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INHIBITION OF TESTOSTERONE 5α-REDUCTASE BY A PROPOSED ENZYME-ACTIVATED, ACTIVE SITE-DIRECTED INHIBITOR

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SUMMARY

RMI 18,341 (5α,20-R)-4-diazo-21-hydroxy-20-methylpregnan-3-one, was designed to be an enzyme-activated irreversible inhibitor of testosterone 5α-reductase. It produced time-dependent, apparently first-order inactivation of the enzyme, which can be antagonized by substrate, indicative of irreversible inactivation occurring at the enzyme active site. Unlike conventional 5α-steroids, RMI 18,341 has a high affinity for the enzyme:apparent $K_i = 3.5 \times 10^{-8}$M. At 25°C, formation of the reversible EI complex is not rate-limiting for enzyme inactivation, and this is expressed as saturation kinetics for the inhibition reaction. RMI 18,341 produces no inhibition of 3α-hydroxysteroid oxidoreductase of rat prostate, in contrast to other 3-keto-5α-steroids. The specificity, irreversibility and high affinity for testosterone 5α-reductase should make RMI 18,341 a useful tool in elucidation of the physiological roles of testosterone metabolites.

Testosterone 5α-reductase (NADPH: Δ^4-3-ketosteroid 5α-oxidoreductase) is found in male accessory sex organs (1,8,22) and in certain other androgen-responsive tissues, notably skin and its appendages (2-5). In these tissues, it catalyzes the reduction of testosterone to 5α-dihydrotestosterone (DHT), which is a more potent androgen than testosterone, and is preferentially bound to the nuclear receptor of these tissues (6-8). Further, 5α-reduction is an obligatory step (9) in the production of the 3α- and 3β-17β androstanediols (ADIOLS), compounds which may mediate specific androgenic functions (10,11). Testosterone 5α-reductase may thus constitute a mechanism for amplification or modification of the androgenic signal in androgen target tissues.

A number of competitive inhibitors of testosterone 5α-reductase have been described (3,12-15), of which androst-4-ene-3-one-17β-carboxylic acid (12) has been most widely investigated. So far, competitive inhibitors have not found extensive application in elucidation of the roles of 5α-reduced steroids in expression of androgenic function, due to their inability to maintain a high level of enzyme

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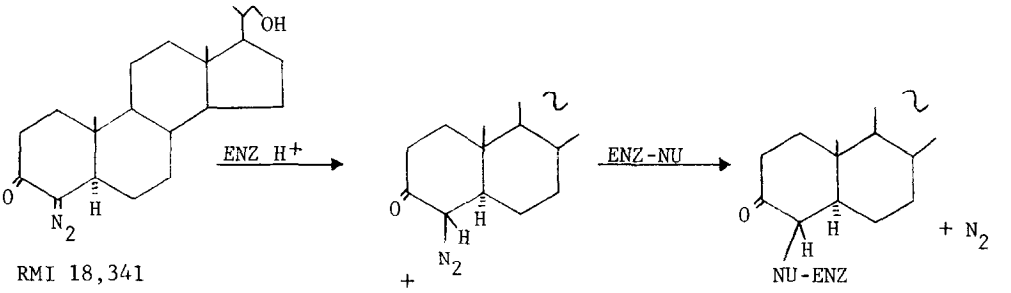

Scheme 1. Proposed Mechanism for Irreversible Inhibition of 5α-Reductase by RMI 18,341. The inhibitor represents an analog of the probable enol intermediate implicit in the reduction mechanism in which the 5α-hydrogen has already been inserted, C-4 has sp_2 hybridization, and awaits introduction of the 4-hydrogen (proton) by the enzyme. This protonation converts the inhibitor to the reactive diazonium species, which is then capable of forming a covalent bond with a nucleophilic group in or near the enzyme active site.

inhibition over an extended period of time. Two allenic secosteroids have been reported (16) to inhibit 5α-reductase non-competitively. However, these compounds also irreversibly inhibit bacterial 5-ene-3-ketosteroid isomerase, hence their specificity with regard to mammalian steroid-metabolizing enzymes may be doubtful. Although the concept of mechanism-based irreversible inhibition offers a powerful approach to the design of specific enzyme inhibitors (29), there is no precedent in this area for the inhibition of NADPH-dependent enzymes which catalyze the saturation of α,β-unsaturated ketones.

In this report we describe the effects of a specific, enzyme-activated irreversible inhibitor of testosterone 5α-reductase. Synthesis of this compound, designated RMI 18,341, is described elsewhere (17). The chemical name is (5α,20-R)-4-diazo-21-hydroxy-20-methylpregnan-3-one; the structure is shown in Scheme 1, which also outlines the mechanism by which RMI 18,341 is proposed to be activated by and react irreversibly with the enzyme.

