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Kinetic analysis of LY320236: competitive inhibitor of type I and non-competitive inhibitor of type II human steroid 5α-reductase

Ann M. McNulty, James E. Audia, Kerry G. Bemis, Robin L. Goode, Vincent P. Rocco, Blake Lee Neubauer*

Lilly Research Laboratories, A Division of Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA

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Abstract

Type I and type II steroid 5α-reductases (5α-R) catalyze the conversion of testosterone (T) to dihydrotestosterone (DHT). LY320236 is a benzoquinolinone (BQ) that inhibits 5α-R activity in human scalp skin ($Ki_{\text{type I}} = 28.7 \pm 1.87$ nM) and prostatic homogenates ($Ki_{\text{type II}} = 10.6 \pm 4.5$ nM). Lineweaver–Burk, Dixon, and non-linear analysis methods were used to evaluate the kinetics of 5α-R inhibition by LY320236. Non-linear modeling of experimental data evaluated $V_{\max}$ in the presence or absence of LY320236. Experimental data modeled to the following equation $1/v = [(In0c + (Km/[S]))/V_{\max} Ki][I] + (1/V_{\max})(1 + Km/[S])$ fixing the *In0c* value equal to 1.0 or 0 are consistent with non-competitive or competitive inhibition, respectively. LY320236 is a competitive inhibitor of type I 5α-R (*In0c* = 0, $Ki = 3.39 \pm 0.38$, RMSE = 1.300) and a non-competitive inhibitor of type II 5α-R (*In0c* = 1, $Ki = 29.7 \pm 3.4$, RMSE = 0.0592). These data are in agreement with linear transformation of the data using Lineweaver–Burk and Dixon analyses. These enzyme kinetic data support the contention that the BQ LY320236 is a potent dual inhibitor with differing modes of activity against the two known human 5α-reductase isozymes. LY320236 represents a class of non-steroidal 5α-R inhibitors with potential therapeutic utility in treating a variety of androgen dependent disorders.

1. Introduction

Dihydrotestosterone (DHT) is essential for organogenesis of the prostate and virilization of the male external genitalia [1,2]. DHT is thought to play a role in the pathogenesis of benign prostatic hyperplasia (BPH), prostatic cancer (PCa), androgenic alopecia, acne, and hirsutism [3]. The enzyme, 5α-reductase (5α-R) is responsible for the NADPH-dependent Δ-4 reduction of testosterone (T) to dihydrotestosterore (DHT) and maintenance of their relative intracellular concentrations. Evidence for two 5α-R isozymes has been reported [2,4–6]. Type I 5α-R (gene — SRD5A1) [6] is found in the peripheral tissues (liver, skin, hair follicles), has a pH optima of 7.0–8.0, and demonstrates relatively moderate affinity for T (*Km*: 10–20 μM). Type II 5α-R (gene SRD5A2) [6] is found in the prostate and male external genitalia, has a pH optima of 5.0–6.0, and demonstrates high affinity for T (*Km*: 50–70 nM) [1,7–9]. Investigators studying intra-prostatic expression of types I and II 5α-R by immunocytochemical techniques have detected only type II 5α-R reactivity [1,10,11], while others have detected both type I and type II mRNA expression and functional activities [7,12–14]. Currently, the only clinically used inhibitor of 5α-R is finasteride (Proscar), a potent type II inhibitor with 200-fold less potency against the type I isozyme. Finasteride has been approved for the treatment of BPH and androgenic alopecia.

Enzyme kinetic studies of 5α-R have been published extensively [8,15–18], but few reports have specifically addressed the inhibition of 5α-R by benzoquinolinone (BQ) compounds. We recently published three reports

* Corresponding author. Tel.: +1-317-276-5675; fax: +1-317-276-5546.

E-mail address: neubauer_blake_l@lilly.com (B.L. Neubauer).

