Mechanism of Time-Dependent Inhibition of 5α-Reductases by Δ^1-4-Azasteroids: Toward Perfection of Rates of Time-Dependent Inhibition by Using Ligand-Binding Energies

Gaochao Tian,*,‡ Robert A. Mook, Jr.,§ Marcia L. Moss,‡ and Stephen V. Frye§

Divisions of Biochemistry and Medicinal Chemistry, Glaxo Wellcome Research Institute, Research Triangle Park, North Carolina 27709

Received April 24, 1995; Revised Manuscript Received August 7, 1995®

ABSTRACT: Finasteride (17β-*N*-*tert*-butylcarbamoyl-4-aza-5α-androstan-1-en-3-one) is a time-dependent, apparently irreversible inhibitor of 5α-reductases, but does not fully inhibit the activity of 5α-reductase in vivo. This limited efficacy has been attributed to its slow rate of inhibition against the type-1 isozyme [Tian, G. (1995) *J. Pharm. Sci.* (in press)]. Here the feasibility of increasing the rate of inhibition of 5α-reductases by providing binding energies with the inhibitor at a site remote from the center of chemical transformation was explored. Substitution of *N*-(2,5-bis(trifluoromethyl)phenyl) group, which had been shown to benefit 6-azasteroids in the binding to 5α-reductases [Frye, S., Haffner, C. D., Maloney, P. R., Hiner, R. N., Unwalla, R. J., Batchelor, K. W., Bramson, H. N., Stuart, J. D., Schweiker, S. L., Van Arnold, J., Bickett, D. M., Moss, M. L., Tian, G., Lee, F. W., Tippin, T. K., James, M. K., Grizzle, M. K., Long, J. E., & Croom, D. K. (1995) *J. Med. Chem. 38*, 2621−2627], for the *N*-*tert*-butyl substituent at C-17 of finasteride did not perturb the mechanism of inhibition but significantly increased the rate of inhibition of type-1 5α-reductase. The rate of inhibition was too fast to determine accurately when the normal substrate testosterone was used in the progress curve analysis as this inhibition rate is approaching the value of k_{cat}/K_m for the enzyme reaction.

Time-dependent inhibitors are attractive as therapeutic agents because of their potential for high potency of inhibition for the therapeutic targets in vivo. However, the potency of time-dependent inhibitors may not be expressed in vivo if the rate of inhibition is slow compared to the rate of inhibitor elimination (Tian, 1995). Given that in vivo factors are often unpredictable and that the lifetime of a drug in the body may be short in many cases, it is generally important that the rate of inhibition for any time-dependent inhibitor be as fast as possible. Consequently, development of methods for improving rates of inhibition is highly desirable.

5α-Reductase (5AR),[1] which exists in two isoforms (Anderson & Russell, 1990; Anderson et al., 1991), catalyzes the NADPH-dependent reduction of testosterone to produce DHT, a potent androgen implicated in benign prostatic hyperplasia (Wilkin et al., 1980; Wilson, 1980; Geller, 1990). Intense research efforts have been devoted to finding inhibitors of both isoforms of the enzyme. Finasteride (Figure 1), a specific inhibitor of 5AR, has been demonstrated to inhibit 5AR apparently irreversibly in a time-dependent fashion (Faller et al., 1993; Tian et al., 1994). The time dependence has been linked to chemical transformations occurring at the Δ^1 double bond in the A-ring of finasteride after binding to the active site, suggesting site-specific modification of the enzyme (Tian et al., 1994, 1995). In clinical tests, finasteride displays a limited efficacy (Vermeulen et al., 1989, 1991; Geller, 1990; Imperato-McGinley et al., 1990; Ohtawa et al., 1991; McConnell et al., 1992), and the dose−response of DHT in plasma is biphasic with about 60% suppression at doses smaller than 5 mg, followed by a less efficient inhibition of DHT formation at doses up to at least 100 mg. It is puzzling why finasteride, an apparently irreversible inhibitor of both types of 5AR, cannot fully suppress the production of DHT in vivo. On the basis of the fact that the rate of inhibition of type-1 5AR is about 70 times slower than the value determined for inhibition of

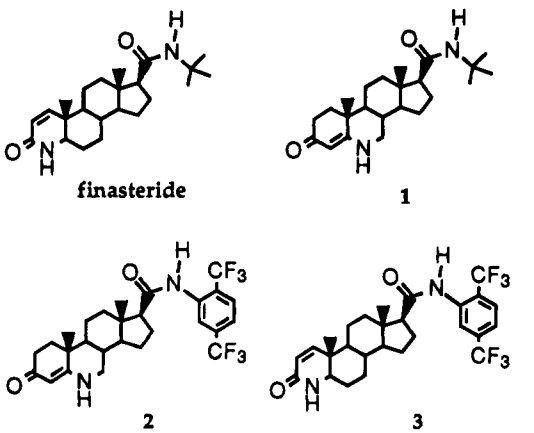

FIGURE 1: Chemical structures of finasteride, **1** (17β-*N*-*tert*-butylcarbamoyl-6-azaandrost-4-en-3-one), **2** (17β-*N*-(2,5-bis(trifluoromethyl)phenyl)carbamoyl-6-azaandrost-4-en-3-one), and **3** (17β-*N*-(2,5-bis(trifluoromethyl)phenyl)carbamoyl-4-aza-5α-androst-1-en-3-one).

* Author to whom correspondence should be addressed. Tel. (919) 990-5678. FAX: (919) 941-4320. E-mail: GT14994@glaxo.com.

‡ Division of Biochemistry.

§ Division of Medicinal Chemistry.

® Abstract published in *Advance ACS Abstracts,* October 1, 1995.

