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DETERMINATION OF RAT 5α-REDUCTASE TYPE 1 ISOZYME ACTIVITY AND ITS INHIBITION BY NOVEL STEROIDAL OXAZOLINES

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The 5α-reductase type 1 isozyme is a key enzyme in the metabolism of the androgen steroid hormones and inhibitors of this enzyme represent a new pharmacological treatment for several androgen dependent diseases. We developed a radiosubstrate *in vitro* incubation method for the determination of 5α-reductase type 1 activity using rat liver microsomes as an enzyme source. With this method we have studied the inhibiting activity of novel (5'*S*)-17β-(4,5-dihydrooxazol-5-yl)androst-5-en-3-one compounds containing various derivatized phenyl substituents coupled to the *exo*-heterocyclic moiety. Tests revealed moderate inhibitory actions compared to finasteride, nevertheless, results provide interesting structure-activity relationship data.

Keywords: 5α-reductase inhibitors – *exo*-heterocyclic steroids – rat liver – finasteride

INTRODUCTION

The 5α-reduction of testosterone (Test) is a key process in the metabolism of the androgen steroid hormones. The 5α-dihydrotestosterone (DHT) formed in this reaction has the highest androgenic potency in many tissues and thus, it controls the function and development of androgen target organs. Nevertheless, 5α-reduction is also a conversion, which opens the way towards the elimination of steroids, making the A-ring of the steroid skeleton susceptible for further catabolic transformations.

Two enzymes with 5α-reductase (EC 1.3.99.5) activity have been identified so far. The isoforms show different tissue distribution and play different physiological roles, too [19, 25]. In human, the 5α-reductase type 1 isozyme is present mainly in the non-genital skin [6, 14], hair follicle [5], bone [8], brain [13] and liver [25] whereas the type 2 isozyme is active predominantly in the prostate, epididymis, seminal vesicle, genital skin, and in the liver [19, 21, 25].

Deficiency or inhibition of the 5α-reductase activity causes androgen withdrawal in the target tissue and in this way the growth, the development and also the function of the target organs might be influenced. The antiandrogenic effect of 5α-reductase inhibitors is modulated by the isozyme specificity of the action. Inhibition of the

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5α-reductase activity may be used in the therapies of androgen dependent diseases, i.e. the cancer or benign hyperplasia of the prostate [10, 27, 30], various skin pathologies, such as acne, androgenic alopecia and hirsutism in women [3, 7]. The 5α-reductase inhibitors are also potential components of a male contraceptive [18]. In the development of a male fetus, however, an accidental inhibition of the 5α-reductase activity disturbs sexual differentiation and causes inborn defects.

Here we report a radiosubstrate *in vitro* incubation method for the determination of rat 5α-reductase type 1 (r5αR1) activity. With this method, we have studied inhibitory action of novel (5'*S*)-17β-(4,5-dihydrooxazol-5-yl)androst-5-en-3-one derivatives exerted towards the r5αR1 activity. The new derivatives bear an oxazoline moiety in the 17β side-chain and phenyl group with various substituents is placed on this heterocycle. The chiral annelation carbon of the heterocycle displays an "*S*" configuration of the compounds (Fig. 1). Our earlier publication describes the synthesis methods and chemical properties of the compounds [17]. Briefly, the Lewis acid-catalyzed reactions of (20*S*)-3β-acetoxy-21-azidomethyl-20-hydroxypregn-5-ene with substituted aromatic aldehydes led to the formation of 3β-acetoxyandrost-5-enes substituted at position 17β with oxazolinyl residues and solvolysis followed by Oppenauer oxidation yielded the corresponding Δ^4-3-ketosteroids. Inhibition of the $C_{17,20}$-lyase inhibition of these compounds were also investigated and published in that paper.

MATERIALS AND METHODS

Steroid standards and other chemicals

Radioactive [4-^{14}C(n)]Test, with specific activity of 46 mCi/mmol was purchased from the Radiochemical Centre, Amersham. Non-radioactive Test and DHT standards were obtained from Sigma and Fluka. Other chemicals and solvents with purity of analytical grade were purchased from Sigma. Reference inhibitor finasteride, *N*-(1,1-dimethylethyl)-3-oxo-(5α,17β)-4-azaandrost-1-ene-17-carboxamide was kindly provided by Dr. Zoltán Tuba (Richter G. Plc., Hungary).

Preparation of rat liver microsomes

Liver tissue was obtained from female rats (12-week old, 200–250 g body mass) via surgeries under ether anesthesia. Tissue specimens were washed with an isotonic solution of NaCl. A 1.00 g pieces of the liver 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 dithiotreitol. The homogenate was centrifuged at 15,000 *g* for 30 min at 4 °C. The supernatant obtained was then centrifuged at 105,000 *g* in a Sorvall Ultra Pro 80 ultracentrifuge for 60 min at 4 °C. The microsomal pellet thus obtained was resuspended in 10.0 ml (2.0 ml/g liver tissue)

HEPES medium, treated briefly with an ultrasonic homogenizer to ensure full dispersion and divided into portions for storage at –70 °C. Protein content of the suspension was determined.

