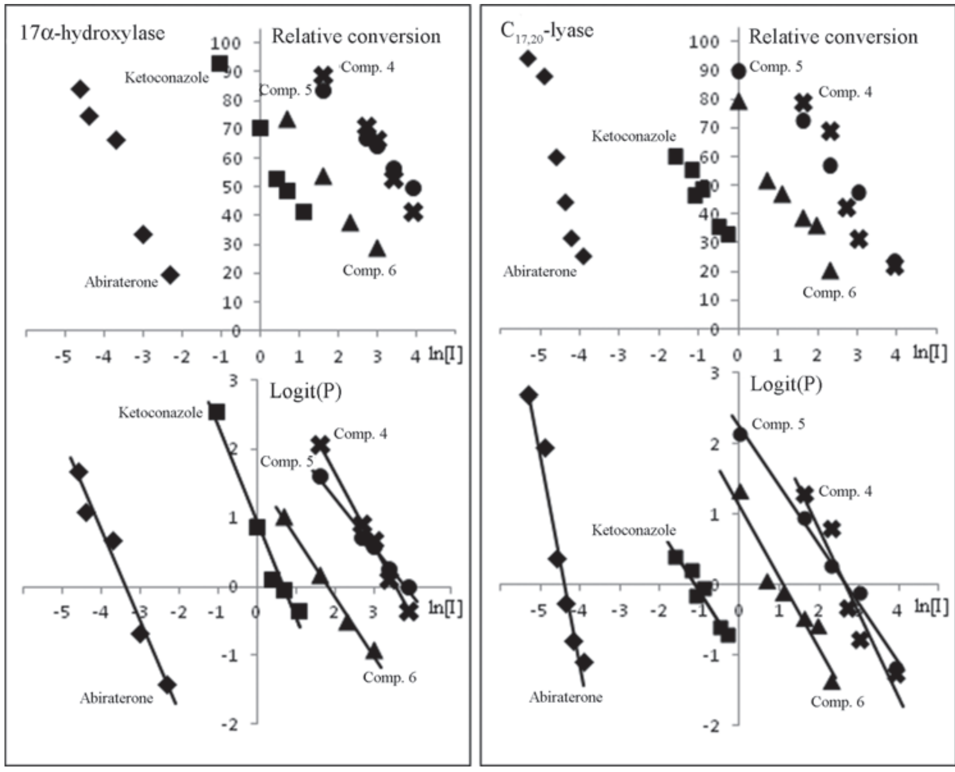

Fig. 2. Concentration dependent inhibition of 17α-hydroxylase and $C_{17,20}$-lyase activities of the $P450_{17\alpha}$ and logit-log transformation with linear regression analysis of inhibition data for the determination of IC_{50} values. P: enzymatic conversion in the presence of inhibitor, P_0: enzymatic conversion of the control incubation in the absence of inhibitor. Relative conversion = P/P_0, Logit(P) = $\ln[P/(P_0 - P)]$. IC_{50} values are derived from intercepts on the axis of ln (inhibitor concentration), as 50% inhibition results in logit parameter equal to zero

Inhibitor tests

The inhibitory effect of the new test compounds was investigated at 0.01–50 μM concentration interval. They were added to the incubate in dimethylsulfoxide solution in a maximal volume of 10 μl. Measurements were performed at least in four to six different concentrations and at least two experiments were done with each concentration. The control experiments were performed without the test substances in every incubation series. Abiraterone and a non-steroidal compound ketoconazole, known potent $P450_{17\alpha}$ inhibitors were used as reference compounds. The inhibitory effects of the compounds investigated in our present work are given in terms of IC_{50} values, i.e. the concentration of inhibitor at which the 17α-hydroxylase or $C_{17,20}$-lyase activity was decreased to 50%. IC_{50} results were calculated by linear regression analysis following a logit-log transformation of the data (Fig. 2), and the standard deviations were determined from the fitted lines. Compounds exerting weaker inhibitory effect were investigated at 50 μM concentration and relative conversions compared to control incubates were determined in these cases. Two experiments were performed and mean values and standard deviations were calculated.

