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Steroid 5α-Reductase: Comparative Study of Mechanism of Inhibition by Nonsteroids ONO-3805 and LY191704

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Two nonsteroids, ONO-3805 and LY191704, were evaluated as inhibitors of the human and rat 5α-reductases (5αR). ONO-3805 was prepared in a 12-step convergent synthesis. This compound is a potent inhibitor of the human and rat 5αRs, with more potent inhibition seen against the rat enzymes. The inhibition patterns of this compound were best fit to an uncompetitive model which suggests binding in a ternary complex with enzyme and $NADP^+$. Apparent K_i values of 27, 31, 1, and 0.5 nM versus testosterone were obtained with human type 1, human type 2, rat type 1, and rat type 2 5αR, respectively. Multiple inhibition studies with ONO-3805 and $NADP^+$ support synergistic binding of these two inhibitors with all isozymes. LY191704 was also evaluated as an inhibitor of the human and rat 5αRs. This compound is a selective, competitive inhibitor of human type 1 5αR. Poor inhibition was observed with human type 2 and rat types 1 and 2 5αR.

INTRODUCTION

Dihydrotestosterone (DHT)[1] rather than testosterone (T) is considered the primary androgenic mediator in the development of benign prostatic hyperplasia (BPH), male pattern baldness, acne, and hirsutism (*1,2*). As a consequence, considerable effort has been devoted toward the design of inhibitors of steroid 5α-reductase (5αR), the enzyme which catalyzes the conversion of T to DHT. This enzyme is found in the androgen-responsive tissues such as prostate, seminal vesicle, epididymis, and skin where DHT rather than T is the active androgen (*3–7*). Two isozymes of the enzyme are known, which are localized in different tissues (*7–10*). The ability to tissue-specifically inhibit the isozymes of 5αR has heightened interest in this enzyme as a therapeutic target.

Various steroidal and nonsteroidal inhibitors of 5αR have been reported, and several are now in human clinical study. Steroidal compounds in the Δ^1-3-oxo-4-aza series such as finasteride (Fig. 1) are potent, competitive, mechanism-based inactivators of the enzyme (*11, 12*). Finasteride, a selective inhibitor of the human type 2 5αR, reduces serum DHT by ~70% and produces beneficial effects in men with BPH (*13*). The 3-carboxy-androstadienes (Fig. 1) were designed to mimic the

[1] Abbreviations used: DHT, dihydrotestosterone; T, testosterone; 5αR, 5α-reductase; BPH, benign prostatic hyperplasia.

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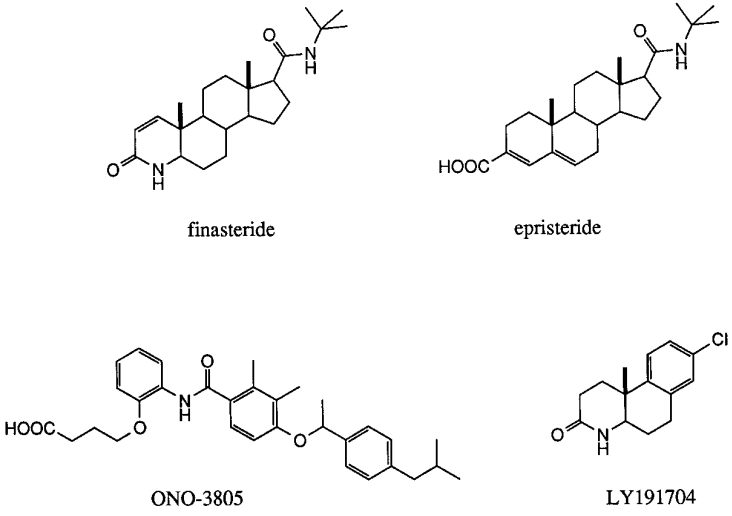


FIG. 1. Inhibitors of 5αR.

enolate transition state and display uncompetitive kinetics indicating binding to E:NADP$^+$ complex (Fig. 2) (*14*). In human clinical trials, epristeride produces similar effects on serum DHT as finasteride, albeit at higher doses (*15*). Long-term clinical effects of epristeride are not yet known.

Among the many other inhibitors of 5αR, there are two major series of nonsteroidal inhibitors: the aryl(oxy)butanoate and benzoquinolinone series. ONO-3805 (Fig. 1) was selected for study as the prototype aryl(oxy)butanoate since it is the earliest and best described in the literature (*16–20*). Although chemical synthesis and enzymological studies are only tersely disclosed, a number of *in vivo* studies have been published. ONO-3805 has been shown to be effective in reducing prostate size in

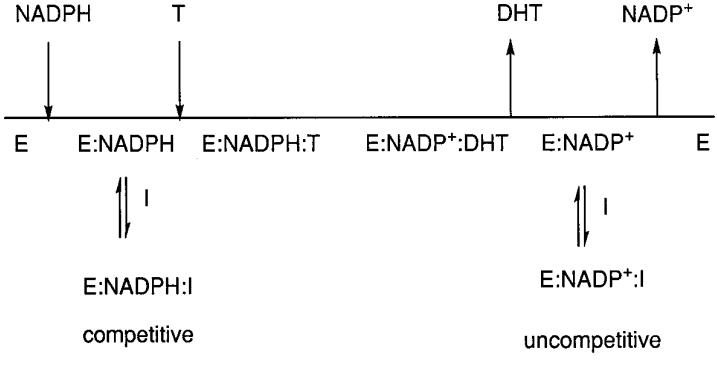


FIG. 2. Comparison of competitive and uncompetitive inhibitors of 5αR.

rats (*21, 22*). Clinical studies in humans are reportedly underway, although recent results are not available (*23, 24*). There are a host of other inhibitors in this structural class including inhibitors from Fujisawa (FK-143) (*25–29*), Kyowa Hakko Kogyo (KF18678) (*30*), and Pfizer (*31, 32*).

The second major nonsteroidal series is the benzoquinolinones, of which LY191704 (Fig. 1) is the prototype (*33, 34*). Although LY191704 is the first example of a selective human type 1 5αR nonsteroidal inhibitor, the *in vivo* evaluation of this compound has not yet been reported.