[1] Abbreviations: DHT, dihydrotestosterone; DTT, dithiothreitol; finasteride, 17β-*N*-*tert*-butylcarbamoyl-4-aza-5α-androstan-1-en-3-one; 5AR, 5α-reductase; 4MA, 17β-(*N*,*N*-diethylcarbamoyl)-4-methyl-4-aza-5α-androstan-3-one; NADPH, reduced form of β-nicotinamide adenine dinucleotide phosphate.

0006-2960/95/0434-13453$09.00/0

type-2 5AR (Tian et al., 1994), it has been hypothesized that the limited endocrinological efficacy of finasteride is due to poor inhibition of type-1 5AR in vivo (Tian, 1995). Inhibitors with an increased rate of inhibition against 5AR 1 might therefore be advantageous.

As the observed rate of inhibition by finasteride involves a relatively poor initial binding step for type-1 5AR (Tian et al., 1994), a variant of finasteride with higher binding affinity would be beneficial for the time-dependent inhibition, although there is a possibility that the structural perturbations may change the inhibition mechanism and eliminate the time dependence such that the potency is compromised. Frye et al. (1993, 1994, 1995) have shown that structural variation at C-17 of 6-azaandrost-4-en-3-ones, a class of reversible inhibitors of 5AR, with more lipophilic substituents significantly enhances the affinity of inhibitors for type-1 5AR. The *N-tert*-butyl group at C-17 of finasteride, on the other hand, is a rather poor substituent in the 6-azasteroid series in terms of type-1 5AR inhibitory potency (Frye et al., 1994). These data and the existing synthetic tools provided an excellent opportunity to explore means of enhancing the rates of time-dependent inhibition of 5AR by increasing ligand-binding energies. Here, we report that replacing the *N-tert*-butyl substituent at C-17 of finasteride with a much more lipophilic *N*-(2,5-bis(trifluoromethyl))phenyl group did not change the inhibition mechanism, but significantly increased the rate of inhibition of type-1 5AR, demonstrating the feasibility of using binding energies to accelerate inhibition rates.

METHODS AND EXPERIMENTAL PROCEDURES

Materials. [1,2,6,7-^{3}H]Testosterone (85−95 Ci/mmol) and [1,2,6,7-^{3}H]progesterone (95 Ci/mmol) were purchased from New England Nuclear. Finasteride was synthesized as described by Rasmusson et al. (1986). 17β-*N*-(2,5-Bis-(trifluoromethyl)phenyl)carbamoyl-4-aza-5α-androst-1-en-3-one was synthesized as described (Batchelor & Frye, 1995). 17β-*N-tert*-Butyl-carbamoyl-6-azaandrost-4-en-3-one and 17β-*N*-(2,5-bis(trifluoromethyl)phenyl)carbamoyl-6-azaandrost-4-en-3-one were synthesized as described recently (Frye et al., 1994, 1995). Testosterone, progesterone, NADPH, DTT, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma. All other reagents purchased were of highest quality possible.

Preparation of Type-1 and Type-2 5AR. Both human 5AR isozymes were prepared according to the procedure described previously (Tian et al., 1994).

5AR Activity Assays. Buffers, solutions, and reaction mixtures were prepared according to the procedure described previously (Tian et al., 1994) except that in this study [1,2,6,7-^{3}H]progesterone was used in the place of [1,2,6,7-^{3}H]testosterone and was kept at 20 nM. All of the assays were performed at pH 7.0, $\mu = 0.3$, and 22 °C unless noted otherwise. These reactions were initiated by addition of enzyme and quenched at desired times with ethanol. The substrate and product were separated by a C-18 reversed-phase column (4.6 × 150 mm) with a mobile phase of 35% water and 65% acetonitrile. The amount of product formation was quantitated by an in-line radiodetector (β-Ram). The reactions catalyzed by type-1 5AR were first order in progesterone ([S] = 20 nM compared to K_m = 690 nM), and the activity of enzyme was expressed as the first-order rate constant (V/K, min^{-1}) for the loss of substrate. For the reactions with 5AR 2, the substrate concentration (20 nM) was higher than its K_m (4.9 nM). Under this condition, the activity of enzyme was expressed as the initial rate (nM/min) at 20 nM substrate.

Titration of Active Sites of 5AR. Microsomes containing type-1 or type-2 5AR were serially diluted (2−0 μM). The mixtures contained either a tight binding 6-aza steroid inhibitor at 20 nM for type-1 5AR or 4MA at 4.5 nM for type-2 5AR. After preincubation for 10 min, the enzyme activity in each reaction was assayed using [1,2,6,7-^{3}H]-testosterone (20 nM) as substrate according to the procedure described above. The activity was then plotted against the reciprocal of the dilution factor, d (data not shown). The concentration of enzyme in the microsomes was calculated as the concentration of inhibitor times the dilution factor corresponding to the x-axis intercept of the plot of activity vs $1/d$. For all subsequent experiments, the enzyme concentration was kept at a value that was at least 10 times less than substrate or inhibitor if present.