MATERIALS AND METHODS

Chemicals. The following reagents were obtained from Sigma Chemical Company, St. Louis, Missouri: NADPH, tetrasodium salt; glucose-6-phosphate (Glc-6-P), monosodium salt; Glc-6-P-dehydrogenase, Type V; 5α-dihydrotestosterone (DHT); 3α,17β-androstanediol (ADIOL); androstane-3,17-dione; bovine serum albumin (BSA), essentially fatty acid free. [4-^{14}C]Testosterone and [1,2-^{3}H]dihydrotestosterone were obtained from New England Nuclear Corporation, and were purified by TLC (18) before use. All other reagents were analytical grade.

Rat Prostate Microsomes. Ventral prostates were dissected from Sprague-Dawley rats (150-250 gm, Charles River Breeding Laboratories, Wilmington, Maine). The minced tissue was homogenized in 5 volumes (w/v) of cold 0.25 M sucrose in a Tekmar Tissuemizer, using three 10-sec bursts. The homogenate was filtered through glass wool and the filtrate further homogenized by 10 strokes of a tight-pestle Dounce homogenizer. This was followed by sonication in a Branson sonicator for three 10-sec periods. Sequential centrifugation at 600, 9000 and 105,000 xg yielded the microsomal pellet, which was resuspended in 0.25 M sucrose-0.05 M phosphate buffer, pH 6.6, to produce a suspension equivalent to 1.5 gm of fresh tissue per ml. This preparation was used immediately, or in some cases stored at -80°C for short periods prior to use.

Rat Prostate Cytosol was prepared by the procedure described for microsomes, except that a 3:1 homogenate was used and the 105,000 xg supernatant was used directly as the source of 3α-hydroxysteroid oxidoreductase.

Time-Dependency of Inhibition. Rat prostate microsomes containing testosterone 5α-reductase were incubated with NADPH, 10^{-3}M and a generating system (Glc-6-P, 5 x 10^{-3}M and Glc-6-P dehydrogenase, 1.2 units) in 0.05 M phosphate buffer, pH 6.6, containing 0.1 percent BSA. The inhibitor was added in 25 μl DMSO to a total volume of 3 ml.

Duplicate incubates were used for each inhibitor concentration. Control incubates received DMSO. Incubations were carried out at 25°C. Portions of 0.5 ml were removed at 0, 5, 10, 20 and 40 min for assay of remaining enzyme activity. The same procedure was used for 3α-hydroxysteroid oxidoreductase, except that rat prostate cytosol was used as the enzyme source, pH was 7.4, and the inhibitor was added in 30 μl of DMSO. Sampling times were extended to 60 min due to the greater stability of this enzyme.

Testosterone 5α-Reductase Assay. [4-^{14}C]Testosterone, 1.45 x 10^5 dpm, was deposited in the assay tube in 5 μl of ethanol solution. To this was added the 0.5 ml incubate sample and NADPH (in 10 μl buffer, sufficient to produce an additional 10^{-3}M). Testosterone final concentration: 5 x 10^{-6}M. The mixture was incubated for 10 min at 25°C; enzyme activity was stopped by addition of $CHCl_3$:methanol (2:1). Extraction and separation of products by thin-layer chromatography, and measurement of radioactivity in testosterone, DHT and ADIOL have been described previously (18). Results were calculated as 5α-reduced products (DHT + ADIOL) formed per mg of microsomal protein (25) per hr or min.

3-Hydroxysteroid Oxidoreductase Assay. This assay was conducted in exactly the same manner as the testosterone 5α-reductase assay, except that the substrate was [1,2-^{3}H]DHT, added to a final concentration of 5 x 10^{-6}M, 0.1 μCi per assay. The labeled product is ADIOL.

Substrate Antagonism of Time-Dependent Inhibition. In this experiment a constant inhibitor concentration (3 x 10^{-8}M) was incubated (25°C) with varying concentrations of labeled testosterone in the presence of microsomes and NADPH (10^{-3}M), with phosphate buffer, pH 6.6, containing 0.1 percent BSA, and the generating system, as above. The incubates were sampled at 10, 30, 50 and 70 min, quenched in $CHCl_3$:MeOH, and substrate conversion to 5α-reduced products determined. Rates of conversion were calculated for each time interval, e.g. 10-30 min, and the rates plotted at the center of the time interval (20 min in this example).

Statistical Methods. Linear portions of curves shown in figures (initial portions of enzyme decay curves) represent least-squares linear regressions. Slopes were compared for statistical significance of differences by paired t-test.

