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STUDIES ON THE MECHANISM OF STEROID 5α-REDUCTASE INHIBITION BY 3-CARBOXY A-RING ARYL STEROIDS

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Summary—The mechanism of interaction between two 3-carboxy A-ring aryl steroids, 17β-(*N*,*N*-diisopropylcarboxamide)-estra-1,3,5(10)-triene-3-carboxylic acid (**1**) and 17β-(*N*-t-butylcarboxamide)-estra-1,3,5(10)-triene-3-carboxylic acid (**2**), with rat hepatic and human prostatic steroid 5α-reductases has been investigated. Dead-end inhibition plots with **1** and **2** versus both substrates (testosterone and NADPH) were linear-uncompetitive using either enzyme, while double-inhibition analyses indicated cooperative binding to enzyme between $NADP^+$ and **1** or **2**. These results were interpreted within the ordered kinetic mechanism of steroid 5α-reductase to result from the preferential association of 3-carboxy A-ring aryl steroids to the enzyme–$NADP^+$ complex. The direct displacement by **2** of a radioligand known to associate to this same enzyme form provides further support for these conclusions.

INTRODUCTION

Steroid 5α-reductase (EC 1.3.1.30) is the NADPH-dependent enzyme that catalyzes the irreversible conversion of testosterone to 5α-dihydrotestosterone. During the past several years, the identification of novel inhibitors of this enzyme has provided a potential approach toward noninvasive therapy of dihydrotestosterone mediated disorders such as benign prostatic hypertrophy (BPH), acne, and male pattern baldness [1–4]. At least one such inhibitor, a 3-oxo-4-aza steroid known as MK-906 (finasteride), is undergoing clinical trials for the treatment of BPH [5].

We recently described the 3-carboxy-17β-carboxamido androstenes (steroidal acrylates) to be potent inhibitors of human and rat prostatic steroid 5α-reductases [3, 6]. Mechanistic studies on the inhibition of steroid 5α-reductase by these 3-carboxy-androstenes, proposed as transition-state analogues, have shown that an anionic C-3 carboxylic acid can serve as a primary structural determinant toward formation of a tight complex with enzyme and $NADP^+$ [3, 6, 7]. In addition to an enzyme tolerance for differing degrees of A-ring unsaturation, comparisons between the inhibitory potency of several structural analogues within this series demonstrated no absolute requirement for the C-19 methyl group [7, 8]. In contrast, a second mode of interaction of reversible steroid 5α-reductase inhibitors has been shown to be operative with the 3-oxo-4-aza steroids involving a ternary complex comprised of enzyme and the reduced form of the cofactor, NADPH [9].

The observations with the steroidal acrylates prompted the synthesis and evaluation of a series of A-ring aromatic steroids, the 17β-carboxamido-1,3,5(10)-estratriene-3-carboxylic acids, as potential steroid 5α-reductase inhibitors. Such compounds, represented by 17β-(*N*,*N*-diisopropylcarboxamide)-estra-1,3,5(10)-triene-3-carboxylic acid (**1**) and 17β-(*N*-t-butylcarboxamide)-estra-1,3,5(10)-triene-3-carboxylic acid (**2**), were shown to inhibit the steroid 5α-reductase activities from human prostate and, to a lesser degree, that from rat prostate [10]; yet, the mode of interaction with the enzyme was not elucidated. In this paper, we report our findings on the mechanism of enzyme inhibition by compounds **1** and **2**.

MATERIALS AND METHODS

[4-^{14}C]Testosterone (55 mCi/mmol) was purchased from Amersham Corporation. Protein

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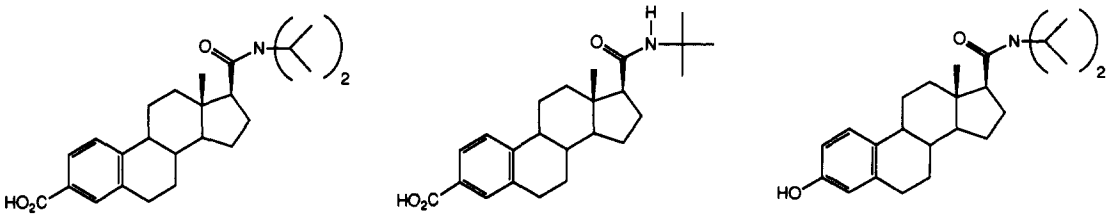


1 2 3

Scheme 1.

concentrations were estimated by the procedure of Bradford [11] using the Bio-Rad protein assay kit with bovine serum albumin as protein standard. Microsomes were prepared from rat livers [12], rat prostates [6], and surgically derived human benign hyperplastic prostatic tissue [13] as described. The separations of steroids by TLC was accomplished using prechanneled glass-backed silica gel TLC plates with preabsorbing region (Si250F-PA 19C, Baker). The syntheses of compounds **1**, **2**, and **3** have been detailed [10].