Kinetic Properties of 5AR Reactions with Progesterone and Testosterone. All of the reactions were run under the same conditions as for the activity assays. The concentration of NADPH was set to 1 mM and maintained at this value by a regenerating system (Tian et al., 1994). For type-1 5AR, the concentration of radioactive progesterone was kept at 20 nM and the total concentration was adjusted with cold progesterone, which was varied from 20 nM to 20 μM. For the type-2 isozyme, the total concentration of substrate was varied from 6 to 600 nM. For the concentrations above 20 nM, the radioactive progesterone was kept at 20 nM while the total concentration was adjusted with the cold material. For the concentration range below 20 nM, the substrate was made only with the radioactive material. The ratio of product formed over the sum of substrate and product was determined by the peak areas of substrate and product that were shown in the HPLC chromatograph, and the concentration of product was then calculated by multiplying this ratio with the known total concentration of substrate. The percent of turnover was maintained at less than 20%. The initial rate, obtained at a single time point, was then calculated by taking the ratio of the concentration of product over the reaction time and expressed as nanomolar per minute. The initial rates at various substrate concentration ([S]) were fit to eq 1

$$v = \frac{V_m[S]}{K_m + [S]} \quad (1)$$

where V_m and K_m are the maximum velocity and the Michaelis−Menten constant, respectively. Kinetic constants for the reaction with testosterone were determined using assay methods described previously (Tian et al., 1994).

Progress Curve Analysis. Reaction mixtures were prepared as described above but contained an inhibitor at a desired concentration. The volume of a reaction mixture was set to 400 μL. Enzyme, at a concentration in the range 0.1−2 nM, was added to initiate the reaction. At different times, 20 μL aliquots were removed and quenched with 40 μL of ethanol. The product formation was monitored as described above.

Table 1: Summary of Kinetic Properties of 5AR with Testosterone and Progesterone, at pH 7.0, 22 °C

5AR	k_{cat}, s^{-1}	K_m, μM	K_m, μM[a]	k_{cat}/K_m, M^{-1} s^{-1}
type-1				
testosterone	0.52 ± 0.03	6.3 ± 0.6		8.2×10^4
progesterone	0.78 ± 0.04	0.69 ± 0.1	1.32 ± 0.24	1.1×10^6
type-2				
testosterone	0.000 10 ± 0.000 03	0.0063 ± 0.0030		1.6×10^4
progesterone	0.017 ± 0.001	0.0049 ± 0.0009	0.0039 ± 0.0019	3.5×10^6

[a] Determined from protection of 5AR by progesterone from the inhibition by **3**.

Table 2: Summary of Kinetic Parameters of the Inhibition of 5AR by Finasteride and **3** and IC_{50} Values of **1** and **2** at pH 7.0

5AR	k_3, s^{-1}	K_i (IC_{50}), nM	k_3/K_i, M^{-1} s^{-1} [a]	T, °C
type-1				
finasteride[b]	$(6.7 \pm 0.8) \times 10^{-4}$	360 ± 40	$(1.9 \pm 0.3) \times 10^3$ [c]	22
	$(1.4 \pm 0.1) \times 10^{-3}$	360 ± 40	$(4.0 \pm 0.6) \times 10^3$	37
3	$(6.7 \pm 0.8) \times 10^{-4}$	6 ± 1[d]	$(7.4 \pm 2.6) \times 10^4$	22
	$(1.1 \pm 0.1) \times 10^{-3}$ [e]		$(1.8 \pm 0.3) \times 10^5$	37
1		(820)[f]		22
2		(4)[g]		22
type-2				
finasteride	$(5.1 \pm 0.7) \times 10^{-3}$	69 ± 1[d]	$(8.3 \pm 0.5) \times 10^4$	22
	$(2.2 \pm 0.5) \times 10^{-2}$ [e]		$(3.2 \pm 0.4) \times 10^5$	37
3	$(3.8 \pm 1.5) \times 10^{-3}$	7 ± 3[d]	$(4.8 \pm 1.5) \times 10^5$	22
	$(4.9 \pm 1.2) \times 10^{-3}$ [e]		$(6.8 \pm 2.9) \times 10^5$	37
1		(0.9)[f]		22
2		(<0.2)[g]		22

[a] The standard errors were computed assuming that the errors from k_3 and K_i propagated independently. [b] From Tian et al. (1994). [c] This value is 2.7 times the one reported previously (Tian et al., 1994), which was calculated by using K_i = 960 nM as estimated from preincubation experiments. [d] Determined from the inhibition of initial rates given by progress curve analysis. [e] Calculated using eq 6 with the K_i value determined at 22 °C, assuming the value is not affected by changing temperature from 22 to 37 °C. This assumption is supported by the data (Tian et al., 1994) for inhibition of type-1 5AR by finasteride (see text). The standard errors were computed using an error function assuming that the error from each component of eq 6 propagated independently. [f] From Frye et al. (1994). [g] From Frye et al. (1995).

RESULTS AND DISCUSSION

A primary goal of this study was to investigate how the rate of time-dependent inhibition of 5AR by finasteride may be enhanced by providing the inhibitor with increased binding energy. To accomplish this, it is necessary that the mechanism of inhibition be elucidated and the kinetic properties determined.

Mechanism of Inhibition of 5AR by Finasteride. The dependence of the observed rate constant, k_{obsd}, on the inhibitor concentration is clearly hyperbolic for inhibition of 5AR 1 by finasteride, as demonstrated by preincubation experiments (Tian et al., 1994), indicating a two-step mechanism of inhibition. As shown by Faller and co-workers using progress curve analysis (Faller et al., 1993), the rate of inhibition of type-2 5AR appears to be linearly correlated with the inhibitor concentration for values less than 150 nM. Since the magnitude (2.3×10^5 M^{-1} s^{-1}) of the pseudo-second-order rate constant, k_{on}, calculated from the linear dependence of k_{obsd} vs [I], is much less than the diffusion limit expected for a true second-order rate constant, the authors concluded that the mechanism may still involve more than one step. Measuring rates of inhibition at higher concentrations of finasteride would aid in elucidating the kinetic mechanism. However, at higher concentrations (>150 nM) of finasteride, the rates of inhibition of 5AR 2 are fast and difficult to measure by progress curve analysis when testosterone is used as substrate. A more sensitive assay can circumvent this difficulty. Since progesterone had been reported to be a faster substrate (Thigpen et al., 1993), we decided to examine its reduction by 5AR via progress curve analysis. The kinetic parameters of 5AR reaction with testosterone and progesterone were analyzed and compared under our experimental conditions. As summarized in Table 1, although the K_m of progesterone with 5AR 2 is similar to that of testosterone, the k_{cat} is much greater and the k_{cat}/K_m is significantly higher. In the case of type-1 5AR, it is the decreased K_m that makes progesterone a faster substrate under k_{cat}/K_m conditions.