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Nomenclature

T	Testosterone	PCa	Prostatic cancer
DHT	Dihydrotestosterone	S.E.M.	Standard error of mean
V_{max}	Maximum velocity	RMSE	Root mean square error
BPH	Benign prostatic hyperplasia		

describing the inhibition of type I 5α-R by the BQs LY306089 and LY191704 [19–21]. Characterization of LY320236 5α-R inhibitory activities described herein utilized graphical and non-linear modeling techniques obtained from in vitro enzymatic assays, human scalp tissue homogenates (type I), and human prostatic tissue homogenates (type II). Taken together, clinical [22] and biological [7,14] data support the existence of types I and II 5α-R in prostatic tissues, making it possible that a dual inhibitor of the two known isozymes of 5α-R will be efficacious in the treatment of androgen-related disorders.

2. Materials and methods

2.1. Chemicals

LY320236, LY306089, and SKF105657 (epristeride) (Fig. 1) were synthesized at Lilly Research Laboratories (Indianapolis, IN), as described previously [23–25]. Bio-Rad protein assay kits (Hercules, CA), and bovine serum albumin standards were used for all protein determinations. Biochemical reagents trizma base, β-nicotinamide adenine dinucleotide phosphate (NADPH), and DL-dithiothreitol were purchased from Sigma (St. Louis, MO). Citric acid was purchased from EM Science (Gibbstown, NJ). Liquid scintillation cocktail was purchased from Packard (Meridien, CT). Radiochemicals, [^{3}H]T NET-370 (spec. act. 92.4 Ci/mmol), [^{3}H]T NET-553 (spec. act. 106.0 Ci/mmol), and [^{14}C]Androstenedione NEC-136 (spec. act. 52.0 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Non-radioactive T and DHT were purchased from Sigma (St. Louis, MO). Non-radioactive androstenedione, androsterone, androstan-3β, 17β-diol and androstan-3α, 17β-diol were purchased from Steraloids (Bayonne, NJ). Isolutes™ 3-ml sample preparation columns (C-18ec) were purchased from Jones Chromatography (Lakewood, CO). A Beckman System Gold™ HPLC (Fullerton, CA) including model #116 pump, model #505 auto-sampler, model #171 radio-isotope detector, and an IBM (Personal System/2, model 70-386) computer were used to collect and analyze the data.

2.2. Analysis of human scalp and prostatic 5α-reductase

Preparation of human scalp and prostatic tissue homogenates used modifications of a previously published protocol [26]. Briefly, we obtained graft recipient site 1.0 mm^2 scalp punches from three male human hair transplant procedures and prostatic tissues from transuretheral resection procedures for infravesicle obstruction secondary to BPH. Dry ice and −80°C storage of the excised tissues were used to prevent degradation of 5α-R activity. Procurements of human specimens were made through appropriate institutional review board and informed consent procedures.

2.3. Enzyme preparation

The type I 5α-R enzymatic preparation contained approximately 75–100 1 mm^2 scalp punches from three human hair transplant patients. The enzyme preparation was composed of approximately 10 g of tissue and 30 ml of ice-cold homogenization buffer (1.0 M Tris–HCl buffer (pH 7.5)) containing 1.0 mM dithiothreitol (DTT). Dissection of subcutaneous tissue from the punches followed by liquid nitrogen freezing and

