Chemical Mechanism of the Covalent Modification of 5α-Reductases by Finasteride As Probed by Secondary Tritium Isotope Effects

Gaochao Tian,*,† Shiang-Yuan Chen,‡
Kevin L. Facchine,§ and Shimoga R. Prakash‡

Departments of Biochemistry, Drug Metabolism, and Analytical Sciences, Glaxo Research Institute Research Triangle Park, North Carolina 27709

Received October 24, 1994

Finasteride is a potent, specific inhibitor of human steroid 5α-reductases and is used for the treatment of benign prostatic hyperplasia, a ubiquitous condition in aging males.[1] The potency of finasteride in inhibiting 5α-reductases is mainly derived from its time-dependent production of an enzyme–inhibitor complex that is apparently irreversible at neutral pH.[2] The enzyme–inhibitor complex formed with 5α-reductase type 1 is stable under a variety of denaturing conditions, including 6 M Gdn-HCl, 1% SDS, boiling, etc., although the complex formed with the type 2 5α-reductase is less stable under these treatments.[3] These data argue for covalent modification of 5α-reductases by finasteride, although the chemistry of this modification has not been established. Since the Δ^1 double bond of finasteride appears to be essential in the time-dependent inhibition of the type 1 isozyme,[2b] it has been proposed that the mechanism of modification may proceed by Michael addition of an enzyme-based nucleophile at C-1 of finasteride.[2b] To further investigate the role of the Δ^1 double bond in this time-dependent event, we synthesized [1,2-^{3}H]finasteride[4] and developed methods for determining the secondary tritium isotope effects on the rate of slow inhibition. Since Michael addition causes sp^2 to sp^3 rehybridization at the unsaturated C-1 and C-2 carbon centers, an inverse isotope effect would be anticipated.

Tritium secondary isotope effects are most commonly determined by internal competition methods. In the current investigation, ^{3}H-labeled finasteride (0.50 μM) and finasteride bearing C-14 at the C-17 *N-tert*-butyl group[5] (0.01 μM) were mixed. This mixture was incubated, at pH 7.0 and 22 °C for 10 h, with either type 1 (0.12 μM) or type 2 (0.06 μM) microsomal 5α-reductase[2b] in the presence of 1 mM NADPH. Following incubation, free finasteride was removed by six consecutive extractions with $CHCl_3$. The ratios of the concentration of ^{1}H-finasteride to that of the ^{3}H species in the aqueous layer before (R_0) and after (R_e) the extraction procedure were calculated by ^{14}C/^{3}H dual counting (Table 1).

Table 1. Data from Tritium Isotope Effect Experiments[a]

isozyme	R_e	R_0	$^Tk_3$
type 1	0.102 ± 0.002	0.144 ± 0.002	0.71 ± 0.02
type 2	0.092 ± 0.002	0.148 ± 0.002	0.63 ± 0.02

[a] R_e and R_0 are the ratios of ^{14}C/^{3}H in finasteride before inhibition and after being attached to 5α-reductase, respectively. $^Tk_3$, the tritium isotope, is calculated by R_e/R_0. Each of the R_e and R_0 values is an average of four replicates.

The inhibition of both 5α-reductases by finasteride has been shown to involve two steps:[2]

$$E + I \overset{K_i}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \quad (1)$$

where K_i is the initial inhibition constant and k_3 the rate constant for the second time-dependent step. For such a two-step kinetic mechanism, and when [I] ≫ [E], the isotope effect on the second, chemical step, $^Tk_3$, can be calculated by[6]

$$^Tk_3 = R_e/R_0 \quad (2)$$

Using the values of R_0 and R_e (Table 1), the apparent kinetic tritium isotope effects ($^Tk_3$) were calculated to be 0.71 ± 0.02 and 0.63 ± 0.02 for the inhibition of 5α-reductases 1 and 2, respectively.

