17β-(*N-tert*-Butylcarbamoyl)-4-aza-5α-androstan-1-en-3-one Is an Active Site-Directed Slow Time-Dependent Inhibitor of Human Steroid 5α-Reductase 1

Gaochao Tian,*,‡ J. Darren Stuart,§ Marcia L. Moss,‡ Paul L. Domanico,‡ H. Neal Bramson,§ Indravadan R. Patel,∥ Sue H. Kadwell,∥ Laurie K. Overton,∥ Thomas A. Kost,∥ Robert A. Mook, Jr.,⊥ Stephen V. Frye,⊥ Kenneth W. Batchelor,§ and Jeffrey S. Wiseman‡

Departments of Molecular Biochemistry, Cellular Biochemistry, Molecular Biology, and Medicinal Chemistry, Glaxo Research Institute, 5 Moore Drive, Research Triangle Park, North Carolina 27709

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ABSTRACT: 17β-(*N-tert*-butylcarbamoyl)-4-aza-5α-androstan-1-en-3-one (finasteride), which has been approved for treatment of benign prostatic hyperplasia, is shown here to be a slow time-dependent inhibitor of human steroid 5α-reductase isozyme 1. This inhibition is characterized by an initial, fast step where the inhibitor binds to the enzyme followed by a slow step that leads to a final enzyme–inhibitor complex (EI*). No recovery of activity from this EI* complex was observed after dialysis for 3 days. The formation of EI* is diminished in the presence of a competitive, reversible inhibitor, indicating that the inhibition is active site-directed. At 37 °C and pH 7.0, the rate constant for the second, slow inhibition step, k_3, is (1.40 ± 0.04) × 10^{-3} s^{-1} and the pseudo-bimolecular rate constant, k_3/K_i, is (4.0 ± 0.3) × 10^3 M^{-1} s^{-1}. This latter rate constant is less than the value of 2.7 × 10^5 M^{-1} s^{-1} determined for the inhibition of 5α-reductase 2 by finasteride [Faller, B., Farley, D., & Nick, H. (1993) *Biochemistry 32,* 5705–5710]. The 1,2-double bond within the A ring of finasteride may be the key structural element required for the slow step, since two other 4-azasteroids, 5α-23-methyl-4-aza-21-norchol-1-en-3,20-dione and 17β-(*N,N*-diethylcarbamoyl)-4-aza-5α-androstan-1-en-3-one, which share the same A ring structure with finasteride, are also slow inhibitors of 5α-reductase 1, whereas 17β-(*N,N*-diethylcarbamoyl)-4-methyl-4-aza-5α-androstan-3-one (4-MA) and 17β-(*N,N*-diethylcarbamoyl)-4-aza-5α-androstan-3-one, which lack the 1,2-double bond in the A ring of finasteride, do not show slow inhibition kinetics. These data suggest covalent modification of 5α-reductase by finasteride.

Inhibitors of steroid 5α-reductase (EC 1.3.99.5), which catalyzes the NADPH-dependent reduction of testosterone to dihydrotestosterone, are of considerable interest for the treatment of those diseases that are at least partially dependent on dihydrotestosterone such as benign prostatic hyperplasia (BPH), acne, and male pattern baldness (Wilson, 1980; Mooradian et al., 1987; Cunha et al., 1987; Metcalf et al., 1987). In man, two isozymes of 5α-reductase, designated types 1 and 2, have been reported (Anderson & Russell, 1990; Anderson et al., 1991). 5α-Reductase 1 has been found mainly in skin and liver but not in prostate, whereas 5α-reductase 2, often referred to as the prostatic 5α-reductase, has been found in prostate, seminal vesicles, liver, and epididymis (Thigpen et al., 1993). Since both isozymes contribute to the plasma level of dihydrotestosterone (Anderson et al., 1991), it is necessary to design potent inhibitors of both the isozymes.

Finasteride,[1] 17β-(*N-tert*-butylcarbamoyl)-4-aza-5α-androst-1-en-3-one, is the only 5α-reductase inhibitor currently approved in the U.S. for treatment of BPH. Several clinical reports have shown that a 5-mg dose of finasteride given daily reduced dihydrotestosterone levels by about 70% (Geller, 1990; Ohtawa et al., 1991; McConnell et al., 1992). Higher doses of finasteride were unsuccessful at further lowering the plasma level of dihydrotestosterone (Imperato-McGinley et al., 1990; Ohtawa et al., 1991). Initial reports showed that finasteride was a competitive, reversible inhibitor of both isozymes of 5α-reductase, where it possessed relatively poor affinity for 5α-reductase 1 and moderate affinity for 5α-reductase 2 (Liang et al., 1985; Anderson et al., 1991; Jenkins et al., 1992). However, a more recent report by Faller et al. (1993) demonstrated that finasteride was, in fact, a slow and potent inhibitor of 5α-reductase 2. This result appears to suggest that the failure to eliminate dihydrotestosterone from plasma by finasteride is due to its inability to inhibit 5α-reductase 1 at the doses used.

This paper will show that finasteride is also a slow and potent inhibitor of 5α-reductase 1 but that the rate constant for the slow inhibition is much less than the value reported for 5α-reductase 2.