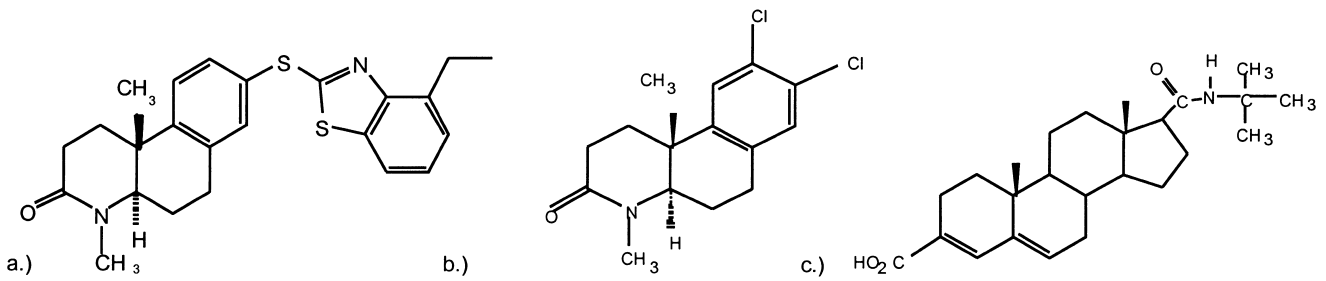

Fig. 1. Chemical structures for (a) LY320236, (b) LY306089 and (c) SKF105657.

pulverization to a powder using a steel pestle and mortar prepared the tissues for isolation of the 5α-R. A Brinkman Polytron™ (Westbury, NY) with a PTA 10-S probe at a setting of eight (four 15 s pulses, on ice) [19] homogenized the preparation. Passage of the homogenate through two layers of cheesecloth separated the filtrate from solid tissue prior to the 1-h centrifugation (100,000 *g*). The pellet was re-suspended in 15 ml of homogenization buffer using a Dounce homogenizer and 0.5 ml aliquots were frozen (−70°C). The type II enzyme preparation was composed of surgically removed prostatic tissue from three patients. Preparation of the homogenate employed the procedures described above for the type I preparation except that the buffer contained 0.4 M citric acid, 1.0 mM DTT, and 1.0 N NaOH to adjust the pH to 5.5 [19]. Characterization of the isolated enzyme preparations began with the determination of the protein content [27] and was completed using HPLC techniques to evaluate SKF105657 (type II) and LY306089 (type I) activity. Long-term storage of the preparations at −70°C maintained stable 5α-R activity for periods exceeding one year.

2.4. Enzyme assay

The 5α-R enzyme assays evaluated the conversion of [^{3}H]T to [^{3}H]DHT by nuclear membrane preparations of 5α-R from human scalp or human prostatic tissues. [^{3}H]T, 8.07×10^{-3} μCi/ml (80 nM, NET-370) and 0.92×10^{-6} M non-radioactive T were combined to give a total T concentration of 1.0 μM in the 200 μl assay volume. The complete buffer for type I 5α-R experiments contained 1.0 μM NADPH, 1.0 M Tris–HCl, (pH 7.5), and the non-radioactive T described above. Complete buffer containing 5.05×10^{-3} μCi/ml [^{3}H]T (50 nM), 1.0 μM NADPH and 400 mM disodium citrate (pH 5.5) were used for the type II enzyme incubations. The addition of 1.0–10,000 nM LY320236, LY306089 or SKF105657 in 5 μl of MeOH (type I) or 10 μl of DMSO (type II) was used to determine inhibitory activity. Addition of scalp (7–12 μg protein/ml) or prostatic (50–60 μg protein/ml) tissue homogenate initiated the reactions. Two hundred μl (type I) or 300 μl (type II) of ice-cold stopping solution terminated the reactions. The reaction mixtures were incubated for 30 min at 37°C (type I) or 25°C (type II).

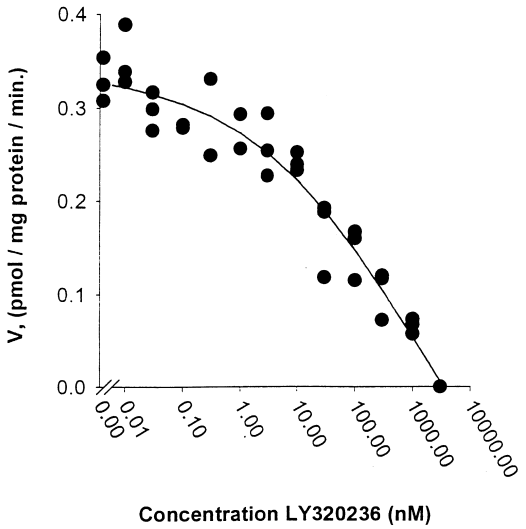

Fig. 2. LY320236 is a potent inhibitor of type I 5α-reductase from human scalp. HPLC methodology was used to assess the conversion of [^{3}H]T (1 μM) to [^{3}H]DHT in the presence and absence of LY320236. The apparent IC_{50} for LY320236 under the experimental conditions (Enzyme Assay) was determined to be 11.6 ± 3.2 nM.