Enzyme assay and inhibition studies

Steroid 5α-reductase activity in the microsomes was determined by following the conversion of testosterone to dihydrotestosterone and 3α-androstanediol. In brief, [^{14}C]testosterone and inhibitors in ethanol were deposited in test tubes and evaporated to dryness. Buffer containing NADPH was added to the tubes and the reactions were initiated by addition of an aliquot of microsomes to final volume of 0.5 ml; activity with the microsomes from human hyperplastic prostatic chips was determined in 50 mM sodium citrate, pH 5.0 and from rat liver in 20 mM sodium phosphate, pH 6.6. As indicated in the legends, a cofactor regenerating system consisting of 1 mM glucose 6-phosphate and 0.5 units of glucose 6-phosphate dehydrogenase was included in the assays. Following 10–30 min incubations at 37°C, the reactions were quenched with 4 ml ethyl acetate containing 0.15 μmol each of testosterone, dihydrotestosterone, and androstanediol as carrier. The radiolabeled steroids were isolated, separated by TLC developing twice with 1:9 acetone–chloroform, and analyzed on a BIOSCAN imaging scanner [6, 12]. In all assays, no more that 20% of testosterone was converted to products. Enzyme activity was calculated from the percent of recovered radiolabel converted to 5α-dihydrotestosterone plus androstanediol. The specific activities of steroid 5α-reductase in microsomes from rat liver and human prostate at 1.0 μM testosterone and 200–400 μM NADPH were 5.0 nmol·(min·mg)$^{-1}$ and 20–40 pmol·(min·mg)$^{-1}$, respectively.

Direct displacement binding assays using [^{3}H]17β-(*N*,*N*-diisopropylcarboxamide)-androst-3,5-diene-3-carboxylic acid as the reference ligand were carried out as described previously [7].

Data analysis

Data from dead-end inhibition analyses were fit to appropriate rate equations as previously described [7, 12] using the programs and criteria established by Cleland [14]. Double-inhibition data were evaluated by equation (1):

$$v_i = v_0/[1 + I/K_I + J/K_J + IJ/(\beta K_I K_J)] \quad (1)$$

where v_0 and v_i are the velocities in the absence and presence of the two inhibitors at concentrations of I and J whose respective apparent dissociation constants are K_I and K_J [15]. The term β is an empirical estimate of the cooperative binding interaction between the two inhibitors on the enzyme. Experimental data was fit to equation (1) with a nonlinear regression procedure using the Marquart algorithm [16] in the SAS statistical analysis software package (SAS Institute Inc., Cary, N.C.).

RESULTS AND DISCUSSION

The initial evaluation of the inhibitory potency of the 3-carboxy A-ring aryl steroids by Dixon analysis, including compounds **1** and **2**, with microsomal-associated human prostatic steroid 5α-reductase [9] has demonstrated potent enzyme inhibition characterized by apparent inhibition constants ($K_{i,app}$) in the range of 20–200 nM. Interactions of the rat prostatic enzyme were less dramatic, with inhibition constants 5–10-fold higher than those against the human derived enzymatic activity. These observations have been confirmed in the more complete analyses with **2** presented in Table 1 where

Table 1. Inhibition of steroid 5α-reductase by 3-substituted A-ring aryl steroids

(A) Dead-end inhibition analyses

Compound	Enzyme source[a]	Variable substrate	Inhibition pattern[b]	Inhibition constant (nM)
1	RL	Testosterone	UC	$K_{ii} = 88 \pm 6$
1	RL	NADPH	UC	$K_{ii} = 172 \pm 9$
2	RL	Testosterone	UC	$K_{ii} = 53 \pm 3$
2	RL	NADPH	UC	$K_{ii} = 111 \pm 4$
2	HP	Testosterone	UC	$K_{ii} = 12 \pm 3$
2	HP	NADPH	UC	$K_{ii} = 27 \pm 2$

(B) Double inhibition analyses with NADP$^+$[c]