RESULTS

Methodological investigations

The optimum conditions for the measurement of 17α-hydroxylase and $C_{17,20}$-lyase activities in rat testicular homogenate were determined in preliminary experiments. Enzyme activities were then measured under conditions of linearity with respect to enzyme concentration, and incubation time. Conversion of the substrate in non-inhibited control incubates was set to 10–15%. We achieved similar turnovers and optimal conversion rates applying higher enzyme concentration for the rate limiting $C_{17,20}$-lyase reaction than for 17α-hydroxylase activity, under the same incubation conditions. In 17α-hydroxylase method we have found that 15–25% of the 17OH-Prog product was consecutively converted to 4-en-dione. This rate was independent from incubation conditions (time, substrate or enzyme concentrations) as the cleavage process takes place without dissociation of 17OH-Prog from the active centre of $P450_{17\alpha}$. Dimethylsulfoxide and propylene glycol were present in the incubates with 5.0 and 3.0 v/v% concentrations, respectively. These organic solvent contents did not reduce the enzyme activities substantially. Procedural loss of the isolation steps was determined by regained radioactivity and the recovery was found around 70% for Prog, 60% for 17OH-Prog, and 80% for 4-en-dione. Coefficients of variation of enzyme activity results in repeated measurements were within ±10%. IC_{50} parameters of references ketoconazole and abiraterone were found 1.86 ± 0.25 μM and 0.034 ± 0.003 μM for 17α-hydroxylase, and 0.32 ± 0.02 μM and 0.0125 ± 0.0015 μM for $C_{17,20}$-lyase in our $P450_{17\alpha}$ inhibition test, respectively. These reference results are in good agreement with values found in the literature [4, 9, 15, 18, 23, 31–32, 34].

Table 1
In vitro inhibition of 17α-hydroxylase and $C_{17,20}$-lyase activities of the rat testicular $P450_{17\alpha}$ enzyme by picolyl (**1–3**) and picolinylidene (**4–6**) androstene compounds

Structural formula, name and number of compound	17α-Hydroxylase inhibition		$C_{17,20}$-Lyase inhibition	
	Relative conversion at 50 μM [%]	IC_{50} [μM]	Relative conversion at 50 μM [%]	IC_{50} [μM]
17α-picolyl-androst-5-en-3β,17β-diol (**1**)	95±0.6	–	NI	–
17α-picolyl-androst-4-en-3β,17β-diol (**2**)	90±4	–	66±13	–
17β-hydroxy-17α-picolyl-androst-4-en-3-on (**3**)	87±10	–	73±11	–

Table 1 (cont.)

Structural formula, name and number of compound	17α-Hydroxylase inhibition		$C_{17,20}$-Lyase inhibition	
	Relative conversion at 50 μM [%]	IC_{50} [μM]	Relative conversion at 50 μM [%]	IC_{50} [μM]
17(*E*)-picolinyliden-androst-5-en-3β-ol (**4**)	45±4	38±1.4	–	8.2±1.8
17(*E*)-picolinyliden-androst-4-en-3β-ol (**5**)	48±2	48±1.7	23±1	14±1.2
17(*E*)-picolinyliden-androst-4-en-3-on (**6**)	–	5.9±1.2	–	2.5±1.4

Results of IC_{50} ± S.D. in μM and relative conversion ± S.D. (n = 2) in %. Non-inhibited control taken as 100%. S.D.: standard deviation. NI: no inhibition

Inhibitiory effect of the new compounds

We investigated three picolyl **(1–3)** and three picolinylidene **(4–6)** androstene compounds (Table 1). Picolyl derivatives exerted no significant inhibition either on the 17α-hydroxylase or the $C_{17,20}$-lyase activities under our experimental conditions. Relative conversions were found between 66–100%. Elimination of the hydroxy group and introduction of a double bond in the side chain of the picolinylidene derivatives enhanced inhibitory actions. The two 3-hydroxy picolinylidene derivatives displayed moderate effect. IC_{50} values of the compounds **4** and **5** were found 38 μM and 48 μM against the 17α-hydroxylase, and 8.2 μM and 14 μM against the $C_{17,20}$-lyase, respectively. Results revealed that compound **6,** a picolinylidene derivative bearing 4-ene-3-oxo structure in its steroidal A-ring, is the best inhibitor among the tested compounds. For this compound values of IC_{50} = 5.9 μM for the 17-hydroxylase activity and IC_{50} = 2.5 μM towards the $C_{17,20}$-lyase activity were determined.