As a continuation of our studies on the mechanism of various inhibitor classes of 5αR, we present results on the mechanism of the nonsteroids ONO-3805 and LY191704. Furthermore, we describe here a synthesis of ONO-3805 which reveals details not available previously (*17*).

EXPERIMENTAL PROCEDURES

[7-^{3}H]Testosterone was purchased from New England Nuclear. Aquasol 2 and Flo-Scint II were obtained from New England Nuclear and Radiomatic Instruments, respectively. All other chemicals were obtained from Sigma Chemical Co. or Aldrich Chemicals. Column chromatography was performed on E. Merck silica gel 60 (70–230 mesh) or grade 62 (60–200 mesh). Mass spectra were obtained with a Varian MAT 731 instrument and were consistent with proposed structures for all chemical intermediates (Scheme 1).

Chemical Synthesis

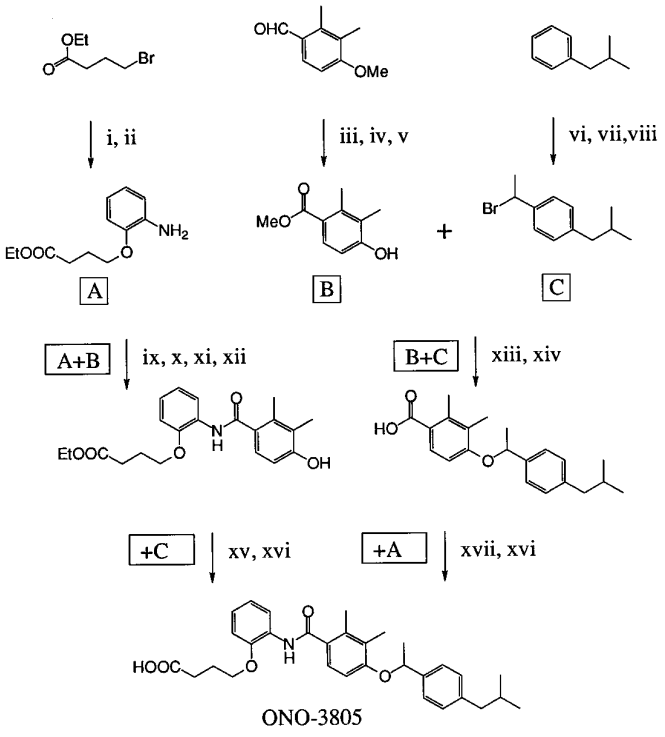
*Synthon **A***

Ethyl 4-(2-nitrophenoxy)butyrate. To a stirred solution of 2-nitrophenol (1.4 g, 10 mmol) and ethyl 4-bromobutyrate (2.1 g, 1.57 ml, 11 mmol) in dry acetone (35 ml) was added anhydrous, ground potassium carbonate (2 g, 14.5 mmol). The resultant orange-colored mixture was then heated under a nitrogen atmosphere at gentle reflux until the color due to the phenol anion had dissipated and a yellow mixture remained. Concentration of the cooled and filtered mixture gave an oil which, on flash chromatography (silica gel; ethyl acetate/hexane or methylene chloride as eluant), yielded 2.4 g (96% yield) of product as an oil.

Ethyl 4-(2-aminophenoxy)butyrate. A solution of the nitro compound (1.27 g, 5.0 mmol) in ethyl acetate (15 ml) containing 5% palladium on carbon (0.20 g) was reacted in a hydrogen atmosphere (40 psi) at room temperature until hydrogen uptake ceased. The mixture was then filtered through a layer of anhydrous sodium sulfate and concentrated *in vacuo* to yield product (1.0 g) as an oil/low-melting solid.

*Synthon **B***

4-Methoxy-2,3-dimethylbenzoic acid. To a stirred solution of 4-methoxy-2,3-dimethylbenzaldehyde (4.92 g, 30 mmol) in glacial acetic acid (25 ml) at ca. 15°C was added a chromium trioxide solution (15 ml); prepared from chromium trioxide (9.9



SCHEME 1. (i) 2-Nitrophenol, K_2CO_3, Me_2CO; (ii) H_2, 5% Pd/C, EtOAc; (iii) CrO_3, HOAc; (iv) BBr_3, CH_2Cl_2; (v) MeOH, H_2SO_4; (vi) AcCl, $AlCl_3$, $C_2H_4Cl_2$; (vii) $NaBH_4$, MeOH; (viii) HBr, PhH; (ix) $PhCH_2Br$, K_2CO_3, Me_2CO; (x) NaOH, aq. MeOH; (xi) $(COCl)_2$, CH_2Cl_2; (xii) H_2, 10% Pd/C, EtOAc-HOAc; (xiii) K_2CO_3, Me_2CO; (xiv) NaOH, aq. MeOH; (xv) K_2CO_3, Me_2CO; (xvi) NaOH, then HCl; (xvii) DCC, DMAP, CH_2Cl_2.

g, 100 mmol) dissolved in water (6 ml) and then diluted with glacial acetic acid (23 ml) dropwise over 15 min. The resultant mixture was allowed to stir with ice-bath cooling until the internal temperature dropped to 15°C, and the bath removed. After stirring overnight at ambient temperatures, the mixture was diluted with water (ca. 150 ml), aged, and filtered, and the solid was washed well with water and dried; 2.2 g of product was obtained as a gray solid.

4-Hydroxy-2,3-dimethylbenzoic acid. (*35*). To a stirred suspension of the 4-methoxy acid (1.8 g, 10 mmol) in dried dichloromethane (20 ml) at −70°C was added boron tribromide (13 ml of 1 N boron tribromide in dichloromethane) dropwise over 8 min. After stirring at this temperature for 3 h the cooling bath was removed and the resultant suspension was allowed to stir overnight at ambient temperatures. The reaction mixture was added to a stirred ice–water mixture (100 ml), the layers were separated, and the aqueous layer was exhaustively extracted with dichloromethane to yield 1.15 g product as a tan solid. More compound could be obtained by concentration of the aqueous solution followed by repeated dichloromethane extractions.