EXPERIMENTAL PROCEDURES

Materials

[1,2,6,7-^{3}H(N)]Testosterone was purchased from Du Pont NEN Research Products. Testosterone, NADPH, DTT, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were products of Sigma. Finasteride was from Lancaster Synthesis Ltd. 17β-(*N,N*-diethylcarbamoyl)-4-methyl-4-aza-5α-androstan-3-one (4-MA), 5α-23-methyl-4-aza-21-norchol-1-ene-3,20-dione, 17β-(*N,N*-diethylcarbamoyl)-4-aza-5α-androstan-3-one, and 17β-(*N,N*-diethylcarbamoyl)-4-aza-5α-androstan-1-en-3-one were synthesized according to known methods (Rasmusson et al., 1986). All other reagents were of the highest quality.

* To whom correspondence should be addressed.
‡ Department of Molecular Biochemistry.
§ Department of Cellular Biochemistry.
∥ Department of Molecular Biology.
⊥ Department of Medicinal Chemistry.
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[1] Abbreviations: finasteride, 17β-(*N-tert*-butylcarbamoyl)-4-aza-5α-androstan-1-en-3-one; 4-MA, 17β-(*N,N*-diethylcarbamoyl)-4-methyl-4-aza-5α-androstan-3-one; HPLC, high-performance liquid chromatography; K_i, inhibition constant; K_m, Michaelis–Menten constant.

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Methods

Plasmid Construction. The plasmid ph5α45 containing a cDNA copy of the human 5α-reductase 1 gene was kindly provided by Dr. David Russell. Synthetic oligonucleotides and site-directed mutagenesis (Sambrook et al., 1989) were used to create *Nhe*I restriction sites immediately 5′ and 3′ of the coding sequence of the cDNA (GCTAGCTATA-AAAATATG—5α-reductase 1 coding sequence—TAA-GTGCTAGC). The coding sequence was then subcloned into the *Nhe*I site of the baculovirus transfer plasmid pJVP10Z (Vialard et al., 1990). In this plasmid, the 5α-reductase 1 gene was placed downstream of the viral polyhedrin promoter, and the bacterial gene coding for the enzyme β-galactosidase was cloned downstream of the viral p10 promoter. This plasmid was designated pJVP10Z-5α1.

Production of the Recombinant Virus. The Sf9 cell line was obtained from the American Type Cell Culture Collection. The cells were maintained in Grace's medium (GIBCO), supplemented with 3.3 μg/mL lactalbumin hydrolysate (Difco), 3.3 μg/mL yeastolate, 50 μg/mL gentamicin (GIBCO), and 0.1% pluronic F-68 (GIBCO) in 125-mL spinner flasks. To generate recombinant baculovirus, Sf9 cells (2×10^6 cells/mL) were cotransfected with 1 μg of linear viral DNA (Invitrogen) and 3 μg of pJVP10Z-5α1 DNA using a cationic liposome transfection kit according to the manufacturer's instructions (Invitrogen). Virus was harvested after 4 days and screened for the recombinants by plaque assay (Summers & Smith, 1987; O'Reilly et al., 1992). Recombinant viruses obtained from this screen were subjected to two further rounds of plaque purification.

Production of 5α-Reductase 1 by Sf9 Cells. For the production of 5α-reductase 1, Sf9 cells were grown in a 5-L airlift fermenter. The fermenter was seeded with 4×10^5/mL Sf9 cells in SF900II (GIBCO). The temperature was maintained at 28 °C, and oxygen was maintained at 50% air saturation by sparging. Cells at a density of 2×10^6 cells/mL were infected with recombinant virus at a multiplicity of infection of 0.1 infectious virus particle per cell. Seventy-two hours after infection, the cells were harvested by centrifugation, rinsed once in phosphate-buffered saline, and then quick frozen in a dry ice–ethanol bath.

Preparation of Microsomal 5α-Reductase 1. Baculovirus-infected Sf-9 cells expressing human 5α-reductase 1 were frozen at −80 °C until microsome preparation. All homogenization steps were performed on ice, and all centrifugations were done at 4 °C. At the time of preparation, the cells (60 mL) were thawed, diluted 5-fold with homogenization buffer (50 mM potassium phosphate, pH 7.4, 0.25M sucrose, 0.1 mM EDTA), and homogenized with a Brinkmann polytron for two, 15-s blasts at 16 000 rpm. The homogenate was further homogenized with a Teflon/glass homogenizer attached to a Con-torque power unit at high speed for 10 strokes. The homogenate was centrifuged at 600*g* for 10 min, and the resulting supernatant was centrifuged at 10400*g* for 15 min. The 10400*g* pellet was resuspended in homogenization buffer using the Teflon/glass homogenizer and then recentrifuged at 10400*g* for 15 min. All 10400*g* supernatants were combined and centrifuged at 101500*g* for 1 h. This resuspension and centrifugation procedure was repeated once. The microsome pellet was resuspended in 12 mL of buffer (50 mM MOPS, pH 7.0, 0.005% Triton X-100, 0.3 mM KCl) using the Teflon/glass homogenizer. The microsomes were stored in small aliquots (<100 μL) at −80 °C, thawed once for use in the enzyme assay, and then discarded.

