J. Steroid Biochem. Molec. Biol. Vol. 43, No. 7, pp. 721–724, 1992
Printed in Great Britain. All rights reserved

0960-0760/92 $5.00 + 0.00
Copyright © 1992 Pergamon Press Ltd

EPITESTOSTERONE—A POTENT COMPETITIVE INHIBITOR OF C_{21}-STEROID SIDE CHAIN CLEAVAGE IN THE TESTIS

MARIE BIČÍKOVÁ, R. HAMPL and L. STÁRKA*
Institute of Endocrinology, Národní 8, 116 94 Praha 1, Czechoslovakia

(Received 21 April 1992)

Summary—Epitestosterone (17α-hydroxy-4-androsten-3-one) inhibits both 17α-hydroxylation and consequent side chain cleavage of the resulting 17α-hydroxyprogesterone in the rat testicular microsomes. The inhibitory activity in terms of the K_i is 2 and 1.5 times as high, respectively, as that of cyproterone acetate.

INTRODUCTION

Antiandrogens such as cyproterone acetate or flutamide do not act only by interacting with the androgen receptors in the target tissues [1] but also at the early stages of androgen biosynthesis in the gonads by inhibiting some steroidogenic cytochrome *P*-450 dependent enzymes [2].

Recently, we have demonstrated that epitestosterone (17α-hydroxy-4-androsten-3-one), besides its ability to compete for an androgen receptor and to inhibit prostatic 5α-reductase [3], also reduced gonadotropin secretion *in vivo* [4]. As a steroid acting at both the peripheral and central level, epitestosterone might be a promising antiandrogenic agent with a lower risk of side effects than the commonly used antiandrogens [5]. Epitestosterone is a natural endogenous C_{19}-steroid. Its production rate measured in normal men averaged 0.2 mg/day [6]. These facts tempted us to investigate the effect of epitestosterone on 17α-hydroxylation and consequent $C_{17,20}$ side chain cleavage of testosterone precursors as key steps in androgen biosynthesis [7, 8]. Though recent reports brought evidence that both activities are caused by one enzyme, 17α-hydroxylase $C_{17,20}$ lyase, the degree of inhibition of the above reactions may be different [9–11].

The experiemnts were performed by using rat testicular microsomes, i.e. the tissue in which 4-ene C_{21} steroids are preferred to 5-ene steroids as substrates for androgen biosynthesis [9].

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]Progesterone, sp. act. 120 Ci (4.4 TBq)/mmol and [1,2,6,7-^{3}H]17α-hydroxyprogesterone, sp. act. 74 Ci (2.7 TBq/mmol were purchased from the Radioachemical Centre (Amersham, England). They were purified and their purity was checked by thin layer chromatography on Alufol F_{254} plates (Merck, Germany) in the systems cyclohexane–ethyl acetate (7:3, v/v) (progesterone) and dichloromethane–methanol (97:2, v/v) (17α-hydroxyprogesterone). Non-radioactive steroids, testosterone, progesterone and androstenedione were obtained from Koch + Light (England), 17α-hydroxyprogesterone, epitestosterone and cyproterone acetate were from Sigma (U.S.A.), NADPH from Calbiochem (Switzerland) and β-mercapto ethanol from Fluka (Switzerland). Other chemicals were of Czechoslovak origin, and of analytical grade.

Preparation of testicular microsomes

The method of Ayub and Levell [2] was used for the preparation of microsomal fractions of the rat testes. Decapsulated tissue was minced and then homogenized in a Potter homogenizer with 50 mmol/l phosphate, pH 7.4, containing 0.25 mol/l sucrose. The homogenate was centrifuged at 10,000 *g* and the supernatant was further centrifuged for 60 min at 105,000 *g* in refrigerated Spinco-Beckman ultracentrifuge Model L-8-55 with Ti 50 Rotor. The pellet (microsomal fraction) was re-suspended in the above buffer and stored frozen in aliquots at

−20°C until used. Protein content was determined by the Lowry method [12].