Progress curve analysis, using progesterone as substrate, was conducted at both 22 and 37 °C. Since the rate of inhibition was slower at 22 °C and easier to determine, experiments were run initially at this temperature in order to fine-tune the experimental conditions. In addition, the K_i obtained at 22 °C would be also useful for calculation of rates of inhibition obtained at 37 °C (see below), since the K_i (Table 2) for finasteride in the inhibition of type-1 5AR does not appear to change upon changing the temperature from 22 to 37 °C (Tian et al., 1994).

For the progress curve analysis of 5AR 2 reactions with progesterone in the presence of inhibitor, the substrate concentration cannot be set to a value much lower than the K_m (4.9 nM), since the sensitivity of progress curve analysis would be poor at such a low substrate concentration. Thus, the progesterone concentration was set to 20 nM, at which the progression of reaction can be readily monitored. As shown in Figure 2, the formation of dihydroprogesterone vs time was linear in the absence of finasteride (dashed line), but curved when it was present. Since the substrate turnover was held under 25%, the extensive curvature observed can only be interpreted as being caused by time-dependent inhibition. The activity of the enzyme was apparently fully inhibited at longer times, and the data were then analyzed by using eq 2 (Morrison & Walsh, 1988)

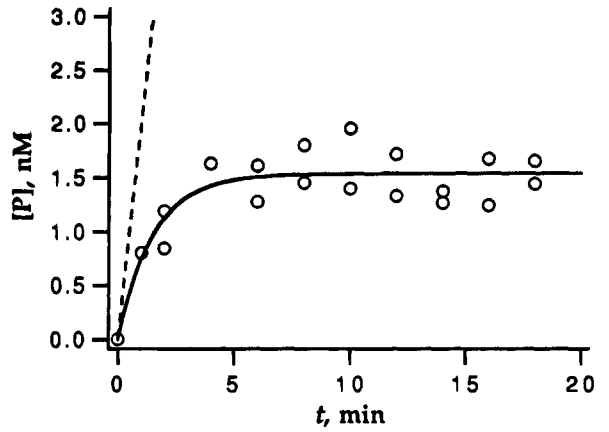

FIGURE 2: Progress curves of reaction of progesterone with type-2 5AR in the presence of 30 nM of finasteride (O). The data were analyzed by eq 2. The dashed line represents the reaction in the absence of inhibitor that was observed numerous times.

$$[P] = \frac{v_i}{k_{obsd}}(1 - e^{-k_{obsd}t}) \quad (2)$$

where [P] is the concentration of product and v_i is the initial rate of reaction at $t = 0$. With progesterone, it was possible to perform progress curve analysis at [I] > 150 nM. As shown in Figure 3A, the plot of k_{obsd} vs [I] becomes hyperbolic as the inhibitor concentration increases beyond 150 nM. In addition, the v_i was reduced upon increases in [I], indicating initial binding of inhibitor to enzyme prior to the time-dependent event. Thus, in support of the conclusion drawn by Faller et al. (1993), the mechanism of inhibition of 5AR 2 by finasteride appears to be identical to the two-step mechanism (eq 3) elucidated for inhibition of type-1 5AR (Tian et al., 1994)

$$E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_3}{\longrightarrow} EI^* \quad (3)$$

where K_i is the inhibition constant for the initial binding step and k_3 is the rate constant for the second, time-dependent step.

Finasteride Inhibits Type-2 5AR More Rapidly than Type-1 5AR. Despite the fact that the inhibition mechanisms are identical, the rates of inhibition of type-1 and type-2 5AR by finasteride differ significantly. To compare the rates of time-dependent inhibition, it is necessary to know both K_i and k_3. Fitting data (Figure 3A) according to eq 4 (Morrison & Walsh, 1988)

$$k_{obsd} = \frac{k_3[I]}{K_i(1 + [S]/K_m) + [I]} \quad (4)$$

yielded a value of (5.1 ± 0.7) × 10^{-3} s^{-1} for k_3 (Table 2) and 62 ± 1 nM for K_i. The K_i value can also be obtained by analyzing the effect of [I] on v_i. The values of v_i at varying [I] were obtained from the same progress curve analysis described above. The degree of inhibition at $t = 0$, R, which is a function of the v_i values, is given by eq 5 (Morrison & Walsh, 1988)

$$R \equiv 1 - \frac{v_i}{(v_i)_{[I]=0}} = \frac{[I]}{K_i(1 + [S]/K_m) + [I]} \quad (5)$$

where $(v_i)_{[I]=0}$ is the initial enzyme activity in the absence of inhibitor. Shown in Figure 3B is the plot of R vs [I]. Fitting the data according to eq 5 by least-squares analysis, with known [S] (20 nM) and K_m (4.9 nM, Table 1), yielded a K_i value[2] of 69 ± 1 nM (Table 2). The progress curve analysis was then conducted at 37 °C at [I] = 207 nM, and the value of k_3 was calculated to be (2.2 ± 0.5) × 10^{-2} s^{-1} (Table 2) by using eq 6.