5α-Reductase 1 Activity Assays. Reaction solution A contained 17.6 mM imidazole, 17.6 mM diethanolamine, and 13.2 mM succinic acid at pH 7.0, and the total ionic strength was adjusted to 0.30 M with KCl. A stock solution of radioactive testosterone was prepared by removing the ethanol from 40 μL of [1,2,6,7-^{3}H(N)]testosterone with a stream of N_2 gas. A 440-μL aliquot of reaction solution A containing 5% Me_2SO was then added to the dry radioactive testosterone to give a 1 μM 91 nCi/μL solution. Reaction solution B contained 10 mM DTT, 10 mM NADPH, and the NADPH regeneration system of 10 mM glucose 6-phosphate and 12 units/mL of glucose-6-phosphate dehydrogenase in reaction solution A. A 5% Me_2SO stock solution in reaction solution A was prepared. This solution was used to maintain a final concentration of 1.5% Me_2SO in the assay. Finally, a 5α-reductase 1 stock solution (0.1–1 mg/mL) was prepared in reaction solution A containing 1 mM DTT and stored at 4 °C.

The reaction mixture (50 μL total) was prepared by adding 5 μL of reaction solution B, 5 μL of the testosterone stock solution, and 5α-reductase 1 to buffer A. The reaction was performed in glass vials at either 22 or 37 °C and initiated by addition of enzyme. The reaction was quenched with 100 μL of ethanol, and the glass vial was vortexed vigorously. A 15-μL aliquot of the quenched solution was injected onto a C_{18} HPLC column, and testosterone and dihydrotestosterone were separated with a water/acetonitrile (52%:48%) mobile phase. The relative amounts of testosterone and dihydrotestosterone were determined by an in-line flow radiochemical detector (Beckman 171-Radiodetector). All the reactions were first order in testosterone, and enzymatic activities were expressed as the first-order rate constant for loss of substrate.

Determination of K_i's for Finasteride and 4-MA. Stock solutions of finasteride and 4-MA were prepared in Me_2SO. Inhibition studies were identical to activity assays except that the reactions were quenched at 1 min or less. The relative inhibition R was fit by nonlinear least-squares analysis to

$$R \equiv 1 - \frac{(V/K)}{(V/K)_0} = \frac{[I]}{K_i + [I]} \qquad (1)$$

where $(V/K)_0$ is the activity in the absence of inhibitor, I, and K_i is the inhibition constant. This equation applies only when the substrate concentration is much less than its K_m.

Progress Curve Analysis. The reaction mixture was scaled up to 400 μL. At different times, 20-μL aliquots of the reaction mixture were removed and quenched with 40 μL of ethanol, and product formation was monitored as above. This procedure was performed with and without finasteride.

Preincubation Experiments. The 5α-reductase 1 solution was incubated for 30 min at a specified temperature in 198 μL of reaction buffer A, containing all the components except testosterone. Then, either 2 μL of Me_2SO or 2 μL of 1 μM 4-azasteroid inhibitor in Me_2SO was added to start the preincubation. At different times, 5 μL of this preincubation mixture was removed and added immediately to 45 μL of the reaction mixture to assay for activity. The reaction was quenched as above.

Recovery of Activity from Inactivated 5α-Reductase 1. 5α-reductase 1 was inactivated by incubating the enzyme with 1 mM NADPH, 1 mM DTT, ±1 μM finasteride, and the NADPH regenerating system in reaction buffer A (200 μL total) for 5 h at 22 °C.

Two procedures were used to remove unbound finasteride. In the first procedure, the enzyme solution was centrifuged at 100000g for 30 min. The microsome pellet was washed

three times and resuspended in 200 μL of fresh buffer. This was repeated once. The final 5α-reductase 1 pellet was resuspended in 100 μL of buffer A, and the 5α-reductase activity was assayed at different time intervals. In the second procedure, 400 μL of the inhibited 5α-reductase 1 was pelleted, washed, and resuspended as in the first procedure; however, this sample was then placed in dialysis tubing with a molecular weight cutoff of 15 000. Dialysis was performed against 400 mL of reaction buffer containing all reaction components except the substrates and the NADPH regeneration system. The samples were dialyzed for 3 days and the dialysis buffer was changed every 24 h. The enzyme activity was determined after dialysis.

RESULTS AND DISCUSSION

Evidence That Finasteride Inhibits 5α-Reductase 1 Slowly. Inhibition studies were performed at pH 7.0. To maintain saturating levels of NADPH (1 mM) and to avoid the accumulation of $NADP^+$, a regenerating system of 1 mM glucose 6-phosphate and 1.2 units/mL glucose-6-phosphate dehydrogenase was employed. The initial concentration of testosterone was set to 100 nM. Since this value is much lower than its K_m (7 μM; Harris et al., 1992), these reactions were first order in testosterone.