Methyl 4-hydroxy-2,3-dimethylbenzoate. To a solution of the benzoic acid (1.15 g, 6.9 mmol) in dry methanol (35 ml) was added five drops of concentrated sulfuric acid (prediluted with a small amount of dry methanol) and the mixture was heated at reflux until TLC analysis indicated the absence of starting material. The methanol was removed *in vacuo*, the residue was stirred with a mixture of water–hexane and filtered, and the resultant solid was washed with water and dried to give 1.1 g product as a tan solid.

*Synthon **C***

4-Isobutylacetophenone. To a stirred solution of isobutylbenzene (13.4 g, 15.7 ml, 0.1 mol) and acetyl chloride (7.82 ml, 0.11 mol) in dried 1,2-dichloroethane (200 ml) at +3°C was added anhydrous aluminum chloride (16.0 g, 0.12 mol) in portions over 22 min. The temperature rose to +8°C during the addition and an orange color developed. After an additional hour at ice-bath temperatures, the bath was removed and the mixture allowed to stir at ambient temperature for 2 days. After cautious addition of the reaction mixture to a stirred mixture of ice, water, and methylene chloride, the mixture was allowed to warm to room temperature and separated, and the organic layer was dried over anhydrous sodium sulfate. Concentration followed by chromatography on a silica gel column using dichloromethane as eluant gave the product as a fluid liquid in 68% yield.

1-(4-Isobutyl)phenylethanol. To a stirred solution of the acetophenone (1.76 g, 10 mmol) in dry methanol (25 ml) at +5°C was added sodium borohydride (0.38 g, 10 mmol) and the resultant mixture was stirred in an ice bath for 10 min. The bath was then removed, and the reaction mixture was stirred at ambient temperatures for 2 h. After removal of the methanol *in vacuo* the residue was covered with methylene chloride (50 ml) and treated with water. When both layers cleared, the organic layer was separated and dried over anhydrous sodium sulfate. Concentration of the filtered solution yielded 1.74 g of product as a clear liquid.

1-(4-Isobutylphenyl)bromoethane. Anhydrous hydrogen bromide was gently bubbled into an ice-cooled stirred solution of the phenylethanol (0.78 g, 4.38 mmol) in dried benzene (30 ml) for 15 min. After an additional 10 min at ice-bath temperatures, the resultant two-phase mixture was stirred at ambient temperatures for 2 h, dry nitrogen was then bubbled through the mixture to displace the excess hydrogen bromide, the mixture was allowed to settle, and the benzene layer was carefully decanted from the water droplets. Removal of the benzene *in vacuo* yielded the product (1.03 g) as a clear, pale-yellow oil that darkened with time.

*Coupling of **A** + **B***

Methyl 4-benzyloxy-2,3-dimethylbenzoate. A mixture of **B** (1.8 g, 10 mmol), benzyl bromide (1.31 ml, 11 mmol) and anhydrous ground potassium carbonate (5.0 g, 36 mmol) in dry acetone (50 ml) was heated and worked up as for **A.** Flash chromatography on silica gel (using 15% ethyl acetate–hexane as eluant) of the residue thus obtained gave 2.1 g of product as a thick oil which solidified on standing.

4-Benzyloxy-2,3-dimethylbenzoic acid. A mixture of the benzyloxy ester (1.51 g, 5.6 mmol), water (4.2 ml), 2.5 N sodium hydroxide solution (7.0 ml, 17.5 mmol),

and methanol (120 ml) was heated at gentle simmer under a nitrogen atmosphere until TLC analysis showed the absence of ester. The methanol was removed *in vacuo*, the residue was stirred with water (150 ml), and the resulting suspension was treated dropwise with 2 N hydrochloric acid (11.2 ml) and aged. Filtration followed by water washing and drying gave 1.39 g of product as a white solid.

Ethyl 4-(2-(4-benzyloxy-2,3-dimethylbenzoylamino)butanoate. To a suspension of the acid (0.256 g, 1.0 mmol) in anhydrous dichloromethane (5.0 ml) at room temperature was added oxalyl chloride (2.0 ml, 23 mmol) dropwise over 2 min. After 1.5 h the volatiles were removed *in vacuo* to yield a crust. This crust was dissolved in dichloromethane (2 ml) and added dropwise to a stirred ice-cold mixture of **A** (0.245 g, 1.1 mmol) and dry pyridine (1.0 ml) in dichloromethane (15 ml) over 1 min. After 30 min the ice bath was removed and the mixture was stirred at ambient temperatures overnight. The mixture was then transferred to a separatory funnel with dichloromethane and washed with 0.5 N hydrochloric acid (2×) and water, dried (sodium sulfate), filtered, and concentrated to 0.47 g of product.

Ethyl 4-(2-(4-hydroxy-2,3-dimethylbenzoylamino)-phenoxy)butanoate. A solution of the benzylated coupling product (0.23 g, 0.5 mmol) in ethyl acetate (20 ml) containing one small drop of glacial acetic acid and 10% palladium on carbon (0.2 g) was reduced under a hydrogen atmosphere (40 psi, room temperature) until no benzyl ether remained by TLC. The filtered and concentrated mixture was then flash chromatographed on silica gel (15–35% ethyl acetate/hexane eluant) to give 0.163 g of product as a thick oil which solidified on standing.

*Coupling of **B** + **C***

Methyl 4-(1-(4-isobutylphenyl)ethoxy)-2,3-dimethyl-benzoate. A stirred mixture of **B** (0.75 g, 4.14 mmol), **C** (1.0 g, 4.14 mmol), and anhydrous, finely ground potassium carbonate (0.75 g, 5.4 mmol) in anhydrous acetone (30 ml) was heated at reflux for 25 h, cooled, and filtered. Concentration of the filtrate followed by flash chromatography on silica gel (5% ethyl acetate/hexane as eluant) gave 0.92 g of product as a pale yellow oil.