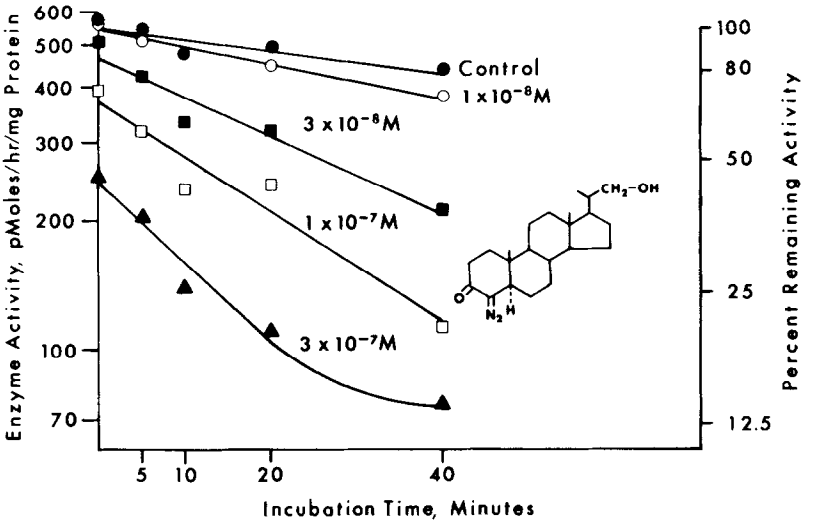

Figure 1. Time Course of Inhibition of Testosterone 5α-Reductase by RMI 18,341. ●——●, Control; ○——○, RMI 18,341, 1 x 10-8M; ■——■, 3 x 10-8M; □——□, 1 x 10-7M; ▲——▲, 3 x 10-7M

RESULTS

Time-Dependency of Inhibition. Testosterone 5α-reductase activity declined with apparent first-order kinetics for nearly 2 half-lives when the enzyme was incubated with RMI 18,341 (Figure 1). Inhibition observed at zero time represents, in part, time-dependent inhibition occurring during the 10 minutes required to assay for activity, but primarily is due to a competitive component of inhibition. Analysis of the time-dependent inhibition data was carried out by the method of Kitz and Wilson (22), as further developed by Jung and Metcalf (23). In this formulation enzyme half-life in the presence of an enzyme-activated, inactivating inhibitor is related to K_i (reversible inhibition constant) and k_{cat} (rat constant for activation of the inhibitor by the enzyme) by the equation:

$$t_{\frac{1}{2}} = \frac{0.69}{k_{cat}} + \frac{0.69}{k_{cat}} \times \frac{K_i}{I}$$

from which it follows that when $t_{\frac{1}{2}} = 0$, $K_i = -I$. It is assumed in this formulation that I>>E and that the rate of reaction of the activated inhibitor with the enzyme e.g., step 2 of Scheme 1, is much greater than the rate of the activation reaction. Figure 2 is a plot of $t_{\frac{1}{2}}$ vs. $^1/_I$, from which it was possible to calculate K_i =

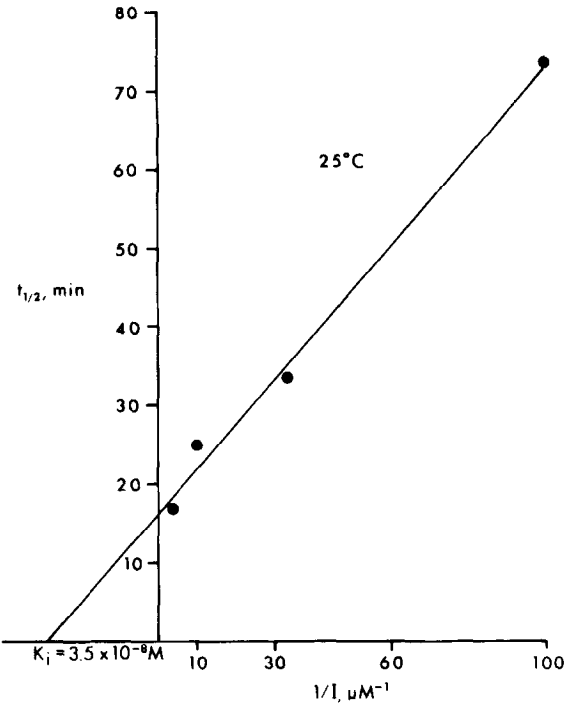

Figure 2. Enzyme Half-Life As A Function of 1/I. See text for explanation.

3.5×10^{-8}M and $k_{cat} = 7.1 \times 10^{4}$ sec^{-1}. The data exhibit saturation kinetics, i.e., at $^{1}/_{I} = 0$, $t_{\frac{1}{2}} > 0$, indicating that a step subsequent to formation of the reversible EI complex (presumed to be the activation step) is rate-limiting.