In vitro *incubation*

Microsome suspension aliquots containing microsomes of 0.23 mg liver tissue (6.0 μg protein) were incubated with 0.2 μM [4-^{14}C(n)]Test (20,000 dpm) in the presence of 1 mM NADPH in HEPES buffer incubation medium. [4-^{14}C(n)]Test was added to the incubate in 20 μl of dimethylsulfoxide solution, which led to a dimethylsulfoxide concentration of 2 v/v% in the 1.0 ml final volume of the incubate. Incubation was carried out at 37 °C for 20 min in air. The enzymatic reaction was stopped by the addition of diethyl ether and freezing. After extraction, unlabeled carriers of Test and DHT were added to the samples to aid visualization of steroid spots on the chromatography plate by ultraviolet light. The two steroids were separated by thin layer chromatography on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick) with the solvent system diisopropyl ether/CH_2Cl_2 (50 : 50 v/v). A Packard Radiochromatogram Scanner and ultraviolet light were used to trace the separated steroids. Test and DHT were found at retention factors 0.30 and 0.53, respectively. Spots were cut out and the radioactivity of the DHT formed and the Test remaining was measured by liquid scintillation counting (Packard Tri-Carb 2200CA). The r5αR1 activity was calculated from the conversion corrected with recovery and expressed in picomoles of DHT formed.

Inhibitor tests

The inhibitory effect of the new test compounds was investigated at 0.01–10 μM concentration interval. Measurements were performed at least in four 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. Finasteride, a known potent 5α-reductase inhibitor already introduced into medical practice was used as a reference compound. The inhibitory effects of the

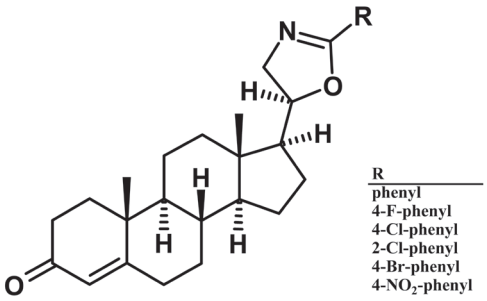

Fig. 1. General structural formula of the (5'*S*)-17β-(4,5-dihydrooxazol-5-yl)androst-5-en-3-one compounds investigated

compounds investigated in our present work are given in terms of IC_{50} values, i.e. the concentration of inhibitor at which the r5αR1 activity was decreased to 50%. The IC_{50} values were calculated by linear regression analysis following a logit-log transformation of the inhibition data (Fig. 2) and the standard deviations were determined from the fitted lines.

RESULTS

Methodological investigations

The optimum conditions for rat liver microsomal r5αR1 activity measurements were determined in preliminary experiments. Enzyme activity was then measured under conditions of linearity with respect to enzyme concentration (microsome amount), substrate concentration, and incubation time. Procedural loss of the isolation steps was determined by the regained total radioactivity and the recovery was usually found in the range of 85–92%. Coefficients of variation of enzyme activity results in repeated measurements were within ±10%. Control incubates without any inhibitor resulted in 20–25 pmol of DHT product, which is equivalent to a 10–12.5% conversion. IC_{50} parameter of finasteride was found 0.0080 μM in our r5αR1 inhibition test, and this result is in good agreement with the IC_{50} reported by Steers [20] (0.011 μM).

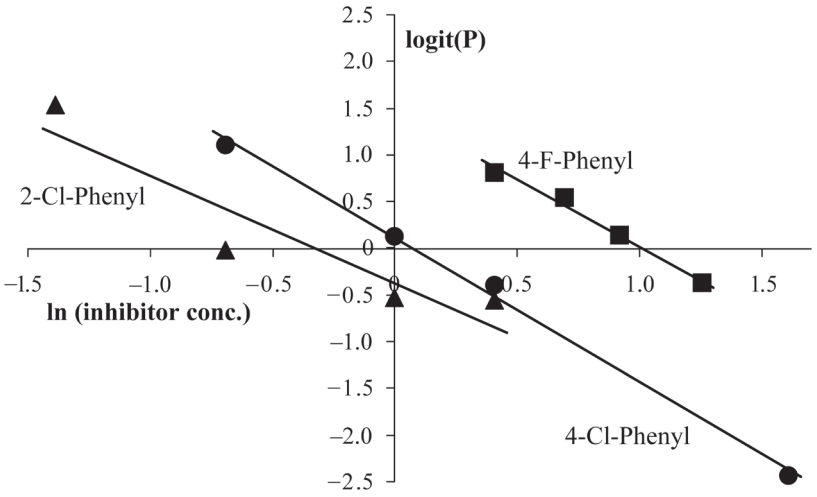

Fig. 2. Logit-log transformation and linear regression analysis of inhibition data for the determination of IC_{50} values. Examples of the 4-fluorophenyl, 4-chlorophenyl and 2-chlorophenyl derivatized (5'*S*)-17β-(4,5-dihydrooxazol-5-yl)androst-5-en-3-one compounds. $Logit(P) = \ln(P/P_0 - P)$. P: enzymatic conversion in the presence of inhibitor, P_0: enzymatic conversion of the control incubation in the absence of inhibitor. IC_{50} values are derived from intercepts on the axis of ln (inhibitor concentration), as 50% inhibition results in logit parameter equal to zero

Inhibition effect of the new compounds

The oxazoline compounds exerted moderate r5αR1 inhibition in our tests: IC_{50} parameters were found between 0.7–2.8 μM (Table 1). The 4-fluorophenyl compound was the weakest inhibitor (IC_{50} = 2.8 μM). The 2-chloro-substituted counterpart proved to be the most potent inhibitor, IC_{50} = 0.72 μM. r5αR1 inhibition of the further oxazolines showed little difference, IC_{50} results of these compounds varied between 1.1–1.5 μM.