For a first-order reaction, the shape of a progress curve is indicative of the type of interaction (slow or fast binding) between inhibitor and enzyme. If there is no time-dependent inhibition of the enzyme, a semilogarithmic plot of the progress curve will be linear; otherwise, time-dependent inhibition will yield a curved semilogarithmic plot. In the absence of finasteride, the progress curve for 5α-reductase 1 was linear at several enzyme concentrations. A typical progress curve for the 5α-reductase 1-catalyzed reaction run at 37 °C in the presence of 1 μM finasteride is shown in Figure 1A. The curvature of this progress curve suggests slow inhibition by finasteride.

To confirm that the inhibition was slow, preincubation experiments were performed. Here, 5α-reductase 1 was preincubated with 1 μM finasteride at 30 °C for various times, and then the remaining activity was determined. For such experiments to be meaningful, the enzyme activity must remain relatively stable throughout the course of the reaction. DTT and KCl at 1 mM and 0.26 M, respectively, were found to stabilize the 5α-reductase 1 activity (data not shown). With finasteride present at 1 μM (Figure 1B), only 10% of the initial 5α-reductase activity remained after 60 min, whereas without finasteride, 95% of the initial activity remained at the same time point. In both cases, the loss of enzymatic activity was first order, where the observed rate constants in the presence and absence of inhibitor were 7×10^{-4} s^{-1} and 3.0×10^{-5} s^{-1}, respectively.

Reversibility of the Slow Inhibition of 5α-Reductase 1 by Finasteride. To determine if the inhibition of 5α-reductase 1 by finasteride was reversible, two experiments were performed. In the first experiment, 5α-reductase 1 was completely inactivated by incubation with finasteride for 5 h. The microsomal enzyme was then separated from the unbound finasteride by repeated centrifugation and resuspension steps. An aliquot of this enzyme sample was then incubated at 37 °C and monitored for recovery of activity (Table 1). In the second experiment, another aliquot of this enzyme sample was dialyzed for 3 days in an attempt to release bound finasteride and then incubated at 37 °C and monitored for recovery of activity (Table 1). No activity (<1% relative to controls) was recovered in either experiment. These data suggest either that the finasteride/5α-reductase 1 complex is irreversible or that the half-life ($k_{off} < 10^{-6}$ s^{-1}) for this complex is greater than 10 days.

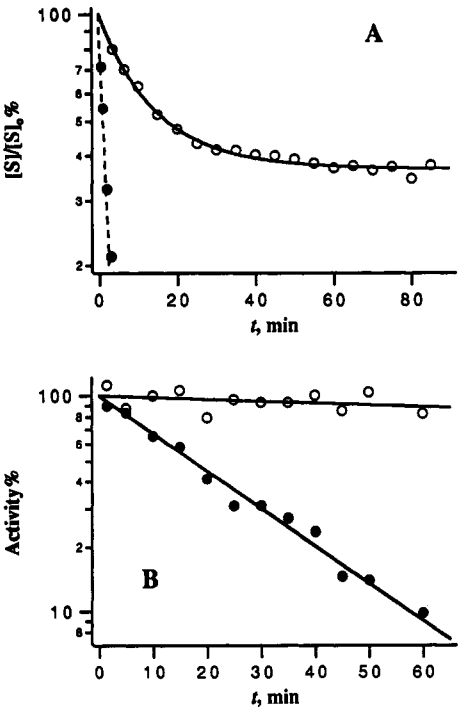


FIGURE 1: (A) Progress curve for inhibition of 5α-reductase 1 by finasteride. The percentage of substrate remaining, $[S]/[S]_o$, was plotted vs time. The reactions were conducted in the presence (○) or absence (●) of 1 μM finasteride. The solid line is a theoretical representation of the data (eq 4). (B) Time-dependent loss of 5α-reductase 1 activity. The microsomal 5α-reductase 1 was preincubated at 37 °C with (●) or without (○) 1 μM finasteride. The activity remaining at different time intervals was determined as described in Methods. The activity vs time data were fit to a first-order decay function by nonlinear least-squares analysis.

Table 1: Reversibility of Inhibition by Finasteride

	5α-reductase activity recovered, %		
method	no inhibitor	4-MA	finasteride
washing[a]	97	91	3.7[c]
dialysis[d]	ND[b]	18	<0.1

[a] α-Reductase 1 was first preincubated in the presence or absence of 1 μM finasteride for 5 h. The unbound finasteride was removed by repeated centrifugation and resuspension. The final pellet was resuspended and incubated at 37 °C. Enzyme activity was determined immediately after washing and again after 24 h. [b] Not determined. The first-order rate constant for the thermoinactivation, at the same enzyme concentration as in dialysis, was ca. 0.02 h^{-1} (unpublished observation), which translates into ca. 77% reduction in the activity in 72 h. [c] This residual activity was seen immediately after the removal of unbound finasteride, but it did not increase with time. [d] 5α-Reductase 1 was inhibited and washed as described above and then dialyzed at 22 °C for 3 days. Enzyme inhibited with the competitive inhibitor 4-MA was treated in parallel.