2.5. Lineweaver–Burk kinetic analysis

The IC_{50} data obtained with LY320236 (Figs. 1 and 2) and the experimentally determined *Km* values of 11.6 ± 1.0 μM (type I) and 49.0 ± 2.7 nM (type II) (data not shown) were integrated into the experimental conditions for optimization of the kinetic analysis. Experimental conditions employed in the type I enzyme kinetic assays used five concentrations of substrate (0.5–15 μM) and five concentrations of inhibitor (0–25 nM). Similarly, type II experiments studied five concentrations of substrate (50–300 nM) and four concentrations of inhibitor (0–20 nM). Results were evaluated using statistical modeling (JMP™, SAS Institute, Cary, NC) and graphical techniques (Sigma Plot®, Jandel Scientific Software, San Rafael, CA).

2.6. Dixon analysis

Dixon analysis of type I 5α-R activity in the human scalp homogenate preparation used modifications of our previously published method [19]. All experiments followed the "Enzyme Assay" procedures described in Section 2.4. Briefly, type I Dixon kinetic experiments evaluated 5α-R activity with substrate concentrations of 0.5 and 1.0 μM T in the presence of 10–50 nM LY320236. Similarly, experiments involving type II 5α-R studied 50 and 100 nM T in the presence of 1.0–25.0 nM LY320236. Determination of experimental results employed graphic analysis techniques (Sigma Plot®).

2.7. Non-linear analysis of models

Experiments performed for non-linear analysis followed the "Enzyme Assay" procedures detailed in Section 2.4. Experiments studied 12 concentrations of substrate ranging from 0.5 to 120 μM (type I) or 50 to 1000 nM (type II). These experiments attempted to differentiate V_{max} in the presence or absence of LY320236. The substrate concentrations used were 40

times greater than the *Km* allowing accurate determination of V_{max}. Determination of experimental conclusions employed statistical modeling of the data to Eqs. (1)–(3), (JMP™) and graphical analysis of the modeled data (Sigma Plot®).

2.8. Analysis of metabolites

Three milliliters of methanol followed by 3.0 ml of deionized water were applied to condition each disposable solid-matrix extraction column (C-18ec reverse phase Isolutes™). Following the addition of the reaction mixture, the columns were washed with 3.0 ml of acetone:H_2O (80%:20%), eluted with 0.8 ml of methanol and samples were collected in 13 × 100 mm glass reaction tubes. Addition of 0.8 ml of deionized H_2O, sample vortexing, centrifugation at 500 *g*, and transfer to HPLC injection vials completed the preparation process. HPLC separation of [^{3}H]T and its metabolites employed a previously published method [28]. The Beckman in-line flow radio-detector in conjunction with Beckman System Gold™ chromatographic analysis software quantitatively determined HPLC peak area. Comparing known amounts of extracted [^{14}C]androstenedione in the stopping solution to non-extracted [^{14}C]androstenedione standards assessed solid-phase column extraction efficiency.