Compound (*I*)	Enzyme source	K_I (nM)	K_J (μM)	β
1	RL	360 ± 10	170 ± 20	0.28 ± 0.05
2	RL	110 ± 10	130 ± 20	0.42 ± 0.07

[a]Assays were conducted with microsomes from rat livers (RL) or human benign hyperplastic tissues (HP) as described under Methods; the cofactor regenerating system was included in all assays in which testosterone was the variable substrate. [b]Inhibition patterns were determined by the best fit of experimental data to linear kinetic models as described by Cleland [14]; all the data sets were best fit to the linear-uncompetitive model (UC). [c]In all double-inhibition analyses the steroidal inhibitor and NADP$^+$ are represented by *I* and *J*, respectively. Data were fit to equation (1) as described under Materials and Methods.

inhibition of the human enzyme is 4–5-fold more favorable than that with the rat liver steroid 5α-reductase. As with the inhibition of 5α-reductase by the steroidal acrylates [7, 8], the presence of an anionic C-3 substituent is critical for achieving a favorable interaction leading to enzyme inhibition. For example, compound **3**, the 3-hydroxy analogue of **1** representing a related structural series which would not be deprotonated under the experimental conditions (pK ~ 19), demonstrates relatively poor inhibition of the human ($K_{i,\,app} = 500$ nM) and the rat ($K_{i,\,app} > 5000$ nM) prostatic enzymes. Increasing the acidity of the A-ring phenol of **3** by introduction of electron-withdrawing ortho-substituents, such as with the C-2 and C-4 chloro-analogues of **3**, demonstrated mixed results supportive of both an increased affinity for steroid 5α-reductase due to lowering of the phenolic pK as well as introduction of steric intolerance at C-4 (data not shown). Clearly, a key element to the tight association of the A-ring aryl steroids to steroid 5α-reductase, like that for the steroidal acrylates, is a functionality at C-3 which is anionic under physiological or assay conditions.

As shown in Fig. 1 for compound **2** with the rat liver enzyme, the dead-end inhibition patterns are best analyzed by a linear-uncompetitive kinetic model [14] versus both testosterone and NADPH. Identical results, summarized in Table 1A, were found using compound **1** with the rat liver enzyme and **2** with the human prostatic activity. Thus, although the inhibition potency of these compounds demonstrate species differences, the mechanism of interaction with steroid 5α-reductase from both species is conserved.

Steroid 5α-reductases from both human prostate and rat liver have been shown to follow a preferentially ordered kinetic mechanism [12, 17]. The cofactor is both the first substrate to bind (NADPH) and the last product to be released (NADP$^+$) from the enzyme's surface (Scheme 1); consequently, both NADPH and NADP$^+$ independently interact with free enzyme giving rise to binary enzyme complexes to which steroidal inhibitors could associate [12]. From this kinetic scheme, the uncompetitive kinetic patterns with **1** and **2** versus both substrates must result from the preferential binding of the 3-carboxy A-ring aryl steroids to enzyme–NADP$^+$.

This proposed mechanism of interaction has been challenged by two additional experiments. First, the binding of [^{3}H]17β-(*N*,*N*-diisopropylcarboxamide)androst-3,5-diene-3-carboxylic acid to steroid 5α-reductase has been shown to depend on the presence of NADP$^+$ [7]. In a competitive binding experiment with rat liver

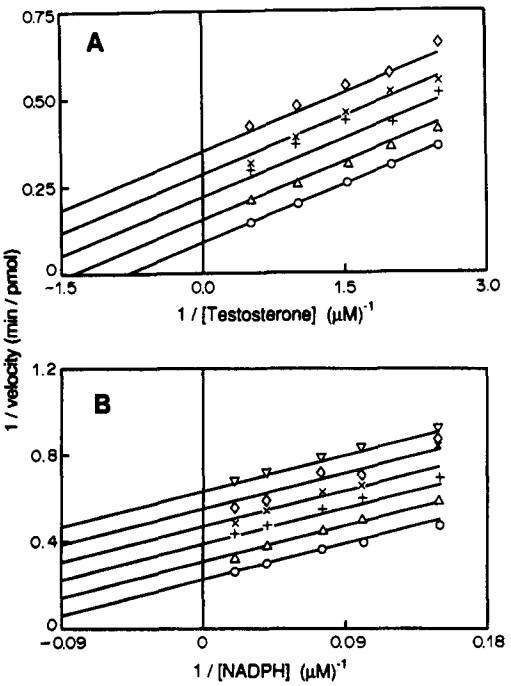
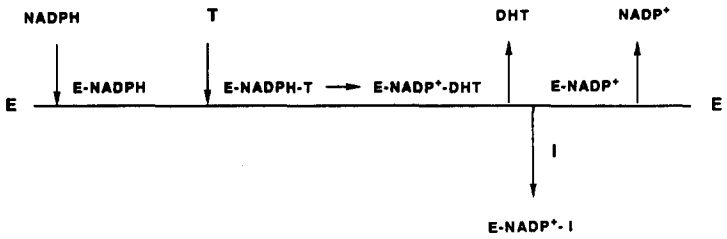