A Two-Step Slow Inhibition by Finasteride. To determine if the inhibition of 5α-reductase 1 by finasteride followed a two step mechanism, preincubation experiments were carried out at different concentrations of finasteride (Figure 2A). Loss of activity was first order in enzyme for the first 4 half-lives of the reaction. The observed rate constants for inhibition, k_{obsd}, were calculated from the slopes of activity vs time plots (Morrison, 1982; Morrison & Walsh, 1988), and these k_{obsd} values were then replotted vs the finasteride concentration (Figure 2B). The fact that the k_{obsd} is a hyperbolic function

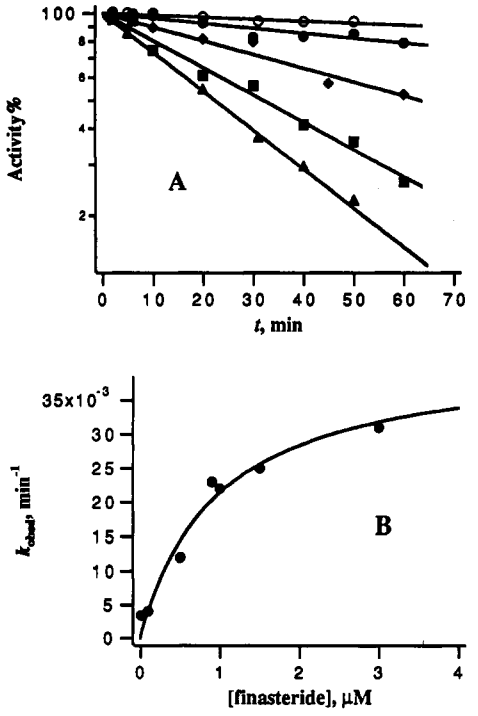


FIGURE 2: Dependence of the rate of inhibition of 5α-reductase 1 on finasteride concentration. (A) Rates of decay of 5α-reductase activity, k_{obsd}, at varying finasteride concentrations. The slow inhibition reactions were carried out at 22 °C in the presence of 0 (○), 0.1 (●), 0.5 (◆), 1.5 (■), or 3 μM (▲) finasteride. The activity remaining was determined as described in Methods. (B) Replot of k_{obsd} vs the concentration of finasteride.

Table 2: Kinetic Parameters for Slow Inhibition of 5α-Reductase 1 by Finasteride

isozyme	k_3, s^{-1}	K_i, μM	k_3/K_i, M^{-1} s^{-1}	T, °C
5α-reductase 1[a]	(6.7 ± 0.8) × 10^{-4}	0.36 ± 0.04[b]	(7.0 ± 3.0) × 10^2	22
	(1.4 ± 0.1) × 10^{-3} [c]	0.36 ± 0.04[b]	(4.0 ± 0.6) × 10^3	37
5α-reductase 2[d]			2.7 × 10^5	37

[a] All the data for 5α-reductase 1 were determined in this work. [b] Determined from independent enzyme inhibition. [c] Estimated by using eq 3 with [I] = 1 μM. [d] From Faller et al. (1993).

of finasteride concentration (Figure 2B) indicates a two-step mechanism:

$$E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_3}{\rightarrow} EI^* \qquad (2)$$

In this mechanism, the equilibrium for the first step is established rapidly whereas the second step is slow. In eq 2, K_i is the dissociation constant for the initial EI complex and k_3 the rate constant for the slow inhibition step. For this mechanism, the k_{obsd} is given by (Shaprio & Riordan, 1984)

$$k_{obsd} = k_3 \frac{[I]}{[I] + K_i} \qquad (3)$$

The values for k_3 (Table 2) and K_i (0.96 ± 0.30 mM) were estimated from nonlinear least-squares analysis.

The K_i was also determined by measuring enzyme inhibition at very short time points (≤1 min). From this experiment, the K_i was 0.36 ± 0.04 μM (Table 2). The discrepancy between this value and the one (0.96 ± 0.30 μM) from slow inhibition experiments was ascribed to systematic errors associated with the methods, and the source of the errors was not investigated. This latter value (0.36 ± 0.04 mM) was reproduced at both