2.9. Statistical analysis

Estimation of IC_{50}, *Km*, and *Ki* values adopted simple graphic and non-linear curve-fitting procedures [29]. A maximum of 50 iterations of the non-linear fitting algorithm provided a best-fit determination. Reciprocal forms of the velocity equation (Eqs. (1) and (2)) were

$$\frac{1}{v} = \frac{Km}{V_{max} Ki[S]}[I] + \frac{1}{V_{max}}\left(1 + \frac{Km}{[S]}\right) \quad (1)$$

$$\frac{1}{v} = \frac{\left(1 + \left(Km/[S]\right)\right)}{V_{max} Ki}[I] + \frac{1}{V_{max}}\left(1 + \frac{Km}{[S]}\right) \quad (2)$$

used to model Lineweaver–Burk kinetic data, provide an estimate of the *Km*, *Ki* values and differentiate competitive from non-competitive inhibition. Comparison of the root mean square error (RMSE) values provided assessment of goodness-of-fit of the data to the model. Accordingly, the lowest RMSE value compared to the model suggested the best fit.

Eq. (3) in Dixon form is the combination of Eqs. (1) and (2) given above, and defines the parameter *In0c* as indicative of competitive (*In0c* = 0 is Eq. (1)) or non-competitive (*In0c* = 1.0 is Eq. (2)) inhibitory activity.

$$\frac{1}{v} = \frac{\left(In0c + \left(Km/[S]\right)\right)}{V_{max} Ki}[I] + \frac{1}{V_{max}}\left(1 + \frac{Km}{[S]}\right) \quad (3)$$

Computer modeling, using experimentally determined constants for V_{max} and *Km*, allowed *Ki* best-fit determination. The assessed mode of inhibitory activity was confirmed by further assigning *In0c* a fixed value of 1.0 and then 0, followed by determination of the RMSE values.

3. Results

3.1. Characterization of 5α-R activity in the tissue homogenate preparations

The pharmacological properties of our human scalp and prostatic tissue homogenates were confirmed by studying the effects of LY306089 and SKF105657. LY306089 (type I-selective) demonstrated an IC_{50} value of (mean ± S.E.M.) 9.7 ± 0.9 nM in human scalp tissue homogenates, while SKF105657 (type II-selective) demonstrated less than 10% inhibition at 1000 nM (data not shown). The human scalp preparations generated a mean V_{max} in the absence of inhibitor of 0.377 ± 0.01 pmol/mg protein/min. SKF105657 inhibited DHT formation (IC_{50} = 14.6 ± 2.4 nM) in human prostatic tissue homogenates, while LY306089 demonstrated 15% inhibition at 1000 nM (data not shown). The mean V_{max} of type II 5α-R enzyme preparation in the absence of inhibitor was 138.12 ± 2.5 pmol/mg protein/min. The affinity of type I and type II 5α-R for T was evaluated in the absence of inhibitor to further confirm the homogenate preparations and optimize ex-

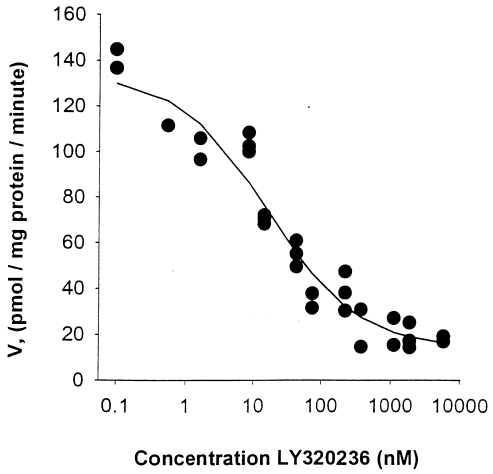

Fig. 3. LY320236 is a potent inhibitor of type II 5α-reductase from human prostate homogenate. HPLC methodology was used to assess the conversion of [^{3}H]T (50 nM) to [^{3}H]DHT in the presence and absence of LY320236.The apparent IC_{50} for LY320236 was determined to be 7.37 ± 1.4 nM. The experimental conditions used to determine the IC_{50} value are described in Section 2.4.

perimental conditions. Type II 5α-R ($Km = 49.7 \pm 2.7$ nM) demonstrated 230-fold greater affinity for T than type I 5α-R ($Km = 11.6 \pm 1.0$ μM) (data not shown).