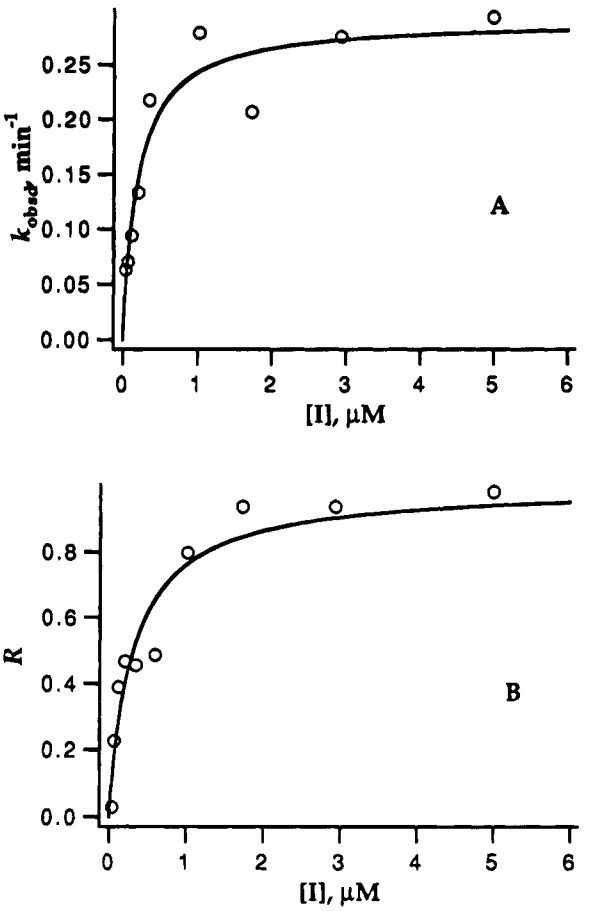

FIGURE 3: (A) The dependence of the rate of inhibition, k_{obsd}, of 5AR 2 on finasteride concentration at pH 7.0, 22 °C. Data were fitted by eq 4. (B) The effect of finasteride concentration on R, the degree of initial inhibition of 5AR 2 at pH 7.0, 22 °C. Data were analyzed by eq 5.

$$k_3 = k_{obsd}\left(1 + \frac{K_i}{[I]}\left(1 + \frac{[S]}{K_m}\right)\right) \quad (6)$$

Subsequently, the pseudo-second-order rate constant, k_3/K_i, was calculated to be (3.2 ± 0.4) × 10^5 M^{-1} s^{-1}, which is in good agreement with the value (2.3 × 10^5 M^{-1} s^{-1}) determined by Faller et al. (1993). This value is about 80 times faster than the value ((4.0 ± 0.6) × 10^3 M^{-1} s^{-1}) measured for the inhibition of type-1 5AR (Table 2).

How Useful Is It To Enhance the Rate of Inhibition of 5AR 1 by Finasteride? At first glance, an 80× difference in the inhibition rate may not seem to be very significant, especially given that the inhibition of either isozyme of 5AR by finasteride appears to be irreversible (Faller et al., 1993; Tian et al., 1994). However, when an irreversible but time-dependent inhibitor is tested in vivo, not only are the

[2] The fact that K_i values obtained from inhibition of initial rates (Table 2) and from variation of k_{obsd} are identical suggests fast equilibrium for the initial binding.

thermodynamic factors important but also the kinetic parameters are crucial for the expression of the inhibitor's potency. As has been discussed (Tian, 1995), the in vivo dose−response in terms of the activity of the target, Act%, may be predicted by eq 7

$$\text{Act\%} = 100e^{-R_{ip}} \quad (7)$$

where R_{ip}, the ratio of partitioning of inhibitor between target inhibition and inhibitor elimination pathways, is given by eq 8

$$R_{ip} = k_{obsd}/k_e \quad (8)$$

in which k_e is the rate constant for drug elimination from the body. Apparently, the R_{ip} value is a function of the concentration of inhibitor. From analysis of available literature data, at a dose of 5 mg this value can be estimated to be 13.6 for inhibition of type-2 5AR but only 0.025 for inhibition of type-1 5AR (Tian, 1995). Consequently, 5AR 2 would be completely suppressed while at most only about 2.5% reduction of the activity of type-1 5AR might occur at this dosage.

Rate Enhancement by Providing Binding Energies. The second, time-dependent step in the inhibition of both type-1 and type-2 5AR by finasteride has been shown to involve chemical transformations at the Δ^1 double bond (Tian et al., 1994; 1995), and a chemical mechanism involving Michael addition of a protein nucleophile to an inhibitor has been proposed (Tian et al., 1995). Where chemical transformations occur, the rates of inhibition may be improved by two different methods. One is to enhance the intrinsic reactivity of the functional group responsible for the time dependence. This approach has been used extensively in the past for studying mechanism-based inhibitors (Walsh, 1978, 1984). Such a method is useful when a further increase in the intrinsic reactivity of inhibitor would not at the same time jeopardize the selectivity due to potential nonspecific modifications. Alternatively, the rates of time-dependent inhibition may be increased by increasing binding energy at a site remote from the reaction center without elevation of the intrinsic reactivity of the functional group. This would be an approach analogous to the "uniform or differential binding" that is thought to operate in nature for evolution of the efficiency of enzyme catalysis (Knowles & Albery, 1977). During evolution, the binding energy derived from variation of the structure of enzyme is utilized to lower the energy of transition state and thereby increase the rate of enzymic reaction. Of course, the enhancement of rates of enzyme inhibition will have to come from variation of the inhibitor structure rather than from variation of enzyme, although the principle is the same as for the evolution of enzyme efficiency.