4-(1-(4-Isobutylphenyl)ethoxy)-2,3-dimethylbenzoic acid. A stirred mixture of the resultant ester from above (0.41 g, 1.2 mmol) in methanol (25 ml), water (1 ml), and 2.5 N sodium hydroxide solution (1.3 ml, 3.25 mmol) was simmered under a nitrogen atmosphere until TLC analysis showed the absence of ester (ca. 48 h). The cooled solution was concentrated *in vacuo*, the resultant crust was stirred with water (75 ml), and the solution was filtered and acidified with 2 N hydrochloric acid. After standing for an hour, the solid was filtered, washed with water, and dried to give 0.37 g of product as a pale, cream-colored solid.

*Coupling of **C** to [**A** + **B**]*

Ethyl 4-(2-(4-(1-(4-isobutylphenyl)ethoxy)-2,3-dimethylbenzoylamino)phenoxy)-butanoate. A mixture of [**A + B**] (0.536 g, 1.44 mmol), **C** (0.40 g, 1.66 mmol), and anhydrous ground potassium carbonate (0.6 g) in acetone (40 ml) was heated and worked up as described above. Flash chromatography on silica gel (10–20% ethyl

acetate/hexane as eluant) of the residue thus obtained gave product (0.72 g) identical to that obtained from the coupling of **A** to [**B + C**].

ONO-3805: 4-(2-(4-(1-(4-Isobutylphenyl)ethoxy)-2,3-dimethylbenzoylamino)phenoxy)-butanoic acid. To a stirred, ice-cooled solution of the above ester (0.72 g, 1.35 mmol) in methanol (35 ml) was added water (seven drops) and 2.5 N sodium hydroxide solution (1.3 ml, 3.3 mmol) dropwise. After 5 min the bath was removed and the mixture was stirred under a nitrogen atmosphere at ambient temperatures until TLC analysis showed no ester (ethyl or methyl, from ester exchange) remained. The methanol was removed *in vacuo* (<25°C), the residue was dissolved in water (90 ml) and filtered, and the filtrate was acidified dropwise with 2 N hydrochloric acid. The resultant precipitate was filtered, washed well with water, and dried to give 0.66 g of product as a waxy solid; mass spectral data and TLC mobility were consistent with published data (*17*). *Anal.* Calcd for $C_{31}H_{37}NO_5$ (503.64): C, 73.93; H, 7.41; N, 2.78. Found: C, 74.09; H, 7.63; N, 2.61.

Coupling of **A** *to* [***B*** + ***C***]

Ethyl 4-(2-(4-(1-(4-isobutylphenyl)ethoxy)-2,3-dimethylbenzoylamino)phenoxy) butanoate. To a stirred mixture of [**B + C**] (0.082 g, 0.25 mmol) and **A** (0.11 g, 0.49 mmol) in dried dichloromethane (5 ml) at room temperature was added *N*,*N*,-dimethylaminopyridine (0.032g, 0.26 mmol) followed by *N*,*N*′-dicyclohexylcarbodiimide (0.069 g, 0.33 mmol), and the mixture was covered with a nitrogen atmosphere. A precipitate of *N*,*N*′-dicyclohexylurea appeared within 5 min. After stirring overnight the mixture was filtered, the filtrate was concentrated, the residue was triturated with small amounts of ether and dichloromethane separately, and the combined ether/dichloromethane extracts were concentrated. Chromatography on a predeveloped silica gel preparative plate using 22% ethyl acetate/hexane as eluant yielded 0.11 g of ONO-3805 ethyl ester as a clear oil.

Steroid 5α-R Assays

Enzyme assays were carried out as described previously (*7*) using baculovirus-expressed human 5α-Rs (*36*) and COS cell-expressed rat 5α-Rs (*37*). Assays of human and rat type 1 5α-R were conducted in 40 mM succinate/imidazole/diethanolamine buffer (SID) (*38*), pH 6.5, while 40 mM SID, pH 5.5, was used to assay the type 2 5α-Rs. NADPH was routinely used at a final concentration of 0.5 mM.

Inhibition Studies

Stock solutions of ONO-3805 and LY191704 were prepared in ethanol and diluted to the appropriate concentration in 10% ethanol. In these studies the final concentration of ethanol was less than 2.5%. IC_{50} values were determined at K_m concentrations of T using a five-point titration ranging in concentration from 0.1 to 1000 nM for type 1 5α-R or from 1 to 10,000 nM for type 2 5α-R.

Data Analysis

Kinetic data were fit to Eqs. [1]–[4] using nonlinear regression analysis. *S* reflects the concentration of T and *I* represents inhibitor concentration. K_i and K_{ii} are the kinetic constants for competitive (Eq. [2]) and uncompetitive inhibitors (Eq. [3]),

respectively. Double inhibition studies were analyzed using Eq. [4] as described by Yonetani and Theorell (*39*). For this analysis, I and J are the concentrations of the variable inhibitors and v_i and v_o are the velocities in the presence and absence of these inhibitors. K_i and K_j are the kinetic constants for the inhibitors and β is the constant which reflects the degree of interaction between I and J.

$$v = V_{max}S/(K_m + S) \quad [1]$$

$$v = V_{max}S/[K_m\,(1 + I/K_i) + S] \quad [2]$$

$$v = V_{max}S/[K_M + S(1 + I/K_{ii})] \quad [3]$$

$$v_i = v_o/[1 + I/K_i + J/K_j + IJ/(\beta K_i K_j)] \quad [4]$$

RESULTS

Chemistry

ONO-3805 comprises three substituted aryl rings: ring A, an aminophenoxybutanoate; ring B, an alkyl-*p*-hydroxybenzamide, and ring C, a *p*-isobutylbenzyl ether. Synthesis of three synthons was devised to support a convergent synthesis of ONO-3805; synthon **A**, ethyl 4-(2-aminophenoxy)butanoate, from 2-nitrophenol and ethyl γ-bromobutyrate; synthon **B**, methyl 2,3-dimethyl-4-hydroxybenzoate, from 2,3-dimethyl-4-methoxybenzaldehyde; and synthon **C**, 1-(4-isobutylphenyl)bromoethane from isobutylbenzene.

