



Human Prostatic Steroid 5α -Reductase Isoforms—A Comparative Study of Selective Inhibitors

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The present study describes the independent expression of the type 1 and 2 isoforms of human 5α -reductase in the baculovirus-directed insect cell expression system and the selectivity of their inhibition. The catalytic properties and kinetic parameters of the recombinant isozymes were consistent with published data. The type 1 isoform displayed a neutral (range 6–8) pH optimum and the type 2 isoform an acidic (5–6) pH optimum. The type 2 isoform had higher affinity for testosterone than did the type 1 isoform ($K_m = 0.5$ and $2.9 \mu\text{M}$, respectively). Finasteride and turosteride were selective inhibitors of the type 2 isoform (K_i (type 2) = 7.3 and 21.7 nM compared to K_i (type 1) = 108 and 330 nM , respectively). 4-MA and the lipido-sterol extract of *Serenoa repens* (LSESr) markedly inhibited both isozymes (K_i (type 1) = 8.4 nM and $7.2 \mu\text{g/ml}$, respectively; K_i (type 2) = 7.4 nM and $4.9 \mu\text{g/ml}$, respectively). The three azasteroids were competitive inhibitors vs substrate, whereas LSESr displayed non-competitive inhibition of the type 1 isozyme and uncompetitive inhibition of the type 2 isozyme. These observations suggest that the lipid component of LSESr might be responsible for its inhibitory effect by modulating the membrane environment of 5α -reductase. Partially purified recombinant 5α -reductase type 1 activity was preserved by the presence of lipids indicating that lipids can exert either stimulatory or inhibitory effects on human 5α -reductase.

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INTRODUCTION

Human steroid 5α -reductase is a membrane-bound enzyme which catalyzes the NADPH-dependent reduction of testosterone (T) into dihydrotestosterone (DHT) in androgen target tissues and which plays a central role in several endocrine disorders such as benign prostatic hyperplasia (BPH) and prostate

cancer. Inhibiting 5α -reductase should cause a significant decrease in circulating and intratissular DHT. Over recent years therefore, considerable efforts have been directed towards the development of inhibitors of this enzyme in order to offer new treatment options. To date, several classes of both inhibitors and modulators have been described including steroidal and non-steroidal compounds as well as lipids and plant extracts.

In order to characterize these compounds interfering with 5α -reductase function fully, in particular in order to evaluate their true selectivity and potential therapeutic use, it is necessary to perform relevant preclinical *in vitro* studies. Two distinct 5α -reductase isozymes, designated type 1 and type 2, have been characterized in the human [1, 2]. They display maximal activity at different pHs (neutral-basic for type 1 and acidic for type 2), are encoded by two different genes [3–5], and display different sensitivity toward

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Abbreviations: $5\alpha\text{R1}$, human steroid type 1 5α -reductase (3-oxo- 5α -steroid: NADP⁺ 4-ene-oxido-reductase, EC 1.3.1.22); $5\alpha\text{R2}$, human steroid type 2 5α -reductase; T, testosterone (17 β -hydroxy-4-androsten-3-one); DHT, dihydrotestosterone (17 β -hydroxy- 5α -androstan-3-one); 4-MA, 17 β -(N,N-diethyl)-carbamoyl-4-methyl-4-aza- 5α -androstan-3-one; finasteride, 17 β -(N-t-butyl)-carbamoyl-4-aza- 5α -androstan-1-en-3-one or MK 906; turosteride, 1-(4-methyl-3-oxo-4-aza- 5α -androstan-17 β -carbonyl)-1,3-diisopropylurea or FCE 26073; LSESr, lipido-sterol extract of *Serenoa repens* fruit or Permixon[®]; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperda*.

inhibitors [6]. In liver and skin, the type 1 isozyme appears to predominate [7]. In the prostate, there is evidence for the expression of both isoforms [8, 9] but their ratio, individual physiological role and contribution toward DHT production in the gland remain unelucidated.