Substrate Antagonism of Time-Dependent Inhibition. Since formation of the EI complex is not rate-limiting, and the apparent K_i for the inhibitor is approximately 20 times lower than the K_m for the substrate, the time-dependent phase of inhibition is relatively insensitive to changes in substrate concentration. Nevertheless, an effect of substrate concentration on the rate of 5α-reductase inhibition could be demonstrated. Figure 3 is a plot of enzyme inactivation at constant inhibitor concentration and varying substrate concentration. Comparison of the slopes of the linear regressions at the different testosterone concentrations (Figure 3) shows that there was a significant protective effect of the substrate against time-dependent inactivation of the enzyme, indicative of involvement of the active site in inactivation of the enzyme.

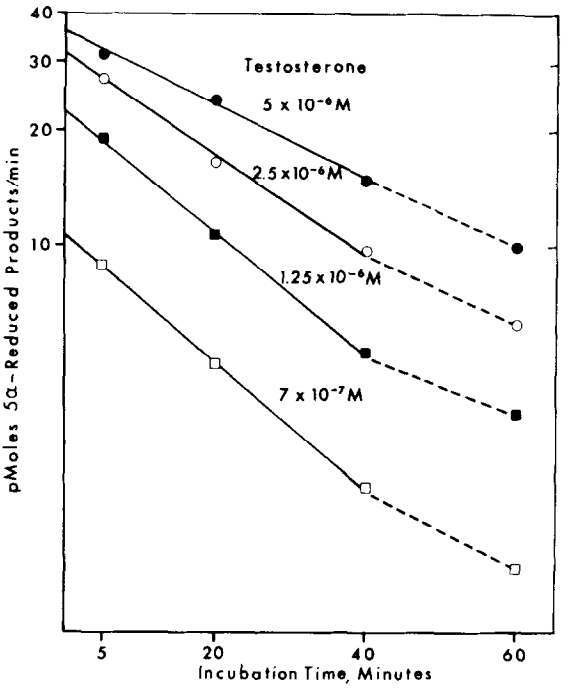

Figure 3. Effect of Substrate Concentration on 5α-Reductase Inactivation by RMI 18,341. Inhibitor Concentration, 3 x 10^{-8}M. □——□, Curve A = 7 x 10^{-7}M testosterone; ■——■, Curve B = 1.25 x 10^{-6}M; ○——○, Curve C = 2.5 x 10^{-6}M; ●——●, Curve D = 5 x 10^{-6}M testosterone. Slopes of Curves A and B are not significantly different; all other slope comparisons are different at the $P < 0.01$ level.

Reversibility and Specificity of Inhibition. Because of instability of 5α-reductase, it was not possible to utilize exhaustive dialysis as a means of investigating reversibility of inhibition. Gel filtration of Sephadex G-30 was explored as a means of removing unreacted inhibitor, utilizing the fact that at 0-4°C there is negligible time-dependency of inhibition. Under these conditions it was still not possible to remove the inhibitory effect of RMI 18,341 completely, even though uninhibited enzyme is slightly activated by this procedure. More definitive experiments on reversibility will have to await availability of radiolabeled inhibitor. When administered to rats intragastrically at 100 mg/kg, RMI 18,341 produced a reduction of prostatic 5α-reductase activity of 60 percent, which was maintained for at least 8 hours.

RMI 18,341 was found to have no effect on 3-hydroxysteroid oxidoreductases present in rat prostate cytosol at concentrations up to 10^{-6}M (data not shown). There was

no effect at concentrations up to 10^{-5}M on A-ring aromatization of testosterone by human placental aromatase preparations (28).

DISCUSSION

Progressive inhibition with time (time-dependency) is usually taken as strong evidence for irreversible enzyme inhibition (19-21), especially when coupled with a rational mechanism as is the case here. Definitive demonstration of irreversibility of the time-dependent phase of inhibition was not possible in this case because of the difficulty in removing even unreacted inhibitor from the microsomal preparation; retention of steroids in such preparations have been noted by others (24). Unfortunately, removal of 5α-reductase from its lipid matrix results in preparations which are too unstable (25) for the kinds of studies required in this work.
The ability of the substrate to antagonize the time-dependent phase of 5α-reductase inhibition by RMI 18,341 is evidence that this phase of inhibition takes place at the enzyme active site. All together, the data strongly support realization of the predicted enzyme-activated, active site-directed mechanism, although final proof will have to await demonstration of covalent attachment of the inhibitor to the enzyme.

Testosterone 5α-reductase exhibits no measurable affinity for conventional 5α-reduced steroids (12,13) and RMI 18,341 is therefore a remarkable exception ($K_i < K_m$ for testosterone). Also, RMI 18,341 exhibited no affinity for 3-hydroxysteroid oxidoreductases, substrates for which are 5α-reduced steroids, e.g. 5α-DHT. These observations support the conception of RMI 18,341 as an analog approaching transition state conformation in the 5α-reductase-catalyzed reaction.

The high affinity for, and apparent irreversibility of inhibition of 5α-reductase by RMI 18,341 suggest that this inhibitor will be a valuable tool in elucidation of the physiological roles of 5α-reduced androgens.

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