In the convergent connection of the three pieces, synthon **A** was coupled to **B** with powdered potassium carbonate in acetone, after protection of the synthon **B** phenol as a benzyl ether and conversion of the acid to the acid chloride. The connection to synthon **C** was then undertaken with anhydrous potassium carbonate in acetone followed by saponification of the ethyl ester to afford ONO-3805. Alternatively, synthon **B** could be coupled to **C** with anhydrous potassium carbonate, the ester saponified, and the product then covalently attached to synthon **A** with carbodiimide in the presence of dimethylaminopyridine followed by saponification of the ester to afford the final product identical in all respects to the material described in the ONO patents (**17**).

Inhibition of 5αR by ONO-3805

The effect of ONO-3805 on the conversion of T to DHT by the human and rat 5αRs is presented in Table 1. IC_{50} values listed in Table 1 represent the concentration of inhibitor required to achieve 50% inhibition of the conversion of T to DHT at K_m levels of T. These studies were conducted at K_m levels of T to minimize effects of differences in mechanism of inhibition on apparent potency. ONO-3805 is an inhibitor of human types 1 and 2 5αR with IC_{50} values of 9–10 nM. Surprisingly, IC_{50} values are >11-fold higher with the human 5αRs indicating ONO-3805 is more potent against the rat isozymes of this enzyme.

TABLE 1
Inhibition of Human and Rat 5αR's by ONO-3805 and LY191704

Isozyme	K_mT (μM)	IC_{50} (nM)	
		ONO-3805	LY191704
Human			
Type 1 5αR	0.3	9.4	3.8
Type 2 5αR	7.5	8.9	>1000
Rat			
Type 1 5αR	1.7	0.8	>1000
Type 2 5αR	0.15	<0.1	390

Mechanism of Inhibition by ONO-3805

ONO-3805 is a potent nonsteroidal inhibitor of both human types 1 and 2 5αR. As presented in Fig. 3, the kinetic patterns obtained with this compound with human type 2 5αR were best fit to a linear uncompetitive model when both T (K_{ii} = 31 ± 2 mM; K_m = 0.63 ± 0.04 mM) and NADPH (K_{ii} = 118 ± 6.8 nM, K_m = 1.7 ± 0.14 mM, data not shown) are used as variable substrates. Uncompetitive inhibition by ONO-3805 was also obtained using human type 1, rat type 1, and rat type 2 5αR as sources of enzyme. However, the magnitude of the inhibition constants

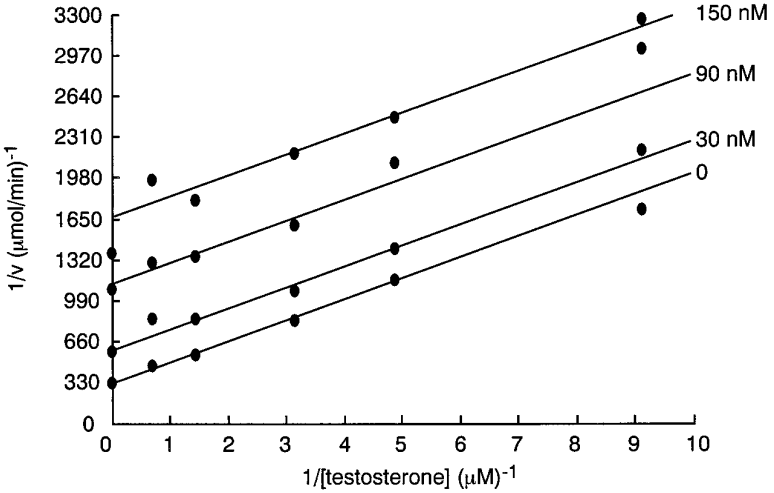


Fig. 3. Uncompetitive inhibition of 5αR by ONO-3805. Human type 2 5αR was assayed with 0.1–3 μM ^{3}H-T in the presence of ONO-3805 at concentration indicated. Following a 30-min incubation, the mixture was quenched with cyclohexane:ethylacetate and T separated from DHT by HPLC as previously described. The data were fit by nonlinear regression to Eq. [3] to give K_m = 0.63 ± 0.04 μM and K_i = 31 ± 2.0 nM. (●) Experimental data, (—) theoretical line.

TABLE 2
Inhibition Analysis of Human and Rat 5αRs by ONO-3805

Enzyme	Variable substrate	K_m (μM)	K_i (nM)	Pattern
Human				
Type 1 5αR	T	17 ± 1.6	27 ± 1.8	UC[a]
Type 2 5αR	T	0.63 ± 0.04	31 ± 2.0	UC
Rat				
Type 1 5αR	T	3.6 ± 0.4	0.7 ± 0.05	UC
Type 2 5αR	T	0.1 ± 0.01	0.5 ± 0.1	UC

[a] UC, uncompetitive.

varied >20-fold with the different isozymes. The values obtained in these experiments are included in Table 2 for comparison.

Uncompetitive inhibition, when interpreted in the context of the ordered binding of substrates by 5αR, indicates that ONO-3805 binds to the E:NADP$^+$ complex (Fig. 2). Multiple inhibition studies as originally described by Yonetani and Theorell were undertaken to support that the inhibitor binds to this form of the enzyme (*39*). In these studies ONO-3805 and NADP$^+$ as second inhibitor were varied at constant levels of T and NADPH and the data were fit to Eq. [4]. In this analysis,

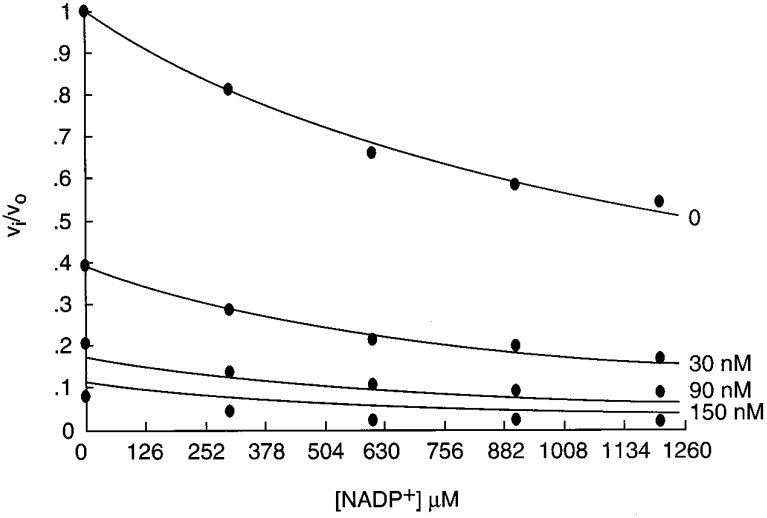


FIG. 4. Multiple inhibition analysis of 5αR with ONO-3805 and NADP$^+$. Human type 2 5αR was assayed at K_m levels of T and NADPH in the presence of variable levels of ONO-3805 and NADP$^+$. The data were fit by nonlinear regression to Eq. [4] to give K_i = 20 ± 1.4 nM and K_j = 1300 ± 70 μM for ONO-3805 and NADP$^+$, respectively. In this analysis β = 0.5 ± 1 reflecting synergistic binding of ONO-3805 and NADP$^+$. (●) Experimental data, (—) theoretical line.