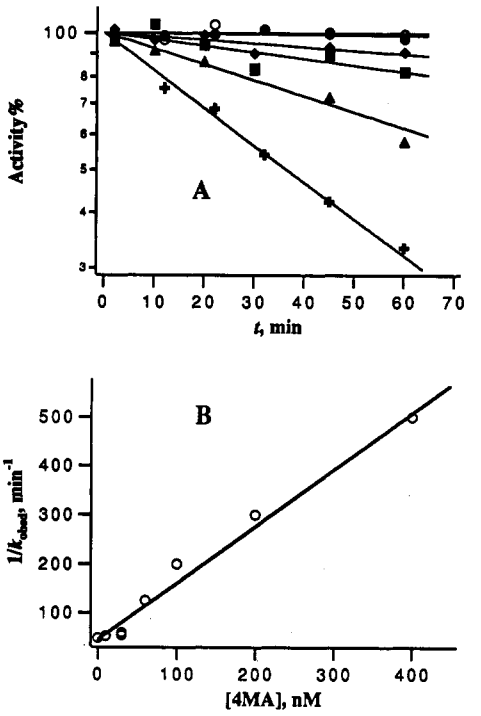


FIGURE 3: 4-MA protects 5α-reductase 1 from slow inhibition by finasteride. (A) Plots of activity remaining vs time. The microsomal 5α-reductase 1 was preincubated at 22 °C in the presence or absence of 1 μM finasteride (I_s) but at different concentrations of 4-MA (I_f). The conditions were $[I_s]$ = 0 μM and $[I_f]$ = 0 μM (○), $[I_s]$ = 0 μM and $[I_f]$ = 0.02 μM (●), $[I_s]$ = 1 μM and $[I_f]$ = 0.4 μM (◆), $[I_s]$ = 1 μM and $[I_f]$ = 0.2 μM (■), $[I_s]$ = 1 μM and $[I_f]$ = 0.1 μM (▲), and $[I_s]$ = 1 μM and $[I_f]$ = 0 μM (+). All the sets of data were fit to a first-order decay function by nonlinear least-squares analysis. (B) Plot of the reciprocal of k_{obsd} vs the concentration of 4-MA. The k_{obsd} values were from the slopes of the plots of activity remaining vs time as given in panel A of this figure.

22 and 37 °C with a relatively small error range, and it was used for further calculations.

The values of k_{obsd} may also be determined from progress curve analysis according to

$$\frac{[S]}{[S]_0}(\%) = \exp\left[\frac{(V/K)_i}{k_{obsd}}(e^{-k_{obsd}t} - 1)\right] \times 100 \qquad (4)$$

where $(V/K)_i$ is the initial activity. A value of (1.04 ± 0.03) × 10^{-3} s^{-1} (37 °C) was obtained from nonlinear least-squares analysis of the data given in Figure 1A. The estimate of k_3 (Table 2) was then obtained by using eq 3 with K_i = 0.36 ± 0.04 μM and [I] = 1 μM.

The Slow Inhibition Is Active Site-Directed. To evaluate whether the slow inhibition occurs at the active site, the effect of a competitive and reversible inhibitor of 5α-reductase, 4-MA, on the rates of slow inhibition by finasteride was examined. From preincubation studies, 4-MA was shown not to be a time-dependent inhibitor of 5α-reductase 1 (Figure 3A, open circles). 4-MA protected 5α-reductase 1 from slow inhibition by finasteride; furthermore, the degree of protection depended on the concentration of 4-MA (Figure 3A). The expression for k_{obsd} in the presence of a rapidly binding, competitive inhibitor is given by (Weiss & Cleland, 1987; Plapp 1987)

$$\frac{1}{k_{obsd}} = \frac{1}{3}\left(1 + \frac{K_{i(s)}}{[I_s]}\right) + \frac{K_{i(s)}}{k_3 K_{i(f)}[I_s]}[I_f] \qquad (5)$$

where $K_{i(s)}$ is the dissociation constant of the initial slow

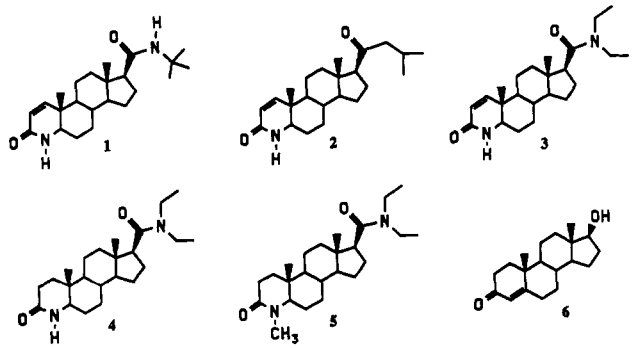


FIGURE 4: Chemical structures: **1**, finasteride; **2**, 5α-23-methyl-4-aza-21-norchol-1-ene-3,20-dione; **3**, 17β-(*N*,*N*-diethylcarbamoyl)-4-aza-5α-androstan-1-en-3-one; **4**, 17β-(*N*,*N*-diethylcarbamoyl)-4-aza-5α-androstan-3-one; **5**, 4-MA; **6**, testosterone.

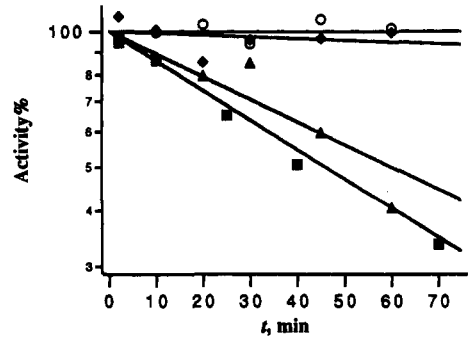


FIGURE 5: Reaction of 4-azasteroids with 5α-reductase 1. The enzyme was preincubated in the absence of inhibitor (○) or in the presence of 1 μM **4** (◆), **3** (▲), or **2** (■), at 22 °C. The activity remaining at different time intervals was determined as described in Methods.