The difficulty in obtaining and manipulating human tissue coupled with the low activity levels of the two isozymes in the prostate have so far limited information concerning the mode of action of potent inhibitors. In the present study, we therefore decided to express both isozymes in a eukaryotic expression system in order to produce sufficient stocks of each to allow us to systematically compare the effects of various inhibitors. We chose the baculovirus-directed insect cell expression system rather than a bacterial, yeast or mammalian system because it produces an abundance of recombinant proteins which are in many cases antigenically, immunogenically, and functionally similar to their authentic counterparts [10]. In addition, unlike mammalian systems, baculoviruses are not pathogenic to vertebrates or plants and do not employ transformed cells or transforming elements.

Since we have already described the expression of human steroid type 1 5α -reductase in the baculovirus-directed insect cell expression system [11, 12], we shall focus in the present study on the expression of the type 2 human isozyme. We shall then compare the abilities of three azasteroids (4-MA [13], finasteride [14], and turosteride [15, 16]) and of the lipido-sterol extract of *Serenoa repens* (LSESr) [17, 18] to inhibit each of these independently expressed isozymes.

MATERIALS AND METHODS

Chemicals

[1,2,6,7- 3 H]T (90–101 Ci/mmol) and [α - 32 P]dCTP (3000 Ci/mmol) were purchased from Amersham (U.K.). Unlabelled steroids were purchased from Sigma (France). The 5α -reductase inhibitors, 4-MA, finasteride (MK906) and turosteride (FCE 26073), were synthesized at the Research Laboratories of Farmitalia Carlo Erba (Milan, Italy). LSESr was supplied by Pierre Fabre Médicament (Castres, France). NADPH was from Boehringer Mannheim (Germany). Restriction enzymes and other DNA modifying enzymes were from New England Biolabs (U.K.) and Boehringer Mannheim (Germany).

Transfected cells

Spodoptera frugiperda (Sf9) (ATCC, CRL1711) cells were maintained at 27°C in Grace's insect medium supplemented with 10% fetal calf serum, 3.3 g/l yeastolate, 3.3 g/l lactalbumin hydrolysate (all from Gibco/BRL), 50 µg/ml gentamycin sulfate and 2.5 µg/ml amphotericin B (Sigma, France). The cells were transfected with *Autographa californica* nuclear

polyhedrosis virus (AcNPV) DNA (Invitrogen Co., CA, U.S.A.) to obtain wild-type viral stocks.

Production and isolation of recombinant baculoviruses

This step has been previously detailed for 5α R1 [11]. The cDNA corresponding to the coding region of human 5α R2 mRNA was inserted into the pVL1392 baculovirus transfer vector (Invitrogen Co., CA, U.S.A.) to yield pVL1392- 5α R2 plasmid. This plasmid was kindly provided by Professor Van Luu-Thé (Quebec, Canada). Transfer of the h 5α R2 cDNA next to the promoter of the polyhedrin gene in the AcNPV genome was accomplished by calcium phosphate cotransfection of pVL1392- 5α R2 and wild-type AcNPV DNA into Sf9 insect cells as described by Summers and Smith [19]. Supernatants collected 6 days post-transfection were screened for recombinant baculovirus by dot-blot hybridization with a 365 bp fragment of the 5α R2 cDNA and enriched by limiting dilution screening for three rounds.

Expression of 5α R1 and 5α R2 proteins by the selected recombinant baculoviruses, designated C5 for 5α R1 and H5 for 5α R2 and pure of polyhedra, was confirmed by 5α -reductase activity assays as described below.

5α -reductase activity assays

Sf9 cells (0.5 – 1×10^6 cells/ml) were infected with the C5 or H5 recombinant baculovirus (at a multiplicity of infection of 5–10 plaque forming units) and cultured in 75 cm² Falcon flasks at 27°C.

The pH optimum of enzyme activity was determined as follows. Cells were harvested by centrifugation 3 days postinfection and the pellets were resuspended in 50 mM Tris-EDTA buffer of different pH strengths. Cells were lysed using a Dounce homogenizer pestel B and sonicated at 4°C for 30 s at 2/3 of maximal power (Ultrasons Annemasse, France). A typical activity assay was performed at 37°C for 10 min with 20 µl of cell homogenate (corresponding to about 150,000 5α R1-expressing cells or 20,000 5α R2-expressing cells) in 50 mM Tris-EDTA buffer of different pH strengths containing substrate (1 µM testosterone and 6×10^5 cpm [3 H]T) and cofactor (5 mM NADPH) mixed to a final volume of 250 µl. The reaction was started by addition of homogenate and was stopped by immersion of the tube into liquid nitrogen. Steroids were extracted twice with ether and the organic phase was evaporated. The 5α -reduced steroids produced were separated from T by HPLC [20] in a methanol-tetrahydrofuran-water (40:13:47) solvent mixture. A Flo-One HP radioactivity detector (Radiomatic Instruments, France) was used for in-line monitoring of the HPLC effluent.