Substitution of *N*-(2,5-bis(trifluoromethyl)phenyl) (**2**) for the *N*-*tert*-butyl group (**1**) at C-17 of 6-azaandrost-4-en-3-ones, a class of reversible inhibitors of 5AR, has been shown (Frye et al., 1994, 1995) to increase significantly the binding affinity for 5AR (see in Figure 1 for the chemical structures of **1** and **2** and Table 2 for the IC_{50} values). Since the C-17 position is far from the A ring, a change in structure at this position was not expected to perturb the inhibition mechanism. Therefore, the strategy to take advantage of the energy for binding to type-1 5AR that is associated with the *N*-(2,5-bis(trifluoromethyl)phenyl) substituent in order to accelerate the relatively slow rate of inhibition displayed by finasteride was pursued.

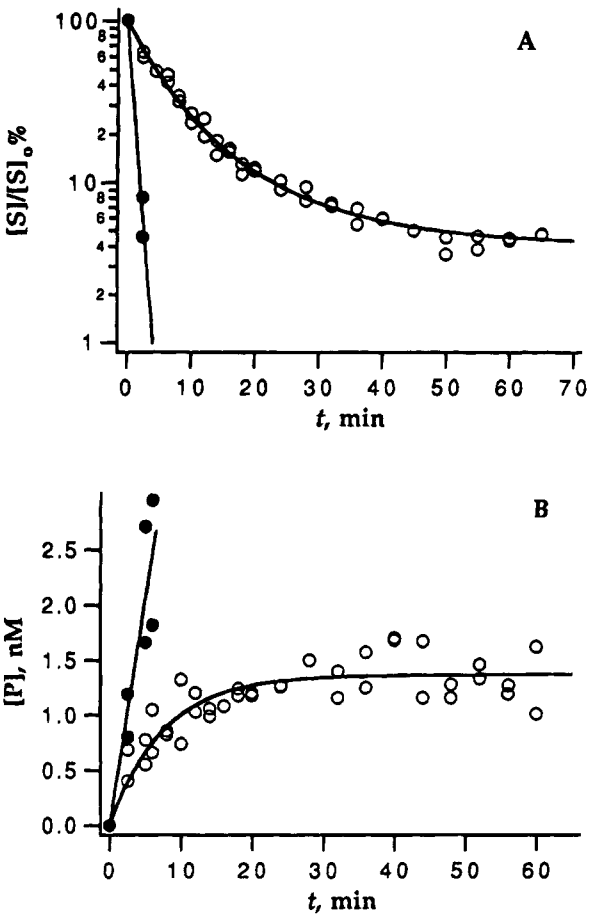

FIGURE 4: Progress curves of reaction of type-1 (A) and type-2 5AR (B) in the absence (●) or presence (○) of 30 nM **3** at pH 7.0, 37 °C. The change of percentage of substrate remaining with time was followed for the type-1 5AR reaction, and the data were analyzed by eq 9, whereas that of product formation was monitored for 5AR 2 and data were fitted to eq 2.

Mechanism of Time-Dependent Inhibition of 5AR by 17β-N-(2,5-Bis(trifluoromethyl)phenyl)carbamoyl-4-aza-5α-androst-1-en-3-one. The new compound, **3** (Figure 1), having *N*-(2,5-bis(trifluoromethyl)phenyl) in the place of *N*-*tert*-butyl at C-17 of finasteride was synthesized as described recently (Batchelor & Frye, 1995). Progress curve analyses of the time-dependent inhibition of 5AR were performed by using progesterone as substrate. For the inhibition of type-1 5AR, monitoring the change in progesterone concentration, rather than in product, is possible since it can be set to a value (20 nM) much lower than its K_m (690 nM) without deteriorating the ease of detecting substrate turnover. As shown in Figure 4A, the semilogarithmic progress curve for 5AR 1 is nonlinear, indicating time-dependent inhibition. The data were then analyzed by using eq 9 (Tian et al., 1994):

$$\frac{[S]}{[S]_0}\% = 100 \exp[(V/K)_i/k_{obsd}](e^{-k_{obsd}t} - 1) \quad (9)$$

where $(V/K)_i$ is the initial enzyme activity. Figure 4B shows the progress curve of the type-2 5AR reaction in terms of product formation vs time as for analysis of the progress curves for inhibition by finasteride. These data were then analyzed according to eq 2. In both cases, the k_{obsd} was measured as a function of [I], and the plot of k_{obsd} vs [I] is