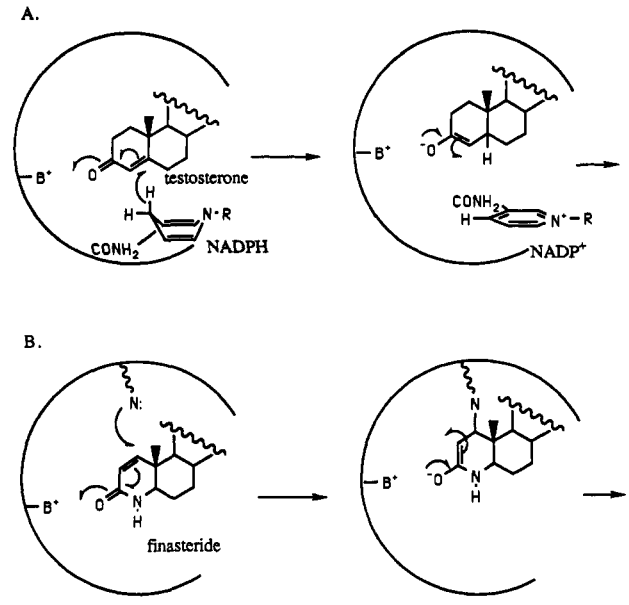
enzyme–inhibitor complex and $K_{i(f)}$ the inhibition constant of the fast inhibitor, and I_s and I_f are the slow and fast inhibitors, respectively. Figure 3B shows the replot of $1/k_{obsd}$ vs the concentration of 4-MA. A linear fit of the data according to eq 5 yielded 44 ± 10 min^{-1} for the intercept (*i*) and 1.18 ± 0.06 $nM^{-1}min^{-1}$ for the slope (*s*). With $K_{i(s)} = 0.36 \pm 0.04$ μM and $[I_s] = 1$ μM, the inhibition constant of 4-MA [$K_{i(f)}$] was then calculated to be 11 ± 3 nM by using the equation:

$$K_{i(f)} = \frac{i/s}{1 + [I_s]/K_{i(s)}} \quad (6)$$

This K_i value is in good agreement with the value of 12 ± 1 nM determined by direct enzyme inhibition experiments. The results strongly suggest that the slow inhibition of 5α-reductase 1 by finasteride is active site-directed.

Why Is Finasteride a Slow Inhibitor of 5α-Reductase? There are several mechanisms that result in slow rates of enzyme inhibition. These mechanisms include steps, such as chemical and conformational transformations, that are slower than the time required for carrying out the experiments (Morrison & Walsh, 1988). The inability to recover 5α-reductase 1 activity after incubation with finasteride suggests that the inhibition is covalent and that this covalent step is responsible for the slow inhibition kinetics. In addition to finasteride (**1**) (for the structure, see Figure 4), 5α-23-methyl-4-aza-21-norchol-1-en-3,20-dione (**2**) and 17β-(*N*,*N*-diethylcarbamoyl)-4-aza-5α-androstan-1-en-3-one (**3**) are also time-dependent inhibitors of 5α-reductase 1 (Figure 5), whereas 17β-(*N*,*N*-diethylcarbamoyl)-4-aza-5α-androstan-3-one (**4**) as well as 4-MA (**5**) are reversible inhibitors. It should be noted that the 1,2-bond within the A ring of **4** and 4-MA is saturated. However, all three compounds (**1**–**3**) that show slow inhibition possess a common structural feature, a 1,2-double bond in the A ring. These results suggest that the double bond within the A ring plays an important role in the slow inhibition. In fact, Petrov and Lark (1981) proposed (Scheme 1A) that the catalytic driving force for the 5α-reductase reaction is the polarization of the α,β-unsaturated carbonyl of testosterone (**6**). This mechanism may also facilitate a chemical transformation between the enzyme and inhibitor, with the α,β-unsaturated carbonyl within the A ring of finasteride acting as a Michael acceptor (Scheme 1B). Confirmation that 5α-reductase 1 is, in fact, covalently modified by finasteride awaits further investigation.

Scheme 1



Comparison between Slow Inhibition by Finasteride of Isozymes of 5α-Reductase. Faller et al. (1993) have demonstrated that finasteride shows slow inhibition kinetics with 5α-reductase 2. The upper limit of the K_i was estimated to be much less than 1 nM by using the value of the association rate constant k_{on} and the estimated limit of the dissociation rate constant k_{off} (Faller et al., 1993). If the value for the dissociation rate of finasteride from the inactivated 5α-reductase 1 is set to 10^{-6} s^{-1} (see before), this apparent K_i would also be less than 1 nM. In conclusion, finasteride is a potent inhibitor of both isozymes of 5α-reductase.