The time-course of enzyme activity was determined as follows. Cells were harvested by centrifugation every 24 h during 5 days postinfection. Cell pellets were resuspended in 50 mM Tris-EDTA (pH 7.4) for

5 α R1 or 40 mM trisodium citrate (pH 5.5) for 5 α R2 (optimum pH for each enzyme). Enzyme activity was assayed as described above.

The effects of potential isozyme inhibitors (4-MA, finasteride, turosteride and LSESr) were studied by adding various concentrations of these compounds to the assay medium. The assays were performed at optimum pH and on homogenate from cells that had been harvested on the day of maximal enzyme production. Inhibitor selectivity was assessed by determining first an IC₅₀ value, i.e. the compound concentration required for 50% inhibition in the presence of 1 μ M testosterone, and, second, an apparent K_i by varying the testosterone concentration (0.1–10 μ M) in the absence or the presence of test-compound at the IC₅₀ value. Kinetic constants were determined from Lineweaver–Burk plots.

All assays were performed under conditions ensuring less than 15% conversion of substrate and cofactor. The “Dose–effect analysis with microcomputers” (Biosoft, Cambridge, U.K.) software was used to determine the kinetic parameters of the enzymes.

Partial purification of recombinant 5 α -reductase 1

Sf9 cells infected with the 5 α R1 expressing baculovirus were harvested 4 days postinfection and homogenized in a high ionic strength buffer (100 mM Tris, pH 7.2, 100 mM sodium citrate, 100 mM KCl, 5 mM DTT, 1 mM EDTA, 20% glycerol) (Buffer A) containing 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml TAME, as previously described [21, 22]. The cell pellet obtained on centrifugation of the homogenate for 1 h at 105,000 g and 4°C was resuspended in buffer A containing 1 μ M testosterone and 5 mM NADPH to obtain a microsomal preparation. A methanol–chloroform solution (2:1) of bovine brain

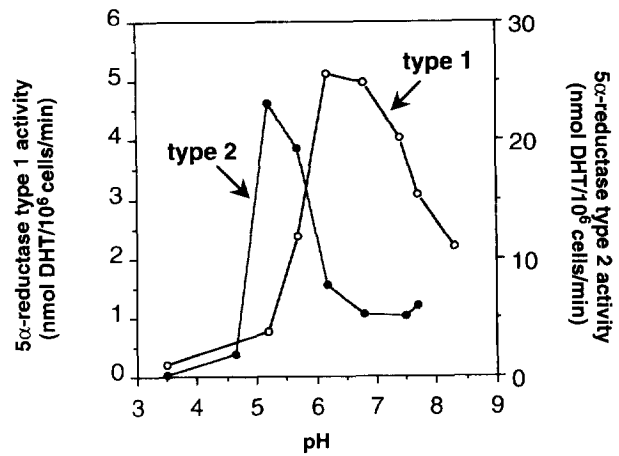


Fig. 1. pH-dependence of types 1 and 2 5 α -reductase activity expressed in the baculovirus/Sf9 cell system. Aliquots of infected cell homogenates were incubated for 10 min at 37°C in the presence of 1 μ M testosterone and 5 mM NADPH at the indicated pHs.

phospholipids (Sigma, France) was evaporated to dryness *in vacuo*, resuspended with sonication in a solution of *n*-octyl β -D-glucopyranoside, and then added directly to the microsomal preparation to give a final concentration of 5 mg/ml lipids and 0.8% w/v octyl glucoside. The mixture was kept on ice for 90 min, stirred from time to time, and then dialysed against 3.5 l of Buffer A at 4°C for 3 days (molecular weight cut-off of Spectrapor tubing = 12,000–14,000). The resulting preparation (liposome entrapped 5 α R1) was used as a source for enzyme kinetic studies. 5 α R1 activity was measured in the initial cell pellet and after liposome entrapment as described above. Protein concentration was measured using the BCA method (Pierce, IL, U.S.A.).