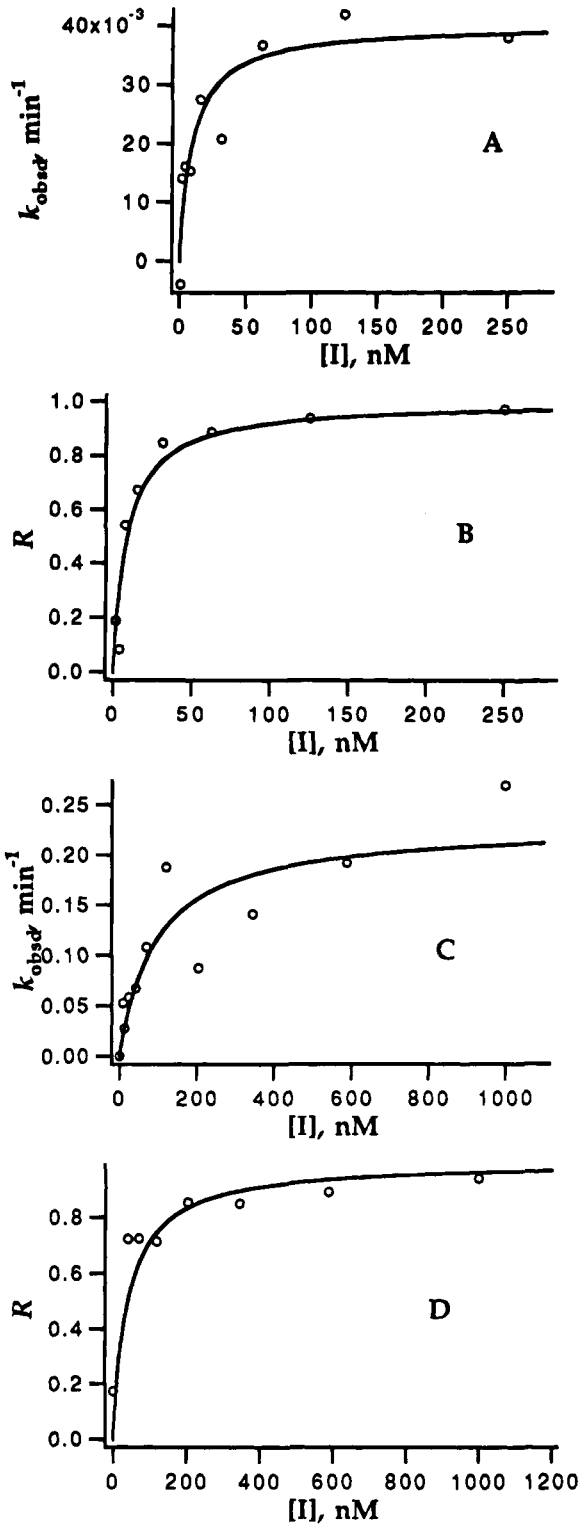

FIGURE 5: (A) The dependence of the rate of inhibition, k_{obsd}, of 5AR 1 on concentration of **3** at pH 7.0, 22 °C. Data were fitted according to eq 3 from Tian et al. (1994). (B) The effect of concentration of **3** on R, the degree of initial inhibition of 5AR 1 at pH 7.0, 22 °C. Data were analyzed by eq 1 from Tian et al. (1994). (C) The dependence of the rate of inhibition, k_{obsd}, of 5AR 2 on concentration of **3** at pH 7.0, 22 °C. Data were fitted by eq 4. (D) The effect of concentration of **3** on R, the degree of initial inhibition of 5AR 2 at pH 7.0, 22 °C. Data were analyzed by eq 5.

apparently hyperbolic, as shown in Figure 5A,C. These data indicate an identical two-step mechanism accounting for the time-dependent inhibition of both the isozymes. Analysis of k_{obsd} data according to eq 3 from Tian et al. (1994) and

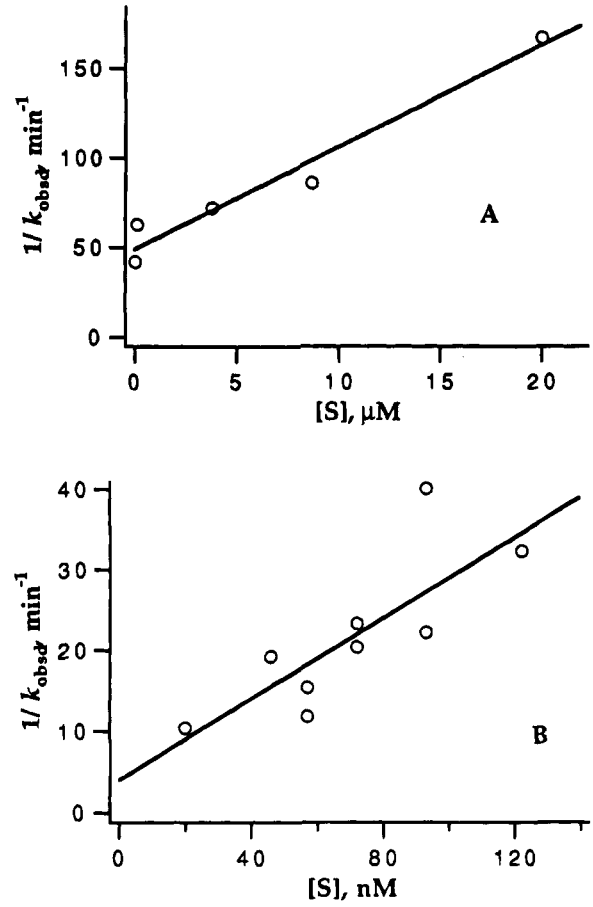

FIGURE 6: Protection of type-1 (A) and type-2 5AR (B) from **3**-induced time-dependent inhibition by progesterone at pH 7.0, 22 °C. The values of k_{obsd} were determined by progress curve analysis at various concentrations of progesterone. Values of $1/k_{obsd}$ were than replotted vs the concentration of progesterone ([S]).

eq 4 of this paper yielded K_i values[2] of 9 ± 3 and 8 ± 2 nM and k_3 values of (6.7 ± 0.8) × 10^{-4} and (3.8 ± 1.5) × 10^{-3} s^{-1} (Table 2) for type-1 and type-2 5AR, respectively.

To evaluate whether the inhibition by **3** occurs at the active site, the effect of substrate concentration on the value of k_{obsd} at a fixed concentration of inhibitor (30 nM for both types of 5AR) was investigated. As shown in Figure 6, the plots of $1/k_{obsd}$ vs [S] for both isozymes are linear. The K_m values for progesterone (Table 1) may then be obtained according to eq 10 (Tian et al., 1994):

$$K_m = \frac{K_i}{sk_3[I]} \qquad (10)$$

where s represents the slope of the plot. The K_m value obtained from the inhibition of type-2 5AR is in good agreement with the value determined independently, whereas the value for type-1 5AR is somehow higher (Table 1). However, the discrepancy may be attributable to systematic errors. These data suggest binding of **3** at the active site prior to the time-dependent event, a step that is also found to be necessary for inhibition by finasteride (Tian et al., 1994).