Enzymatic assay

Radioactive substrates (400,000 dpm/tube), admixed with unlabelled steroids to desired concentrations and inhibitors were dissolved in ethanol (50 μl) and buffer (0.5 ml of 50 mmol/l sodium phosphate, pH 7.4) and pre-incubated at 37°C for 5 min. The reaction was initiated by addition of a microsomal preparation (equivalent of 590 μg of protein) and the final volume of the incubation medium was adjusted to 1 ml with the buffer. The control samples for each concentration of the substrate, containing neither microsomal preparation nor inhibitors were processed in all experiments.

17α-Hydroxylase. [^{3}H]progesterone–progesterone mixtures in duplicate (62.5, 125, 250 and 500 nmol/l, respectively) were incubated with microsomal preparations in the presence of increasing concentrations of epitestosterone or cyproterone acetate (as a standard) (0, 50, 100, 200 and 400 μmol/l, respectively) in the system containing NADPH (60 mmol/l), at 37°C, under shaking, for 3 min.

$C_{17,20}$-lyase. [^{3}H]17α-hydroxyprogesterone diluted with non-radioactive substrate to concentrations of 50, 100, 200 and 400 nmol/l, respectively, was incubated with the microsomal preparation and NADPH (100 μmol/l) in the presence of the same concentrations of epitestosterone or cyproterone acetate as above, at 37°C, for 5 min. In each case the incubation was terminated by the addition of dichloromethane (2 ml). Following extraction and evaporation of the solvent the standards (progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone, 10 μg each) was added to the samples which were then chromatographed on a thin layer of silica gel using the systems cyclohexane–ethyl acetate (7:3, v/v) for 17α-hydroxylase and chloroform–acetone (185:15, v/v) for $C_{17,20}$-lyase, respectively, at 4°C. The standards were visualized under u.v. light (240 nm). The distribution of radioactivity corresponding to added standards was measured on the automatic TLC-linear analyzer (Tracemater Berthold, Germany), enabling us to calculate the percentage of radioactivity in the regions of interest.

The rate of enzymatic 17α-hydroxylation was expressed as a sum of 17α-hydroxyprogesterone, androstenedione and testosterone formed from the known concentration of substrate per 1 min. Similarly, the yields of $C_{17,20}$-lyase were calculated from the sum of androstenedione and testosterone formed from 17α-hydroxyprogesterone substrate.

Michaelis' constants at various concentrations of inhibitors (including zero) were derived from Lineweaver–Burk plots.

RESULTS

The effect of epitestosterone on two enzyme activities involved in the testosterone biosynthesis in the rat testis, namely on 17α-hydroxylase/$C_{17,20}$-lyase was compared with that of cyproterone acetate as a standard. Since the

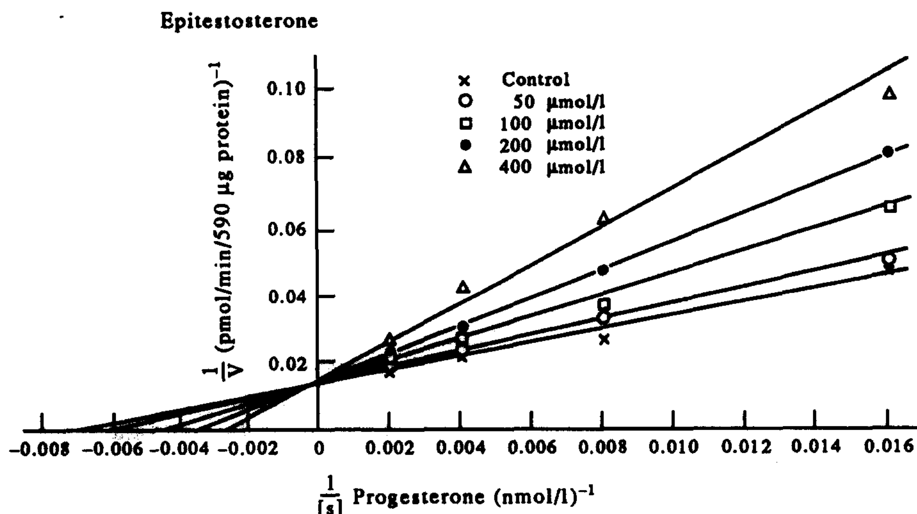

Fig. 1. Inhibition of 17α-hydroxylase by epitestosterone. Lineweaver–Burk plot of 17α-hydroxylation of progesterone at increasing concentrations of epitestosterone. Each point represents the mean of duplicate incubations from three independent experiments.