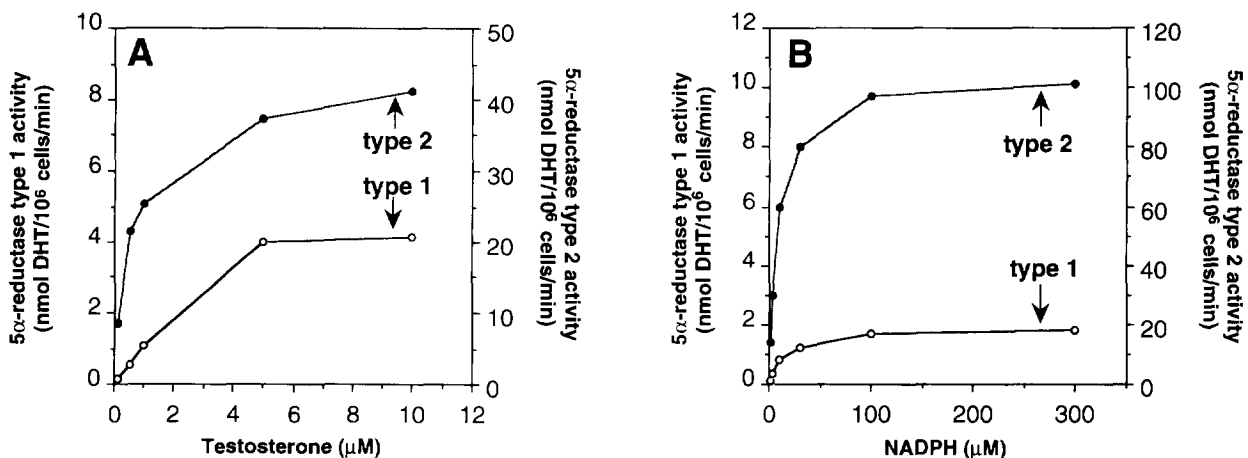


Fig. 2. 5 α -reductase activity in Sf9 cells. Sf9 cells were infected with the C5 or H5 baculovirus containing, respectively, the type 1 and type 2 5 α R cDNA. After 4 days (5 α R1) or 3 days (5 α R2) of infection, cell homogenates were assayed for enzyme activity in the presence of (A) the indicated concentrations of T and 5 mM NADPH or (B) the indicated concentrations of NADPH and 1 μ M T. 5 α R1 activity was measured at pH 7.4 and 5 α R2 activity at pH 5.5. Each value represents the mean of three experiments.

Table 1. Characterization *in vitro* of human 5 α -reductases expressed in the baculovirus expression system

	5 α -reductase type 1 activity (at pH 7.4)				5 α -reductase type 2 activity (at pH 5.5)			
	K_m (μ M)	V_{max} (nmol/10 ⁶ cells/min)	K_i (nM)	IC ₅₀ (nM)	K_m (μ M)	V_{max} (nmol/10 ⁶ cells/min)	K_i (nM)	IC ₅₀ (nM)
<i>Substrate/cofactor</i>								
Testosterone	2.9 \pm 0.8	4.2 \pm 1.4			0.5 \pm 0.2	57.6 \pm 19.9		
NADPH	21 \pm 7				6.7 \pm 0.7			
<i>Competitive inhibitors</i>								
4-MA			8.4 \pm 2.3	9.3 \pm 2.6			7.4 \pm 3.4	5.3 \pm 1
Finasteride			330 \pm 9	400 \pm 100			7.3 \pm 2.8	10.7 \pm 0.3
Turosteride			108 \pm 12	183 \pm 15			21.7 \pm 4.3	18.3 \pm 0.6
<i>Modulator</i>								
LSEsr			7.2 \pm 7.7*	4 \pm 1*			4.9 \pm 1.2*	7 \pm 2*

Mean (\pm SD) of at least three experiments. *Values expressed in μ g/ml; the *n*-hexane extract of *Serenoa repens* fruit (LSEsr) cannot be expressed in nM units because of the presence of components (free fatty acids and sterols) of different molecular weight.