The large size of the apparent kinetic isotope effects indicates chemical transformations at the Δ^1 double bond, supporting the view of covalent modification of 5α-reductases by finasteride.[2b,3] Further, the inverse nature is anticipated for sp^2 to sp^3 rehybridization, and the results are therefore consistent with a chemical mechanism where a nucleophile at the enzyme active site adds to the Δ^1 double bond of finasteride after the inhibitor binds to the 5α-reductases.

It has been reported[7] that 5α-reductase also catalyzes reduction of finasteride to dihydrofinasteride (*N*-(1,1-dimethylethyl)-3-oxo-4-aza-5α-androstan-17β-carboxamide). In principle, such reduction alone could also give rise to an inverse isotope effect. Since the formation of dihydrofinasteride occurs only after the formation of the tight-binding complex (EI*),[7] the rate of formation of dihydrofinasteride is limited by the off-rate ($k_{off} = 8 \times 10^{-7} s^{-1}$)[7] of EI*. This off-rate ($t_{1/2}$ = 10 days) is too slow to influence the isotope effects determined in this study. If the reduction had any appreciable effect, it would have reduced the content of tritium attached to enzyme and therefore made the measured kinetic effects normal rather than inverse.

Calculation of equilibrium secondary isotope effects using known values on model reactions may allow further mechanistic insights. In the current study, the situation is more complicated. As assessed by tritium NMR,[4] the synthetic procedure employed unavoidably produces more than one tritium species. In general, the apparent equilibrium isotope effect, ^PK_e, with the superscript P designating for a heavy isotope such as D (deuterium), T (tritium), ^{14}C, ^{18}O, etc., arising from a mixture of n heavy atom species can be predicted by[6]

$$^PK_e = 1/\sum_i^n (f_i/^PK_i) \quad (3)$$

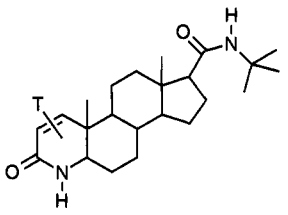
where i represent the ith isotopic species, and f_i and ^PK_i represent respectively the molar fraction of and the equilibrium isotope effect caused by the ith species.[8] Tritium finasteride used in this study was composed of three labeled species,[4] [1-^{3}H]-finasteride, [2-^{3}H]finasteride, and [1,2-^{3}H]finasteride with molar fractions of 0.10, 0.54, and 0.36, respectively (Table 2). The

† Department of Biochemistry.
‡ Department of Drug Metabolism.
§ Department of Analytical Sciences.
* Author to whom correspondence should be addressed: phone (919) 990-5678; FAX (919) 941-4320; e-mail GT14994@glaxo.com.

(1) Geller, J. *J. Am. Geriat. Soc.* **1991**,*39*, 1208–1216.
(2) (a) Faller, B.; Farley, D.; Nick, H. *Biochemistry* **1993**, *32*, 5705–5710. (b) Tian, G.; Stuart, D. N.; Patel, I. R.; Kadwell, S.; Overton, L. K.; Kost, T. A.; Mook, R.; Frye, S. V.; Batchelor, K. W.; Wiseman, J. *Biochemistry* **1994**, *33*, 2291–2296.
(3) Moss, M. L.; Tian, G., unpublished data.
(4) [1,2-^{3}H]finasteride was synthesized by reduction of finasteride with tritium gas followed by reoxidation of the reduced compound with benzeneseleninic anhydride (details to be published elsewhere). ^{14}C-Labeled finasteride was prepared by minor modifications of a published procedure.[5] Treatment of the 2-pyridylthio ester of 3-oxo-4-aza-5α-androst-1-en-17β-carboxylic acid with a mixture of [stem-^{14}C]*tert*-butylamine hydrochloride and sodium hydride in THF at 50 °C for 9 h followed by preparative HPLC purification provided finasteride with ^{14}C label at the C-17 N-*tert*-butyl group.
(5) Prakash, S. R.; Ellsworth, R. L.; Mertel, H. E.; Rasmusson, G. H. In *Proceedings of the 3rd international symposium on synthesis and applications of isotopically labeled compounds*; Baillie, T. R.; Jones, J. R.; Eds.; Elsevier Science: InnsBruck, Austria, 1989; pp 619–623.
(6) The derivation of this equation is available as supplementary material.
(7) Baginsky, W. F.; Harris, G.; Bull, H. *FASEB J.* **1994**, *8*, A638.
(8) This principle also holds for kinetic isotope effects.