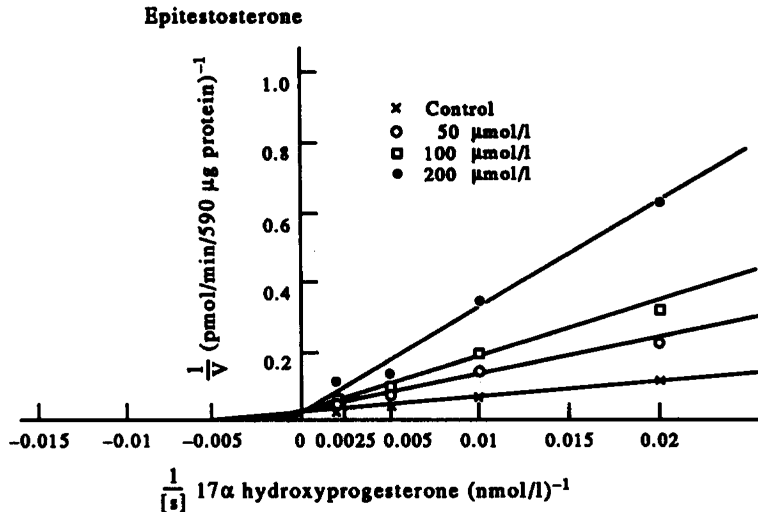

Fig. 2. Inhibition of $C_{17,20}$-lyase by epitestosterone. Lineweaver–Burk plot of $C_{17,20}$-side chain cleavage of 17α-hydroxyprogesterone at increasing concentrations of epitestosterone. Each point represents the mean of duplicate incubations from three independent experiments.

4-ene pathway is the main route leading to androgens in the rat, 4-en-3-oxo substrates were used in the experiments [9].

Lineweaver–Burk plots for rat microsomal 17α-hydroxylase and $C_{17,20}$-lyase activities in the presence or absence of increasing concentrations of epitestosterone are known in Figs 1 and 2, respectively. In Fig. 3 the K_m values thus obtained were plotted as a function of epitestosterone concentration and the respective inhibition constants were derived from the resulting straight lines. The inhibition constants for cyproterone acetate were obtained in the same way (the data not shown). The results are summarized in Table 1.

DISCUSSION

As demonstrated, epitestosterone acted as a strong competitive inhibitor of the side chain cleavage of 17α-hydroxyprogesterone—almost twice as strong—and a 1.5 times more effective inhibitor of progesterone 17α-hydroxylation than cyproterone acetate.

The antiandrogenic potential of cyproterone acetate is believed to be due to its complex effect on both the androgen biosynthesis and on the interaction with the androgen receptor. Our experiments with epitestosterone demonstrate that this endogenous steroid might be a good candidate for antiandrogenic