RESULTS

Characterization of recombinant enzyme activity

In homogenates of infected Sf9 cells, the type 1 isozyme displayed a neutral pH optimum in the range of 6–8 whereas the type 2 isozyme had an acidic pH optimum in the range of 5–6 (Fig. 1).

Maximal activity was displayed on the third and fourth days postinfection for the type 2 (data not shown) and type 1 [11] isozymes, respectively. These were the durations postinfection selected in all subsequent experiments.

Higher levels of enzyme activity were recorded using the virus expressing the type 2 (pH 5.5) than type 1 (pH 7.4) isozyme (V_{max} = 57.6 and 4.2 nmol of DHT/10⁶ cells/min, respectively). No activity was detected in uninfected cells or in cells infected with the wild type baculovirus as previously described [11]. The type 2 isozyme exhibited a higher affinity for substrate (T) and cofactor (NADPH) than did the type 1 isozyme (Fig. 2, Table 1).

Inhibitor selectivity and type of inhibition

A 50% decrease in type 1 isozyme activity was obtained with 183 nM turosteride compared to 400 nM finasteride [Fig. 3(A)] indicating that turosteride is a more potent inhibitor of this isozyme than finasteride. On the other hand, finasteride (10.7 nM) appeared to be a fractionally better inhibitor than turosteride (18.3 nM) of the type 2 isozyme [Fig. 3(B)]. 4-MA was the most potent inhibitor of both type 1 and 2 isozymes (IC₅₀s of 9.3 and 5.3 nM, respectively). The *n*-hexane extract of *Serenoa repens* fruit (LSEsr) inhibited both isoforms with about the same IC₅₀s (4 μ g/ml for type 1 and 7 μ g/ml for type 2) (Fig. 4).

The three azasteroids are competitive inhibitors of both isoforms [Fig. 5(A, C)] whereas inhibition of the type 1 isozyme by LSEsr was non-competitive (panel B) and of the type 2 isozyme uncompetitive (panel D). Kinetic parameters and inhibition constants are summarized in Table 1.

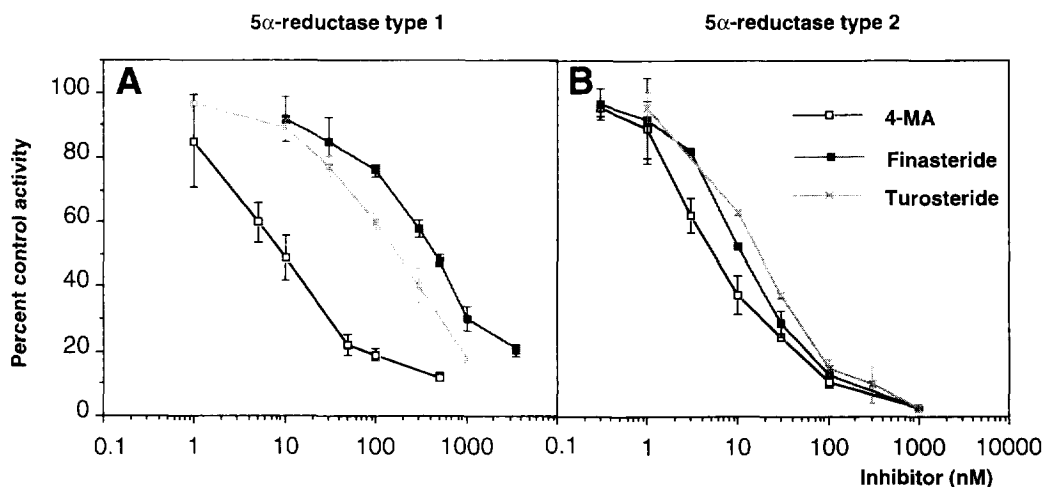


Fig. 3. Inhibition of 5 α -reductase type 1 (A) and type 2 (B) activity expressed in Sf9 cells. Enzyme activity in the presence of 1 μ M T but in the absence of inhibitor is defined as 100% activity (0.7–2 nmol of DHT/10⁶ cells/min for the type 1 isozyme; 34–38 nmol of DHT/10⁶ cells/min for the type 2 isozyme). Results are the mean (\pm SD) of three experiments.