TABLE 3
Multiple Inhibition Analysis

Enzyme	*I*	*J*	K_i (nM)	K_j (μM)	β
Human					
Type 1	ONO-3805	$NADP^+$	14 ± 1.0	1200	0.5 ± 0.1
Type 2	ONO-3805	$NADP^+$	20 ± 1.4	1300 ± 70	0.5 ± 0.1
Rat					
Type 1	ONO-3805	$NADP^+$	1.1 ± 0.1	79 ± 6	0.4 ± 0.1
Type 2	ONO-3805	$NADP^+$	0.3 ± 0.04	90 ± 12	0.5 ± 0.1

β reflects the degree of cooperativity between the two inhibitors. If $\beta < 1$, there is a positive interaction between the inhibitors, indicating that they bind synergistically with respect to each other. As β approaches infinity (∞), the inhibitors bind to the enzyme in a mutually exclusive manner. Results of double-inhibition studies with human type 2 5αR, ONO-3805, and $NADP^+$ are presented in Fig. 4, which yielded $K_i = 20 \pm 1.4$ nM, $K_j = 1300 \pm 70$ μM, and $\beta = 0.5$ (see also Table 3). This result is consistent with the synergistic binding of ONO-3805 and $NADP^+$ to the enzyme as expected for an uncompetitive inhibitor of 5αR. Similar results were obtained with human type 1 5αR as well as rat types 1 and 2 5αR (Table 3).

Inhibition by LY191704

LY191704 represents another series of nonsteroidal inhibitors of 5αR. As indicated in Table 1, LY191704 is a potent, selective inhibitor of the human type 1 5αR. Poor inhibition of human type 2 and rat types 1 and 2 5αR was observed.

Given the structural similarity of the A and B ring of the benzophenone to the azasteroid, it is reasonable to assume that LY191704 can compete with T for the substrate binding site on the enzyme. If this assumption is correct, then LY191704 should be a competitive inhibitor of the human type 1 5αR versus T. The mechanism of inhibition of this compound was reinvestigated to probe the mode of binding to 5αR. Despite previous reports of noncompetitive inhibition of the human type 1 5αR (*33, 34*), the data in Fig. 5 are best fit to competitive inhibition by LY191704 with $K_i = 4.6 \pm 0.3$ nM.

DISCUSSION

In the present study we have compared the mechanism of inhibition of human and rat 5αRs by two nonsteroidal inhibitors. The results indicate that ONO-3805 inhibits both human types 1 and 2 5αR while LY191704 selectively inhibits human type 1 5αR. Large differences are observed in IC_{50} values for human and rat 5αRs with these two nonsteroids. Although species differences in apparent inhibitor potency are well established in the steroidal series of inhibitors such as 4-azasteroids (e.g., finasteride) (*40*) and 3-carboxyandrostadienes (e.g., epristeride) (*41*), this is

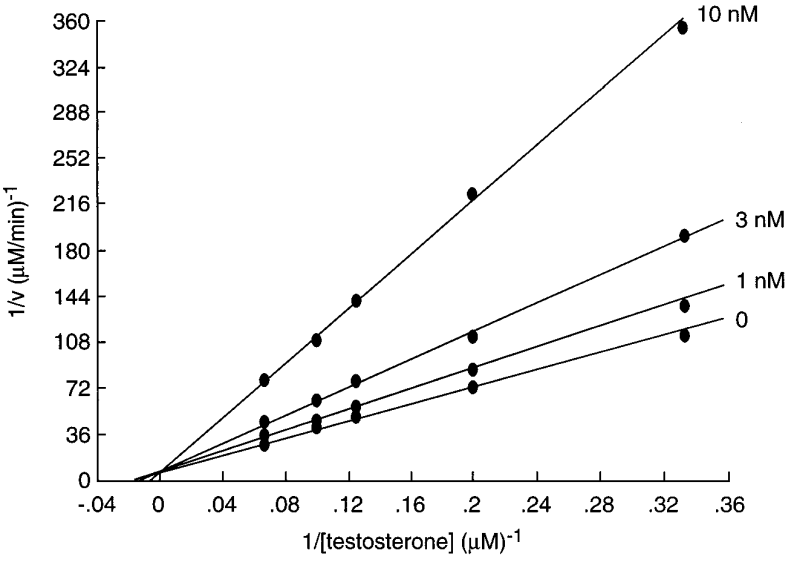


FIG. 5. Competitive inhibition human type 1 5α-R by LY191704. Human type 1 5α-R was assayed with 1–15 μM ^{3}H-T in the presence of LY191704 at the concentrations indicated. Following a 20-min incubation, the mixture was quenched with cyclohexane:ethylacetate and T separated from DHT by HPLC as previously described. The data were fit by nonlinear regression to Eq. [2] to give K_m = 4.3 ± 0.7 μM and K_i = 4.6 ± 0.3 nM. (●) Experimental data, (—) theoretical line.

the first direct comparison of the inhibition of human and rat isozymes of 5α-R by nonsteroidal inhibitors. From the results in Table 1, it is clear that differences in potency and isozyme selectivity between human and rat presents a challenge in the preclinical evaluation of these compounds.