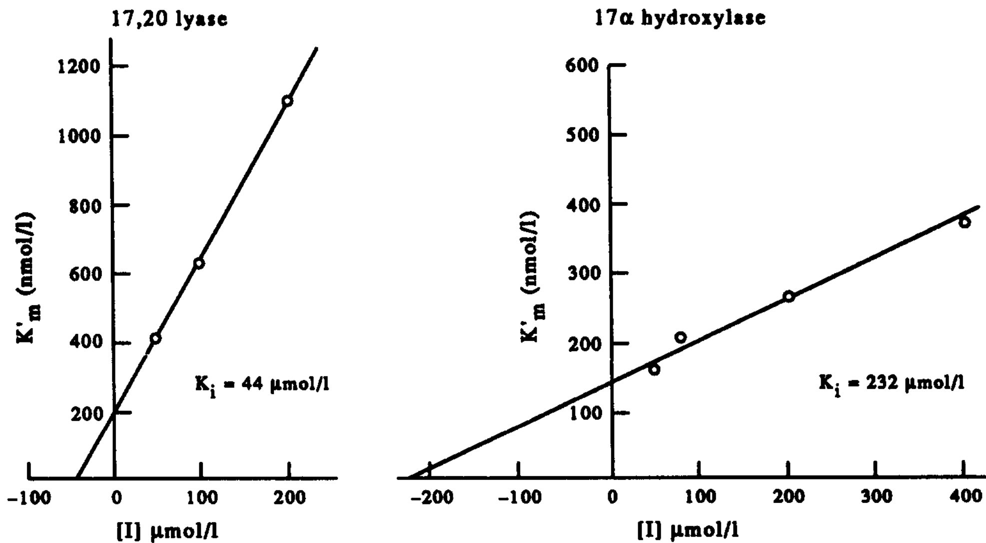

Fig. 3. The plot of the apparent K_m values (K'_m) versus the inhibitor concentrations (I) from which the inhibition constants (K_i) were read.

Table 1. K_i values of inhibition of rat testicular microsomal 17α-hydroxylase/$C_{17,20}$-lyase by antiandrogens

Enzyme activity	Substrate	K_m (nmol/l)	Antiandrogen K_i (μmol/l)	
			Epi-Te	Cy-Ac
17α-Hydroxylase	Progesterone	147 ± 1.3	232 ± 4.1	360 ± 5.4
$C_{17,20}$-lyase	17α-Hydroxyprogesterone	200 ± 0.3	44 ± 0.1	86 ± 3.0

The K_i for antiandrogens and for the substrates were determined from Lineweaver–Burk plots. Results are given as means ± SEM. Abbreviations: Epi-Te = epitestosterone; and Cy-Ac = cyproterone acetate.

treatment, which combines action directed to target tissues as a competitor for androgen receptor [3], to gonadotropin secretion [4] as well as to androgen biosynthesis and metabolism.

REFERENCES

1. Steinsapir J., Mora G. and Muldoon T. G.: Effect of steroidal and non-steroidal antiandrogens on the androgen binding properties of the rat ventral prostate androgen receptor. *Biochim. Biophys. Acta* **1094** (1991) 103–112.
2. Ayub M. and Levell M.: Inhibition of rat testicular 17α-hydroxylase and 17,20-lyase activities by anti-andorgens (Flutamide, Hydroxyflutamide, RU 23908, Cyproterone acetate) *in vitro*. *J. Steroid Biochem.* **28** (1987) 43–47.
3. Stárka L., Bičíková M. and Hampl R.: Epitestosterone—an endogenous antiandrogen? *J. Steroid Biochem.* **33** (1989) 1019–1021.
4. Stárka L., Bičíková M. and Hampl R.: Antiandrogenic action of epitestosterone. *Front. Horm. Res.* (Karger, Basel) **19** (1991) 109–118.
5. Raynaud J. P., Azadian-Boulanger G., Bonne C., Perronet J. and Sakiz E.: Present trends in antiandrogen research. In *Androgens and Antiandrogens* (Edited by L. Martini and M. Motta). Raven Press, New York (1977) pp. 281–293.
6. Wilson H. and Lipsett M. B.: Metabolism of epitestosterone in man. *J. Clin. Endocr. Metab.* **26** (1966) 902–914.
7. Vermoulen A.: Biosynthesis of androgens. In *Hormones in Blood* (Edited by G. H. Gray and V. H. T. James). Academic Press, New York, Vol. 3 (1979) pp. 356–359.
8. Nakajin S. and Hall P. F.: Side-chain cleavage of C_{21} steroids by testicular microsomal cytochrome *P*-450 (17α-hydroxylase/lyase): involvement of heme. *J. Steroid Biochem.* **19** (1983) 1345–1348.
9. Hall P. F.: Cytochrome *P*-450 C_{21scc}: one enzyme with two actions: hydroxylase and lyase. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 527–532.
10. Hall P. F.: Cytochrome *P*-450 and the regulation of steroid synthesis. *Steroids* **48** (1986) 133–196.
11. Payne A. H.: Hormonal reuglation of cytochrome *P*450 enzymes, cholesterol side-chain cleavage and 17α-hydroxylase/C_{17-20} lyase in Leydig cells. *Biol. Reprod.* **42** (1990) 399–404.
12. Lowry O. H., Rosebrough N. S., Farr A. L. and Randall R. S.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.