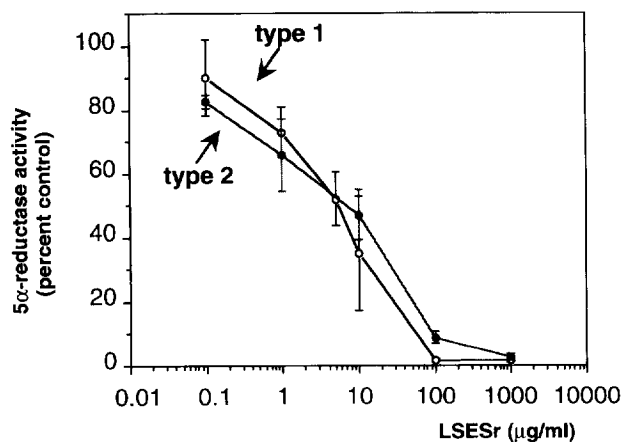


Fig. 4. Inhibition by LSESr of 5 α -reductase type 1 and type 2 activity expressed in Sf9 cells. (For further details, see legend to Fig. 3.)

Partial purification of the type 1 isozyme by lipid entrapment

Liposome entrapment of the type 1 isozyme preserved activity and affinity and led to modest purification. The entrapped isozyme had an apparent K_m of 1.8 μ M and a specific activity of 6.5 nmol/mg protein/min compared to 2.9 μ M and 2.4 nmol/mg pro-

tein/min before entrapment, i.e. a slight but not significant fall in K_m and a near 3-fold increase in specific activity.

DISCUSSION

In the present study, we produced human 5 α -reductase isoforms 1 and 2 in a baculovirus-directed insect cell expression system. When the pellets of infected Sf9 cells were stored at -80°C , these enzyme isoforms remained stable and active for more than 1 y. Our object was to obtain sufficient quantities of human isozymes to perform systematic and comparative *in vitro* studies on the binding affinities of various potential inhibitors in order to predict selectivity of action although, in the absence of further data, there is no reason, yet, to presume that it may not be necessary to inhibit both isozymes simultaneously to treat endocrine disorders involving DHT overproduction.

The type 2 isozyme proved to be the more active of the two isozymes but this observation on recombinant proteins may not apply to a biological situation. The type 1 isozyme displayed a neutral (range 6–8) pH optimum and the type 2 isozyme an acidic (range 5–6) pH optimum. These results are in agreement with those previously reported using cell lysates from