3.2. LY320236 inhibits type I and type II 5α-reductase

Our data are consistent with the observation that LY320236 is a potent inhibitor of both type I and type II 5α-R. LY320236 concentration-response experiments (0.01–10,000 nM) generated IC_{50} values of 11.6 ± 3.2 nM (type I 5α-R)) and 7.37 ± 1.4 nM (type II 5α-R) (Figs. 2 and 3). LY320236 completely inhibited type I 5α-R at concentrations ≥ 3000 nM, while 10,000 nM LY320236 in type II preparations produced a maximal inhibition of 85%. Incorporation of the experimentally determined IC_{50} values into the enzyme kinetic assays allowed targeting of optimal LY320236 concentration ranges.

3.3. Enzyme kinetics analysis: Lineweaver–Burk method

Lineweaver–Burk linear transformation techniques evaluated the mode of inhibitory activity by LY320236 in type I and type II 5α-R preparations. Type I 5α-R kinetic experiments studied the metabolic formation of [^{3}H]DHT from 0.5 to 15.0 μM [^{3}H]T in the presence or absence of LY320236. LY320236 inhibited type I 5α-R with an apparent *Ki* of 28.7 ± 1.87 nM, consistent with a competitive mode of action (Fig. 4). In a similar type II kinetic experiment evaluating 50–300 nM T substrate concentrations, LY320236 inhibited prostatic 5α-R with an apparent *Ki* value of 10.6 ± 4.5 nM and an apparent non-competitive mode of action (Fig. 5).

3.4. Enzyme kinetics analysis: Dixon plot method

Dixon analysis techniques confirmed the Lineweaver–Burk evaluation of LY320236 inhibitory activities. In these studies, 1.0 and 2.0 μM T (type I) or 50 and 100 nM T (type II) substrate concentrations were employed in the presence or absence of LY320236. LY320236 inhibited the type I isoform with an apparent *Ki* of 25.7 ± 8.7 nM consistent with competitive inhibition (Fig. 6). LY320236 inhibited the type II activity with an apparent *Ki* value of 17.6 ± 4.5 nM in an apparent non-competitive fashion (Fig. 7).

3.5. Enzyme kinetics analysis: non-linear analysis method

Experiments designed to differentiate competitive from non-competitive inhibition used concentrations of substrate 40 (type II) to 70 (type I) times greater than the determined *Km* values. Under these non-Michaelis–Menton experimental conditions (excess substrate), the amount of substrate present should not be a limiting factor in the reaction. Hence, the ability of LY320236 to compete with T in the presence of excess

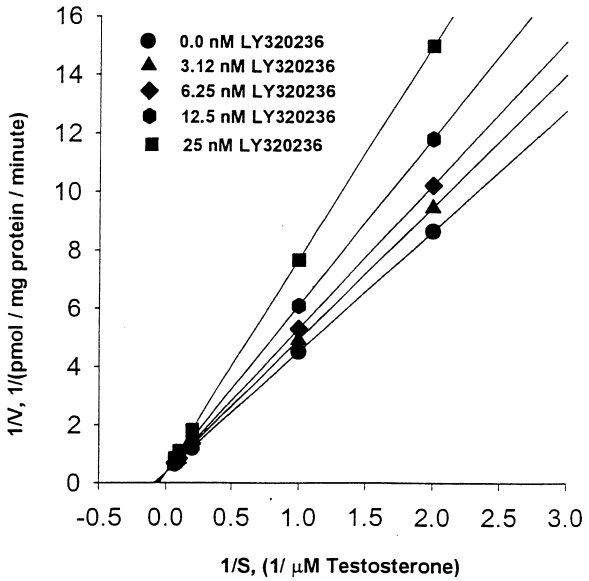

Fig. 4. LY320236 is a competitive inhibitor of type I 5α-reductase (lines intersect *y*-axis at common point). This figure represents data modeled to Eq. (1), the Lineweaver–Burk transformation of non-linear data. The experiment evaluated five concentrations of T (0.5–15 μM) and five concentrations of LY320236 in triplicate. All experimental values were evaluated in Eq. (1) resulting in the determined best-fit points and best-fit lines displayed in this figure. The experimental conditions are described in Section 2.4.