0002-7863/95/1517-2369$09.00/0

Table 2. Calculation of the Equilibrium Tritium Isotope Effect on the Slow Inhibition of 5α-Reductases by Finasteride



isotopic species	$^{D}K_e$	$^{T}K_e$[a]	f_i
[2-^{3}H]finasteride	0.89[b]	0.85	0.34
[1-^{3}H]finasteride	0.75[c]	0.66	0.56
[1,2-^{3}H]finasteride	0.67[d]	0.56	0.10
mixture	0.62[e]	1.00	

[a] Calculated using the Swan equation.[12] [b] Estimated $^{D}K_e$ on the β-position of fumarate.[9] [c] Calculated after allowance for the effect of attachment to an oxygen atom.[10] [d] The product of the $^{D}K_e$ values for species with deuterium at C-1 and C-2. [e] Calculated using $^{T}K_e = 1/(f_1/^{T}K_1 + f_2/^{T}K_2 + 2f_3/^{T}K_3)$ (see text).

deuterium isotope effect at C-2 of finasteride may have a value of 0.89 as a consequence of the sp^2 to sp^3 rehybridization.[9] In addition to undergoing the bond rehybridization, the C-1 carbon of finasteride also would become attached to a heteronuclear atom as the result of the nucleophilic addition. It has been predicted that replacement of H on a carbon center with N or O would enrich deuterium at the carbon center by factors of 1.15 and 1.18, respectively.[10] If the heteronuclear atom from the attacking nucleophile is sulfur, then the enrichment of deuterium in the resulting molecules would be about 1.07.[11] Therefore, a fractionation factor of 1.18 is the highest possible for nucleophilic substitutions if the nucleophile is enzyme-based (S, N, or O). Thus, a limiting value of 0.75 (in the inverse sense) for the deuterium isotope effects arising from the C-1 position can be obtained by taking the ratio of the isotope effect caused by the sp^2 to sp^3 rehybridization (0.89) over the fractionation factor of 1.18 for the C-1 carbon becoming attached to oxygen. For the inhibitor labeled at both C-1 and C-2, the deuterium isotope effect would be 0.67, the product of the isotope effects arising individually from C-1 and C-2 (0.89 × 0.75). The corresponding tritium isotope effects (Table 2) can be calculated from the Swan relationship.[12]

A further complication is that the isotopic ratios, to be computed as the ratio of *molar* concentrations of H over T species, cannot be truly represented by $^{14}C/^{3}H$ in this study, as the dual label species [1,2-^{3}H]finasteride has twice as many counts as do the other two singly labeled compounds. To account for the difference, the molar fraction of the dual label species was artificially increased by a factor of 2 in the final calculation using eq 3. This led to a value of 0.62 (Table 2) for $^{T}K_e$.