Although the results in this report strongly support an uncompetitive mode of inhibition by ONO-3805, it is worth noting that there are conflicting reports concerning the mechanism of inhibition ONO-3805 and another related nonsteroid, FK143. Nagamoto *et al.* (*42*) reported that ONO-3805 is noncompetitive with respect to inhibition of rat pituitary 5α-R, which they concluded was the type 1 5α-R. Additionally, Hirosumi *et al.* (*28*) reported that FK143 (4-[3-[3-[bis(4-isobutylphenyl)methylamino]benzoyl]-1*H*-indolyl-1-yl]butyric acid) inhibits both the rat and human prostatic 5α-Rs in a noncompetitive fashion. The difference in the mode of inhibition compared to that reported in this report may reflect the difficulty in carrying out complex kinetic experiments with tissue sources of 5α-R containing relatively low levels of the enzyme of interest.

Our finding of uncompetitive inhibition by ONO-3805 suggests that this compound binds to the enzyme in a ternary complex with $NADP^+$ (Fig. 2). Multiple inhibition with studies with $NADP^+$ support synergistic binding of ONO-3805 with $NADP^+$, as expected for a compound with this mode of inhibition. In this analysis, a $\beta < 1$, the factor which reflects the degree of cooperativity between the inhibitors,

indicates that inhibition by ONO-3805 is enhanced in the presence of $NADP^+$. It is reasonable to assume that ONO-3805 forms a charge transfer complex with $NADP^+$ and in effect mimics the enolate. Similar results have been reported with other carboxylic acid steroidal and nonsteroidal inhibitors of 5αR including epristeride, benzophenonecarboxylic acid, and KF18678 ((*E*)-4,4′-{2-[[3-(indol-5-yl)-1-oxo-2-butenyl]amino]-difluorobenzhydryl} butyric acid), another of the aryl-butanoate class related to ONO-3805 (*14, 30, 43*). This strongly suggests that carboxy groups of the members of the nonsteroidal arylbutanoate series may overlay the carboxy group of the carboxyandrostadiene series in their charge transfer complex interactions with $NADP^+$.

As indicated previously, LY191704 is the most widely studied of the nonsteroidal inhibitors of 5αR. Despite this fact, it has not previously been described as a competitive inhibitor of the human type 1 5αR. The reason for the discrepancy in the mode of inhibition as reported originally by Hirsch *et al.* is not clear at present (*34*). However, as presented in Fig. 5, this inhibitor clearly displays competitive inhibition of the human type 1 5αR versus T. This finding may suggest that the type 1 isozyme recognizes the benzoquinolone as an abbreviated steroid rather than as a nonsteroid, thus allowing it to bind at the substrate binding site. This result may have implications for other tricyclic inhibitors of 5αR. Recently, a tricyclic aryl acid (7-bromo-9,10-dihydrophenanthrene-2-carboxylic acid) was described as a potent selective inhibitor of the human type 1 5αR (*44*). It is reasonable to assume that addition of the carboxy group allows the inhibitor to form a complex with enzyme and $NADP^+$, as is well established for epristeride (*45*). If so, the mode of inhibition will change from competitive to uncompetitive as found with 4-azasteroids and diene acids.

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REFERENCES

1. Imperato-McGinley, J., Guerrero, L., Gautier, T., and Peterson, R. E. (1974) *Science* **27,** 1213–1215.
2. Peterson, R. E., Imperato-McGinley, J., Gautier, T., and Sturla, E. (1977) *Am. J. Med.* **62,** 170–191.
3. Anderson, K. M., and Liao, S. (1968) *Nature* **219,** 277–279.
4. Bruchovsky, N., and Wilson, J. D. (1968) *J. Biol. Chem.* **243,** 2012–2021.
5. Takayasu, S., and Adachi, K. (1972) *J. Clin. Endocrinol. Metab.* **34,** 1098.
6. Wilson, J. D., and Walker, J. D. (1969) *Clin. Endocrinol. Metab.* **34,** 1098–1101.
7. Harris, G., Azzolina, B., Baginsky, W., Cimis, G., Rasmusson, G. H., Tolman, R. L., Raetz, C. R. H., and Ellsworth, K. (1992) *Proc. Nat. Acad. Sci. USA* **89,** 10787–10791.
8. Andersson, S., Bishop, R. W., and Russell, D. W. (1989) *J. Biol. Chem.* 16249–16255.
9. Andersson, S., Berman, D. M., Jenkins, E. P., and Russell, D. W. (1991) *Nature* **354,** 159–161.