However, the *in vitro* potency of finasteride does not appear to translate into *in vivo* effects. As mentioned earlier, a normal dose response of dihydrotestosterone level in plasma occurs up to 5 mg of finasteride, but the response plateaus off at higher doses (Imperato-McGinley et al., 1990; Ohtawa et al., 1991). Such a biphasic behavior is typical of situations where the therapeutic target exists in two isozymic forms and one of the isoforms is insufficiently inhibited. Since the *in vitro* potency of finasteride is now no longer an issue, the difference between the rates of inhibition of the isozymes may account for the *in vivo* observations. The slow inhibition of 5α-reductase 2 by finasteride is characterized by an apparent one-step mechanism whose second-order inhibition rate constant, k_{on}, is 2.7×10^5 $M^{-1}s^{-1}$ at 37 °C and pH 7.0 (Faller et al., 1993), whereas, under the same conditions, the slow inhibition of 5α-reductase 1 by finasteride is typical of a two-step mechanism and the pseudo-bimolecular constant k_3/K_i is estimated to be 4.0×10^3 M^{-1} s^{-1} (Table 2). This means that it takes 70 times longer for finasteride to completely inhibit 5α-reductase 1 than to inhibit 5α-reductase 2. Im-

perato-McGinley and co-workers (1990) determined that, after a 5-mg dose, finasteride in plasma reached a maximum concentration of 100 nM. Since 90% of finasteride in plasma is bound to serum proteins (Lee et al., unpublished data), the effective maximum concentration would be about 10 nM. In addition, the half-life of finasteride in plasma is only about 5 h as determined by pharmacokinetics (Imperato-McGinley et al., 1990). Given these measurements, the 5α-reductase 2 inhibition would happen within minutes, whereas 5α-reductase 1 would be inhibited no more than 50% after 5 h, consistent with the observations that, at therapeutic doses, finasteride fails to eliminate dihydrotestosterone from plasma.

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REFERENCES

Anderson, S., & Russell, D. W. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3640–3644.

Anderson, S., Bishop, R. W., & Russell, D. W. (1989) *J. Biol. Chem. 264*, 16249–16255.

Anderson, S., Berman, D. M., Jenkins, E. P., & Russell, D. W. (1991) *Nature 354*, 159–161.

Cunha, G. R., Donjacour, A. A., Cooke, P. S., Mee, S., Bigsby, R. M., Higgins, S. J., & Sugimura, Y. (1987) *Endocr. Rev. 8*, 338–362.

Faller, B., Farley, D., & Nick, H. (1993) *Biochemistry 32*, 5705–5710.

Geller, J. (1990) *J. Clin. Endocrinol. Metab. 71*, 1552–1555.

Harris, G., Azzolina, B., Baginsky, W., Cimis, G., Rasmusson, G. H., Tolman, R. L., Raetz, C. R. H., & Ellsworth, K. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10787–10791.

Imperato-McGinley, J., Shackleton, C., Orlic, S., & Stoner, E. (1990) *J. Clin. Endocrinol. Metab. 70*, 777–782.

Jenkins, E. P., Anderson, S., Imperato-McGinley, J., Wilson, J. D., & Russell, D. W. (1992) *J. Clin. Invest. 89*, 293–300.

Liang, T., Cascieri, M. A., Cheung, A. H., Reynolds, G. F., & Rasmusson, G. H. (1985) *Endocrinol. 117*, 571–579.

McConnell, J. D., Wilson, J. D., George, F. W., Geller, J., Pappas, F., & Stoner, E. (1992) *J. Clin. Endocrinol. Metab. 74*, 505–508.

Metcalf, B. W., Levy, M. A., & Holt, D. A. (1989) *Trends Physiol. Sci. 10*, 491–495.

Mooradian, A. D., Morley, J. E., & Korenman, S. G. (1987) *Endocr. Rev. 8*, 1–28.

Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102–105.

Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.

Ohtawa, M., Morikawa, H., & Shimazaki, J. (1991) *Eur. J. Drug Metab. Pharmacokinet. 16*, 15–21.

O'Reilly, D. R., Miller, L. K., & Luckow, V. A. (1992) *Baculovirus expression vectors. A laboratory manual*, W. H. Freeman.

Petrov, V., & Lack, L. (1981) in *The Prostatic Cell: Structure and Function* (Murphy, G. P., Sandberg, A. A., & Karr, J. P., Eds.) Part B, pp 283–297, Alan R. Liss, New York.

Plapp, B. (1983) *Methods Enzymol. 87*, 469–499.

Rasmusson, G. H., Reynolds, G. F., Steinberg, N. G., Walton, E., Patel, G. F., Liang, T., Cascieri, M. A., Cheung, A. H., Brooks, J. R., & Berman, C. (1986) *J. Med. Chem. 29*, 2298–2315.

Sambrook, J., Fritsch, E. F., & Maniatis, T. A. (1989) *Molecular Cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shaprio, R., & Riordan, J. F. (1984) *Biochemistry 23*, 5234–5240.

Summers, M. D., & Smith, G. E. (1987) *A manual of methods for baculovirus vectors and insect cell culture procedures*, Texas Agric. Exp. Sta. Bull. 1555.

Thigpen, A. E., Silver, R. I., Guileyardo, J. M., Casey, M. L., McConnell, J. D., & Russell, D. W. (1993) *J. Clin. Invest. 92*, 903–910.

Vialard, J., Lalumiere, M., Vernet, T., Bredis, D., Alkhatib, G., Henning, D., Levin, D., & Richardson, C. (1990) *J. Virol. 64*, 37–50.

Weiss, P. M., & Cleland, W. W (1987) *Anal. Biochem. 161*, 438–441.

Wilson, J. D. (1980) *Am. J. Med. 68*, 745–756.