Table 1
Inhibition of r5αR1 enzyme activity by the derivatized phenyl substituted (5'*S*)-17β-(4,5-dihydrooxazol-5-yl)androst-5-en-3-one compounds. IC_{50} results measured by *in vitro* incubations

Derivatized phenyl substituent R	r5αR1 inhibition IC_{50} ± S.D. (μM)
Phenyl	1.18 ± 0.15
4-F-Phenyl	2.75 ± 0.10
4-Cl-Phenyl	1.09 ± 0.05
2-Cl-Phenyl	0.72 ± 0.13
4-Br-Phenyl	1.05 ± 0.05
4-NO_2-Phenyl	1.52 ± 0.10
Reference: Finasteride	0.0080 ± 0.0008

S.D.: standard deviation.

DISCUSSION

The 5α-reductase type 1 enzyme plays a crucial role in the androgenic actions via the catalyzed Test–DHT conversion. Assessment of 5α-reductase type 1 inhibitory potential is therefore an inevitable test of new pharmacons or other novel synthetic compounds. Experiments using rat tissue preparations are widely acknowledged *in vitro* tests for the investigation of a presumed 5α-reductase inhibitory activity. The rat and human type 1 enzymes share 60% identity in amino acid sequence. They display similar steroid substrate preference, although, the two enzymes may show somewhat different sensitivity to certain inhibitors [1, 16]. In liver microsomes of the rat the type1 but not the type 2 isozyme of the 5α-reductase is expressed [15, 19]. In females, the lower level of endogenous testosterone is accompanied by a ten times higher r5αR1 activity as compared to males [4, 28, 29]. Liver microsome preparation made from liver of female rats therefore may be used as a feasible source of r5αR1 for *in vitro* experiments.

Enzymatic conversion may be detected by the photometry of the NADPH coenzyme consumed in stoichiometric amount during the 5α-reduction reaction [23]. Incubation with radiosubstrate followed by the isolation of the labeled product is

another way for measuring the enzyme activity [22]. This method yields a qualitative information on the enzyme product and on the direction of the enzymatic reaction.

The 5α-reduction itself involves the region of the A-ring of the steroid skeleton, however, the modulation of activity and isozyme selectivity towards the 5α-reductase type 1 isozyme can be performed by changing the substituent at the position 17 [16]. The D-ring region of the steroid ligand is supposed to interact with a flexible N-terminal binding domain (which is different in the type 1 and type 2 5α-reductase isozymes) [2, 24]. Several steroidal 5α-reductase type 1 inhibitors including the best known dutasteride, (5α, 17β)-*N*-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-azaandrost-1-ene-17-carboxamide bear a 17β side chain containing a carboxamide bond and an aromatic ring [9, 11, 12, 26]. Our new compounds own similar moieties; therefore it is reasonable to investigate their inhibitory effect towards the r5αR1.

We developed an *in vitro* radiosubstrate incubation method for r5αR1 inhibition tests using rat liver microsomal preparation as enzyme source. In this study, (5'*S*)-17β-(4,5-dihydrooxazol-5-yl)androst-5-en-3-one derivatives bearing different derivatized phenyl substituents adjacent to the heterocyclic ring were to be investigated.

Our investigations have found that the oxazoline compounds exerted moderate inhibitory effect towards the r5αR1. The reference pharmacon finasteride has a 100-times higher inhibitory potential than the best inhibitors among the new compounds. (It should be noted that the inhibitory action might be improved by several modifications in the A- or the B-ring of the steroid skeleton, but these modifications were not among the aim of the recent studies.) The 2-chlorophenyl substituent of the oxazolines significantly increases the inhibitory potential. The presence of fluorine in the position 4 of the phenyl ring decreases the r5αR1 inhibition. These effects, however, have relatively little extent. Our further test compounds bear phenyl substituents with considerably different structure and chemical nature, too, but changes in the r5αR1 inhibitory potential are even less pronounced. r5αR1 inhibition, therefore, seems to be independent in some degree from the chemical properties of the substituent placed on the phenyl ring adjacent to the 17β-oxazoline heterocycle.

Our results indicate that the phenyl substituted 17β-oxazoline or other related 17β-*exo*-heterocyclic steroidal compounds may possibly be promising target molecules of the 5α-reductase type 1 isozyme inhibitor research. Synthesis of further 17β-*exo*-heterocyclic steroids and investigations to explore presumed antiandrogenic properties of the new compounds are to be performed.

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