10. Thigpen, A. E., Silver, R. I., Guileyardo, J. M., Casey, M. L., McConnell, J. D., and Russell, D. W. (1993) *J. Clin. Invest.* **92,** 903–910.
11. Bakshi, R. K., Rasmusson, G. H., Patel, G. F., Mosley, R. T., Chang, B., Ellsworth, K., Harris, G. S., and Tolman, R. L. (1995) *J. Med. Chem.* **38,** 3189–3192.
12. Bull, H. G., Garcia-Calvo, M., Andersson, S. A., Baginsky, W. F., Chan, H. K., Ellsworth, D., Miller, R., Stearns, R. A., Bakshi, R. K., Rasmusson, G. H., Tolman, R. L., Myers, R. W., Kozarich, J. W., and Harris, G. S. (1996) *J. Am. Chem. Soc.* **118,** 2359–2365.
13. Gormley, G. J. (1995) *Biomed. Pharmacother.* **49,** 319–324.
14. Levy, M. A., Brandt, M., Heys, R., Holt, D. A., and Metcalf, B. W. (1990) *Biochemistry* **29,** 2815–2824.
15. Audet, P., Nurcombe, H., Lamb, Y., Jorkasky, D., Lloyd-Davies, K. Morris, R. (1993) *Clin. Pharm. Therapeut.* **53,** 231.
16. Nakai, H., Terashima, H., and Arai, Y. (1988) Novel Cinnamoylamide Derivs Have 5α-Reductase Activity, Ono Pharmaceutical Co. *Chem. Abstr.* **110**(13), 1144752.
17. Nakai, H., Terashima, H., and Arai, Y. (1988) Benzoylaminophenoxybutanoate derivs., Ono Pharmaceutical Co. *Chem. Abstr.* **110**(23), 212384t.
18. Nakai, H., Terashima, H., and Arai, Y. (1988) New N-Subst'd and Ring Subst'd 2- or 3-Methylcinnamoylamide Cmpd Having Inhibitory Activity on 5α-Reductase, Ono Pharmaceutical Co. *Chem. Abstr.* **111**(3), 23231a.
19. Nakai, H., Terashima, H., and Arai, Y. (1988). New 4-(2-Benzoylamino-phenoxy, thio or sulphinyl)Butanoic Acids Useful as 5α-Reductase Inhibitors for Treating e.g. Alopecia, Ono Pharmaceutical Co. *Chem. Abstr.* **111**(1), 7074w.
20. Nakai, H., Konno, M., Kosuge, S., Sakuyama, M. T., Toda, M., Arai, Y., Obata, T., Katsube, N., Miyamoto, T., Okegawa, T., and Kawasaki, A. (1988) *J. Med. Chem.* **31,** 84–91.
21. Imai, K., Takahashi, O., Watanabe, K., Nakazawa, Y., Nakata, S., and Yamanaka, H. (1991) *Acta Urol. Jpn.* **37,** 1669–1676.
22. Takahashi, O., Imai, K., Watanabe, K., Nakazawa, Y., Nakata, S., Kurita, M., Saito, Y., Kubota, H., and Yamanaka, H. (1992) *Acta Urol. Jpn.* **38,** 305–310.
23. Anonymous (1990) Epalrestat's Importance for Ono, *Scrip.* 1514, **May 16,** p. 32.
24. Anonymous (1991) Ono's Full-Year Results, *Scrip*. 1634, **July 17,** p. 14.
25. Okada, S. Sawada, K., Kuroda, A., Watanabe, S., and Tanaka, H. (1993) Indole Derivative as Testosterone 5-Alpha-Reductase Inhibitors, Fujisawa Pharmaceutical Co. *Chem. Abstr.* **120**(11), 134279c.
26. Hirosumi, J. Nakayoma, O., Kojo, H., Takahashi, S., Chida, N., and Notsu, Y. (1994) *Jpn. J. Pharmacol.* **64**(Suppl. 1), 287.
27. Kojo, H. Nakayama, O., Hirosumi, J., Chida, N., Notsu, Y., and Okuhara, M. (1995) *Mol. Pharmacol.* **48,** 401–406.
28. Hirosumi, J. Nakayama, O., Fagan, T., Sawada, K., Chida, N., Inami, M., Takahashi, S., Kojo, H., Notsu, Y., and Okuhara, M. (1995) *J. Steroid Biochem. Mol. Biol.* **52,** 357–363.
29. Hirosumi, J. Nakayama, O., Chida, N., Inami, M., Fagan, T., Sawada, K., Shigematsu, S., Kojo, H., Notsu, Y., and Okuhara, M. (1995) *J. Steroid Biochem. Mol. Bio.* **52,** 365–373.
30. Kumazawa, T., Takami, H., Kishibaashi, N., Ishii, A., Nagahara, Y., Hirayama, N., and Obase, H. (1995) *J. Med. Chem.* **38,** 2887–2892.
31. Blagg, J. Finn, P. W., Greengross, C. W., and Maw, G. N. (1993) Indole Derivatives as 5-Alpha-Reductase Inhibitors, in WO 95/05375. Pfizer, Limited. *Chem. Abstr.* **120**(9), 106982h.
32. Maw, G., Blagg, J., and Rawson, D. J. (1994) Indole Derivatives as Steroid 5α-Reductase Inhibitors, in WO 94/27990. Pfizer Limited. *Chem. Abstr.* **122**(13), 160689x.
33. Neubauer, B. L., Gray, H. M., Hanke, W., Hirsch, K. S., Hsiao, K. C., Jonew, C. D., Kumar, M. V., Lawhorn, D. E., Lindzey, J., McQuaid, L., Tindall, D. J., Toomey, R. E., Yao, R. C., and Audia, J. E. (1996) *J. Clin. Endocrinol. Metab.* **81,** 2055–2060.
34. Hirsch, K. S., Jones, C. D., Audia, J. E., Andersson, S., McQuaid, L., Stamm, N. B., Neubauer, B. I., Pennington, P., and Toomey, R. E. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5277–5281.
35. Raphalen, D. (1965) *Comp. Rend.* **261,** 2234–2235.
36. Chan, H. K., Geissler, W. M., and Andersson, S. (1994) *in* Sex Hormones and Antihormones

in Endocrine Dependent Pathology: Basic and Clinical Aspects (M. Motta and M. Serio, Eds.), pp. 67–76, Elsevier, Amsterdam.
37. Ellsworth, K., Azzolina, B. A., Bull, H., and Harris, G. S. (1996) *FASEB J.* **10,** 2211.
38. Ellis, K. J., and Morrison, J. F. (1982) *Methods Enzymol.* **87,** 405–426.
39. Yonetani, T., and Theorell, H. (1964) *Arch. Biochem. Biophys.* **106,** 243–251.
40. Normington, K., and Russell, D. W. (1992) *J. Biol. Chem.* **267,** 19548–19554.
41. Levy, M. A., Brandt, M., Sheedy, K. M., Holt, D. A., Heaslip, J. I., Trill, J. J., Ryan, P. J., Morris, R. A., Garrison, L. M., and Bergsma, D. J. (1995) *J. Steroid Biochem. Mol. Biol.* **54,** 307–319.
42. Nagamoto, A., Noguchi, K., Murai, T., and Kinoshita, Y. (1994) *J. Androl.* **15,** 521–527.
43. Holt, D. A., Yamashita, D. S., Konialian-Beck, A. L., Luengo, J. I., Abell, A. D., Bergsma, D. J., Brandt, M., and Levy, M. A. (1995) *J. Med. Chem.* **38,** 13–15.
44. Abell, A. D., Brandt, M., Lexy, M. A., and Holt, D. A. (1996) *Bioorg. Med. Chem. Lett.* **6,** 481–484.
45. Levy, M. A., Brandt, J., and Greway, A. T. (1990) *Biochemistry* **29,** 2808–2815.