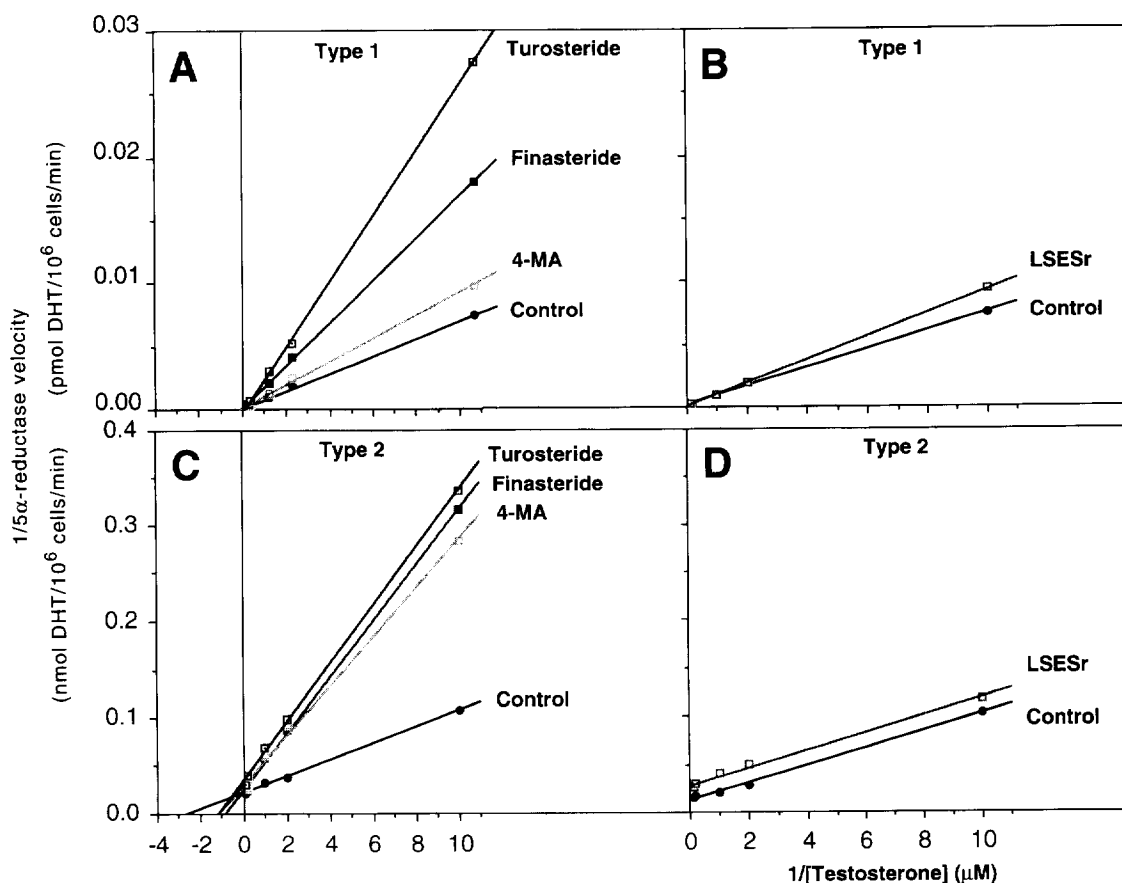


Fig. 5. Double reciprocal plots of the inhibition by 10 nM 4-MA, 400 nM finasteride, 180 nM turosteride and 2 μ g/ml LSESr of type 1 (A, B) and by 5 nM 4-MA, 10 nM finasteride, 18 nM turosteride and 6 μ g/ml of type 2 (C, D) 5 α -reductase activity expressed in infected Sf9 cells.

transiently transfected 293 cells [2]. In addition, the pH optimum for the type 2 isozyme was identical to that measured using the microsomal fraction of human BPH tissue [23].

Three compounds and one plant extract were screened for inhibition of the activity of each isozyme. We confirmed that 4-MA is the most potent inhibitor of both isoforms whereas finasteride inhibits the type 2 isozyme as effectively as 4-MA but is a poor inhibitor of the type 1 isoform. Turosteride, a more recent compound, inhibited the type 1 isozyme better than finasteride but remained nevertheless 12 times less efficient than 4-MA. All three compounds had equivalent activity toward the type 2 isozyme and all three were competitive inhibitors of both isozymes. The plant extract, LSESr, displayed distinctive behaviour because its inhibition of the type 1 isozyme was non-competitive and of the type 2 isozyme uncompetitive. However, insofar as it inhibited both isozymes to similar extents, it can be most likened to 4-MA. Another analogy that is of interest is with epristeride, a steroid acrylate, that has recently been found to be a specific uncompetitive inhibitor of the type 2 isozyme [24].

The inhibitory action of LSESr can doubtless be partly attributed to a modulatory action of its lipid component on the environment of the enzyme. 5α -reductase is a nuclear membrane-associated enzyme and many fatty acids are essential component of mammalian membranes. Specific aliphatic fatty acids can inhibit 5α -reductase activity [25] whereas phospholipids such as phosphatidylserine and phosphatidylcholine can either stimulate or inhibit activity [25, 26]. As previously described [22] and confirmed by this study, partial purification of 5α -reductase requires the presence of lipids to recover and stabilize enzyme activity. Furthermore, as suggested by the experiments with LSESr, both 5α -reductase isoforms appear highly sensitive to their environment. Enzyme access to its cofactor depends on the conformational state of the protein which in turn depends upon membrane composition. This observation could explain the discrepancies in affinity of the enzymes for NADPH recorded by different authors [this study, 5, 6, 24] which seem to vary with the expression system used.

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