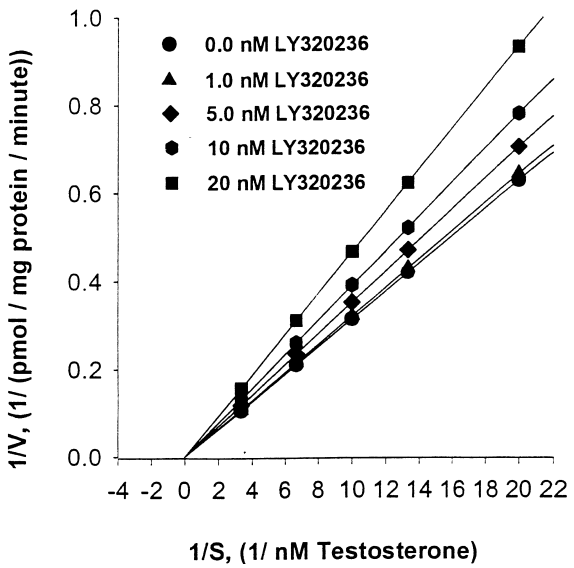

Fig. 5. LY320236 is a non-competitive inhibitor of type II 5α-reductase (lines intersect *x*-axis at same point). This figure represents data modeled to Eq. (1), the Lineweaver–Burk transformation of non-linear data. The experiment evaluated five concentrations of T (50–300 nM) and five concentrations of LY320236 in triplicate. All experimental values were evaluated in Eq. (1) resulting in the determined best-fit points and best-fit lines displayed in this figure. The experimental conditions are described in Section 2.4.

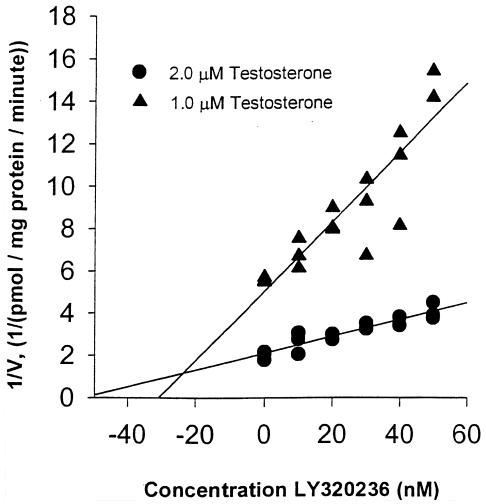

Fig. 6. LY320236 produces competitive inhibition of type I steroid 5α-reductase from human scalp homogenate. Qualitative assessment of competitive inhibition is demonstrated by the intersection of the regression lines above the *x*-axis. All experimental data points that related to 1.0 and 2.0 μM testosterone combined with five concentrations of LY320236 are represented in this figure and the regression lines were determined by Sigma Plot®. The apparent *Ki* value is 25 nM. The experimental details are described in Section 2.4.

substrate can be evaluated. LY320236 is a competitive inhibitor of type I 5α-R (V_{max} values are equal) and a non-competitive inhibitor of type II 5α-R (V_{max} values are different) (Figs. 8 and 9). JMP™ modeling of 5α-R experimental data to Eq. (3) allowed best-fit modeling determination of type I and type II *Ki*, while *In0c* equals either 0 or 1. Computer modeling of LY320236