Taken together, we conclude that the replacement of *N-tert*-butyl with *N*-(2,5-bis(trifluoromethyl)phenyl) at C-17 of finasteride does not perturb the mechanism of inhibition of 5AR. Since time dependence has also been observed for

other Δ^1-4-azasteroids (Tian et al., 1994), this mechanism of inhibition may be generally true for Δ^1-4-azasteroids.

Toward Perfection of Rates of Time-Dependent Inhibition of 5AR. To further evaluate the K_i values for the binding step, the initial activity of 5AR was also obtained from the progress curve analyses at 22 °C as described above, and the degree of inhibition was plotted vs [I] (Figure 5B,D). The data from inhibition of type-2 5AR were analyzed according to eq 5 whereas the data obtained with type-1 5AR were evaluated according to eq 1 from Tian et al. (1994). These analyses yielded a K_i value[2] of 6 ± 1 and 7 ± 3 nM for type-1 and type-2 5AR, respectively (Table 2). The progress curve studies at a fixed [I] were then performed at 37 °C, and the k_3 values (Table 2) were calculated as described above.

As expected, the k_3 value for the inhibition of 5AR 1 is hardly perturbed by the new substituent of **3**, but the binding affinity is increased considerably (40−60 times, Table 2). As a result, the second-order rate constant, k_3/K_i (Table 2), appears to be enhanced solely from the binding energy associated with the new substituent, demonstrating the feasibility of the approach which we have taken to increase the rate of time-dependent inhibition in this particular case. For the inhibition of type-2 5AR, again the initial binding is enhanced (ca. 10×) upon the C-17 substitution, but the k_3 is somehow decreased. Nevertheless, the k_3/K_i is still increased by a factor of 2−4 (Table 2). In both cases, the rate of inhibition (Table 2) is so fast that it is approaching the rates of turnover for the enzymic reactions with natural substrates (Table 1).

Preliminary clinical tests using **3** have indicated maximum suppression (>90%) in DHT at 40 mg (D. J. Hermann, personal communications), supporting the concept and approach toward inhibiting type-1 5AR via development of faster time-dependent inhibitors.

ACKNOWLEDGMENT

Dr. D. J. Hermann is acknowledged for providing unpublished data.

REFERENCES

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

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

Batchelor, K. W., & Frye, S. V. (1995) WO95/07927, March 23, 1995.

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

Frye, S. V., Haffner, C. D., Maloney, P. R., Mook, R. A., Jr., Dorsey, G. F., Jr., Hiner, R. N., Batchelor, K. W., Bramson, H. N., Stuart, J. D., Schweiker, S. L., Van Anold, J., Bickett, D. M., Moss, M. L., Tian, G., Unwalla, R. J., Lee, F. W., Tippin, T. K., James, M. K., Grizzle, M. K., Long, J. E., & Schuster, S. V. (1993) *J. Med. Chem. 36*, 4313−4315.

Frye, S. V., Haffner, C. D., Maloney, P. R., Mook, R. A., Jr., Gorsey, G. F., Jr., Hiner, R. N., Cribbs, C. M., Wheeler, T. N., Ray, J. A., Andrews, R. C., Batchelor, K. W., Bramson, H. N., Stuart, J. D., Schweiker, S. L., Van Anold, J., Croom, S., Bickett, D. M., Moss, M. L., Tian, G., Unwalla, R. J., Lee, F. W., Tippin, T. K., James, M. K., Grizzle, M. K., Long, J. E., & Schuster, S. V. (1994) *J. Med. Chem. 37*, 2352−2360.

Frye, S. V., Haffner, C. D., Maloney, P. R., Hiner, R. N., Unwalla, R. J., Batchelor, K. W., Bramson, H. N., Stuart, J. D., Schweiker, S. L., Van Arnold, J., Bickett, D. M., Moss, M. L., Tian, G., Lee, F. W., Tippin, T. K., James, M. K., Grizzle, M. K., Long, J. E., & Croom, D. K. (1995) *J. Med. Chem. 38*, 2621−2627.

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

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

Knowles, J. R., & Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105−111.

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

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.

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.

Thigpen, A. E., Kristine M. C., & Russell, D. W. (1993) *J. Clin. Invest. 92*, 903−910.

Tian, G. (1995) *J. Pharm. Sci.* (in press).

Tian, G., Chen, S., Facchine, K. L., & Prakash, S. R. (1995) *J. Am. Chem. Soc. 117*, 2369−2370.

Tian, G., Stuart, J. D., Moss, M. L., Domanico, P. L., Bramson, H. N., Patel, I. R., Kadwell, S. H., Overton, L. K., Kost, T. A., Mook, R. A., Jr., Frye, S. V., Batchelor, K. W., & Wiseman, J. S. (1994) *Biochemistry 33*, 2291−2296.

Vermeulen, A., Giagulli, V. A., De Schepper, P., Buntinx, A., & Stoner, E. (1989) *Prostate 14*, 45−53.

Vermeulen, A., Giagulli, V. A., De Schepper, P., & Buntinx, A. (1991) *Eur. Urol. 20*, 82−86.

Walsh, C. (1978) *Annu. Rev. Biochem. 47*, 881−931.

Walsh, C. (1984) *Annu. Rev. Biochem. 53*, 493−535.

Wilkin, R. P., Bruchovsky, N., Shintka, T. K., Rennie, P. S., & Comeau, T. L. (1980) *Acta Endocrinol. 94*, 284−288.

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

BI950906V