Fig. 1. Dead-end inhibition patterns of microsomal rat liver steroid 5α-reductase with compound **2**. Steroid 5α-reductase activity in rat liver microsomes was determined at variable concentrations of testosterone (panel A) or NADPH (panel B); the concentrations of **2** were 0 (○), 40 (△), 80 (+), 120 (×), 160 (◇), and 200 (▽) nM. Each set of experimental data was analyzed by the UNCOMP program [13]; kinetic constants are summarized in Table 1 (A).



Scheme 2. Proposed kinetic mechanism of steroid 5α-reductase inhibition by the 3-carboxy A-ring aryl steroids. In the scheme E, T, DHT, and I represent enzyme, testosterone, 5α-dihydrotesterone, and inhibitor (**1** or **2**), respectively.

enzyme, we have shown that **2** will displace the radioligand from the enzyme–$NADP^+$ complex with a dissociation constant ($K_d \sim 150$ nM) comparable to the inhibition constants presented in Table 1. This result supports the association of **1** and **2** to an enzyme–$NADP^+$ complex. Second, multiple inhibition data between $NADP^+$ and either **1** or **2** using the rat liver enzyme was analyzed with equation (1). As shown for the interaction between **2** and $NADP^+$ in Fig. 2, the binding of oxidized cofactor and steroid is cooperative and the values for β, presented in Table 1B, are significantly less than unity. This term (β) represents an empirical estimate of the cooperative binding interaction between the two inhibitors; when the value of β is less than unity, association of the two inhibitors to enzyme is synergistic [7]. Thus, **1** and **2** preferentially associate to the $NADP^+$ complex (Scheme 2), and upon binding of $NADP^+$ to steroid 5α-reductase, affinity of the steroid for the enzyme is enhanced.

The results from the kinetic experiments and the filtration-binding displacement assays support the association of **1** and **2** in a ternary complex with enzyme and $NADP^+$. The mechanism of inhibition by these 3-carboxy A-ring aryl steroids is the same as previously shown for the steroidal acrylates [3, 6, 7] and confirms that the C-3 anionic functionality is a primary factor in targeting an inhibitor toward the steroid 5α-reductase–$NADP^+$ complex and in determining inhibitor potency. Taken with the results of inhibition by the steroidal acrylates observed previously [7], we further conclude that the positioning and degree of A-ring unsaturation contributes a significantly lesser role in these determinants than does the C-3 anionic functionality. In contrast, the neutral 3-oxo-4-aza-steroids (e.g. MK-906) inhibit steroid 5α-reductase through formation of a ternary complex with enzyme and NADPH [9, 13]. Selected 3-carboxy A-ring aryl steroids currently are undergoing preclinical assessment for their utility in the treatment of dihydrotestosterone mediated disorders.

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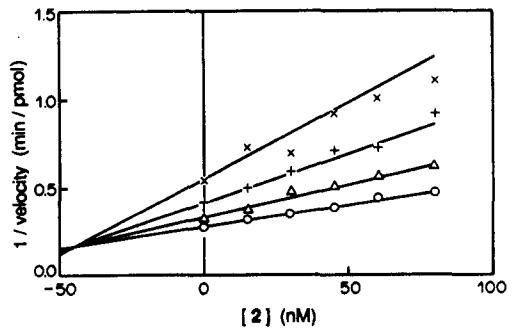

Fig. 2. Double inhibition analysis of compound **2** and $NADP^+$. Rat liver steroid 5α-reductase was evaluated at constant concentrations of testosterone (1.0 μM) and NADPH (200 μM) in the presence of variable concentrations (0–80 nM) of **2** and 0 (○), 25 (△), 63 (+), and 125 (×) μM $NADP^+$. The experimental data were fit to equation (1) as described under Methods; results from this analysis are summarized in Table 1 (B).

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