The kinetic isotope effects (0.71 and 0.63, Table 1) are close in size to the calculated equilibrium effect (0.62, Table 2), suggesting a later, product-like transition state.[13,14] Therefore,

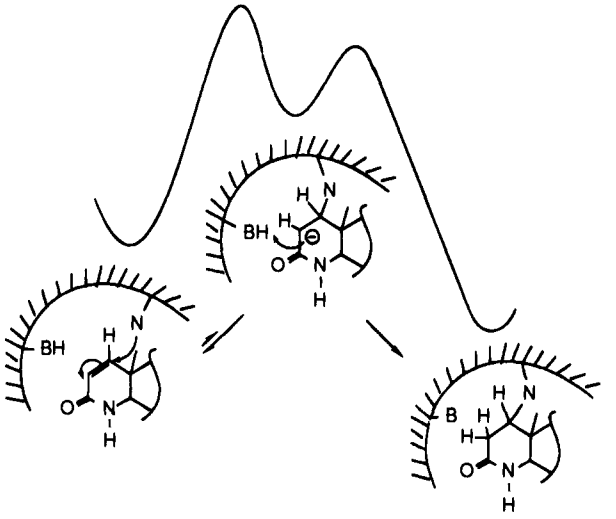


Figure 1. Energetics of proposed chemical mechanism of inhibition of 5α-reductases by finasteride. A partial, six-membered ring structure of finasteride is shown bound at the active site of 5α-reductase. In the first step of the chemical modification, an enzyme-based nucleophile (N) attacks at C-1 of finasteride to generate a carbanion intermediate. This step involves a late, high barrier transition state. The second, protonation step is fast, leading to an apparently irreversible covalent complex.

a mechanism involving only a single chemical step, where the addition of the nucleophile at C-1 and protonation at C-2 occur simultaneously, is considered unlikely as such a mechanism would be contradictory with the apparent irreversibility of the inhibition of 5α-reductases by finasteride.[2] However, the large size of the kinetic effects is consistent with a two-step mechanism in which a high barrier addition of the attacking nucleophile at C-1 precedes a low barrier protonation at C-2 (Figure 1). Such energetics allows near full expression of the equilibrium effect on the first, isotope-sensitive step without violating the observed apparent irreversibility of the overall reaction. This mechanism is supported by the fact that the rate of inhibition of 5α-reductase 1 is not appreciably retarded in a D_2O buffer (unpublished observations).

A noteworthy feature of the proposed chemical mechanism of the modification of 5α-reductases by finasteride is that the partitioning of the activated intermediate, which occurs in the mechanism-based inhibition,[15] is essentially zero. The partitioning undermines the potency of the inhibitor and possibly causes adverse effects in vivo. Considering that the safety of the drug has been assessed clinically, this study may have implications for design of novel covalent inhibitors for use as pharmaceutics.

Acknowledgment. Professor W. W. Cleland is acknowledged for useful discussions.

Supplementary Material Available: Deviation of eqs 2 and 3 (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA943445U

(9) This value was calculated for the sp^2 to sp^3 rehybridization at the β-carbon of fumarate on its hydration to form malate (Cleland, W. W. *Methods Enzymol.* **1980**, *64*, 104−125) and confirmed experimentally later (Cook, P. F.; Blanchard, J. S.; Cleland, W. W. *Biochemistry* **1980**, *19*, 4853−4858).

(10) Schimerlik, M. I.; Rife, J. E.; Cleland, W. W. *Biochemistry* **1975**, *14*, 5347−5354.

(11) Cleland, W. W., personal communication.

(12) Swan, C. G.; Stivers, E. C.; Reuwer, J. F. Jr.; Schaad, L. J. *J. Am. Chem. Soc.* **1958**, *80*, 5885−5893.

(13) Schowen, R. L. *Prog. Phys. Org. Chem.* **1972**, *9*, 275−332.

(14) Klinman, J. P. *Adv. Enzymol.* **1978**, *46*, 415−494. Cleland, W. W. *CRC Crit. Rev. Biochem.* **1982**, *13*, 385−428.

(15) Seiler, N.; Jung, M. J.; Koch-Weser, J. *Enzyme-Activated Irreversible Inhibitors*; Elsevier: Amsterdam, 1978. Abeles, R. *Chem. Eng. News* **1983**, *61*, 48−56. Walsh, C. *Annu. Rev. Biochem.* **1984**, *53*, 469−535.