DISCUSSION

$P450_{17\alpha}$ lies at the crossroads of steroid hormone biosynthesis. Its inhibition reduces testosterone production and provides a feasible way for androgen withdrawal therapy. *In vitro* $P450_{17\alpha}$ inhibition test is an essential step in the development of new antiandrogen pharmacons and it is applied furthermore in toxicology and in screening of xenobiotics or endocrine disruptors [11, 21, 29, 40].

Various laboratory techniques have been developed in the latest decades for the investigation of 17α-hydroxylase and $C_{17,20}$-lyase activities of the $P450_{17\alpha}$. Biotransformations are more often monitored via conversion of ^3H or ^{14}C labelled substrate steroids. Following the enzyme reaction labelled product steroids are isolated with chromatography and then quantified by radiometric detection [3, 6, 10–11, 21–22, 29, 40]. Other versions of the radiosubstrate method use specifically labelled substrates. Hydroxylation of the [17α-^{3}H]Prog eliminates a tritiated water molecule [30, 39], whereas cleavage of labelled C-17 side chain of 17OH-Prog produce labelled acetic acid [19, 23, 24, 28, 30]. In these techniques, enzyme activities, can be detected via measurement of labelled water or acetic acid which remains in the incubate water phase following an organic extraction. Further incubation techniques apply non-labelled substrate steroids. Enzyme products in these methods can be either isolated by high performance liquid chromatography and detected by ultraviolet spectrophotometry [20, 34–35] or can be directly quantified by specific immunoassays [9]. Technologies based on quantification of radio-labelled steroidal enzyme products, nevertheless, are acknowledged superior to other methods in sense of both precision and qualitative identification.

Here we report an improved radiosubstrate method for specific determination of the 17-hydroxylase and $C_{17,20}$-lyase activities of $P450_{17\alpha}$. The newly developed technique bears numerous advantages compared to our earlier radiosubstrate methodologies [13, 37–38]. New procedures use small volume incubates, and apply simple and

efficient isolation techniques making these methods suitable for rapid and cost effective estimation of 17-hydroxylase and $C_{17,20}$-lyase activities and their inhibition by test substances.

In this study, steroidal 17-picolyl and 17-picolinylidene derivatives were investigated against 17-hydroxylase and $C_{17,20}$-lyase activities of $P450_{17\alpha}$. It has been found that the 17-picolinylidene-androstene compounds exerted stronger inhibition than their 17-picolyl counterparts. 17-Picolinylidene-androstenes bear trigonal C-17 centre, whereas this region displays tetrahedral geometry in the 17-picolyl compounds [8]. Compounds bearing heterocyclic side chain attached in trigonal geometry to the steroid C-17 are more potent inhibitors, in general, than those containing tetrahedral C-17 junction [5]. The rigid planar structure of our 17-picolinylidene groups, in particular, stabilizes the nitrogen in a position which is preferred for co-ordination to the heme of the enzyme [1]. Double bond at C17-C20 in the 17-picolinylidene compounds extends π-electron conjugation of the heterocyclic aromatic ring. This electron delocalization may also promote binding to the heme iron and enhance inhibitor affinity, as it is reported about several Δ^{16} unsaturated C-17 heterocyclic steroidal compounds [5, 17, 24]. The 17-picolinyliden-androst-4-en-3-on compound **(6)** exerted effective inhibition towards both the 17α-hydroxylase and $C_{17,20}$-lyase activities of the $P450_{17\alpha}$.

Our results demonstrate that the presented methods provide feasible ways for determination of activities and inhibitions of 17α -hydroxylase and $C_{17,20}$-lyase of the $P450_{17\alpha}$. Our findings indicate that steroidal 17-picolinylidene compounds may possibly be promising target molecules of $P450_{17\alpha}$ inhibitor research. Synthesis of further 17-picolinylidene steroids and investigations to explore presumed antiandrogenic properties of the new compounds are to be performed.

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