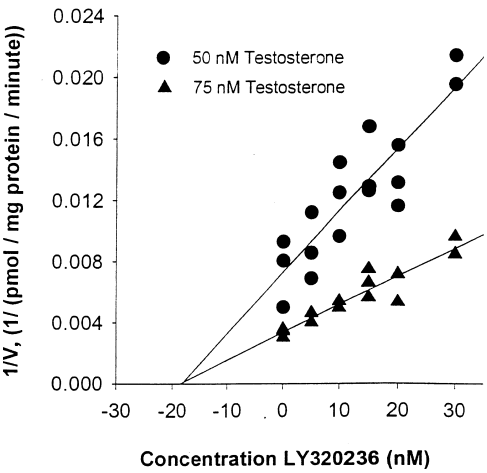

Fig. 7. LY320236 inhibits type II steroid 5α-reductase (human prostatic tissue homogenate) in a non-competitive fashion. Qualitative assessment of non-competitive inhibition is demonstrated by the intersection of the regression lines at the *x*-axis. All experimental data points that relate to 50 and 75 nM testosterone combined with five concentrations of LY320236 are represented in this figure and the regression lines were determined by Sigma Plot®. The apparent *Ki* value is 17.6 nM. The experimental details are described in Section 2.4.

data from type I experiments generated *Ki* values (*In0c* = 0) 3.3 ± 0.38 and (*In0c* = 1) 3.8 ± 0.47. Computer modeling of LY320236 data from type II experiments generated *Ki* values of (*In0c* = 0) 29.7 ± 3.4 and (*In0c* = 1) 8.6 ± 0.8. Further evaluation of the fit of the data to model Eq. (3) compared RMSE terms confirming the above experimentally determined competitive (type I) and non-competitive (type II) inhibitory models (Table 1).

4. Discussion

Experimental results with MK906 and SKF105657 support the contention that prostatic homogenate preparations contain primarily type II 5α-R activities [2,4–6]. A published clinical report evaluating BPH patients concluded that daily administration of finasteride (MK906) resulted in a 30% reduction in prostatic volume and 72% reduction in serum DHT, compared to baseline values [22]. The incomplete reduction of serum DHT levels and the incomplete resolution of symptomatic BPH support the contention that alternative functional 5α-R isoforms exist in the

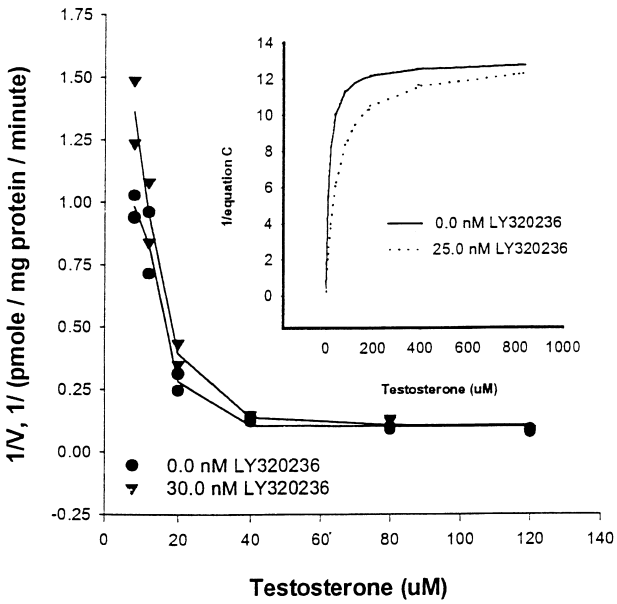

Fig. 8. LY320236 is a competitive inhibitor of type I 5α-R. The plot of the data indicated that V_{max} values in the presence or absence of LY320236 are the same. All experimental data points that relate to multiple (0–120 μM) T concentrations combined with two concentrations of LY320236 are represented in this figure. Modeling of the data to a modification of the Dixon reciprocal form of the velocity equation (1/Eq. (3)) evaluated both competitive and non-competitive inhibition (figure insert). The insert in this figure represents the best-fit line as determined by 1/Eq. (3) using JMP™ statistical software. The root mean square error (RMSE) terms for competitive (1.3000) and non-competitive (1.35883) inhibition provided conformation of the fit of the data to 1/Eq. (3) and provided further evidence for competitive inhibition.

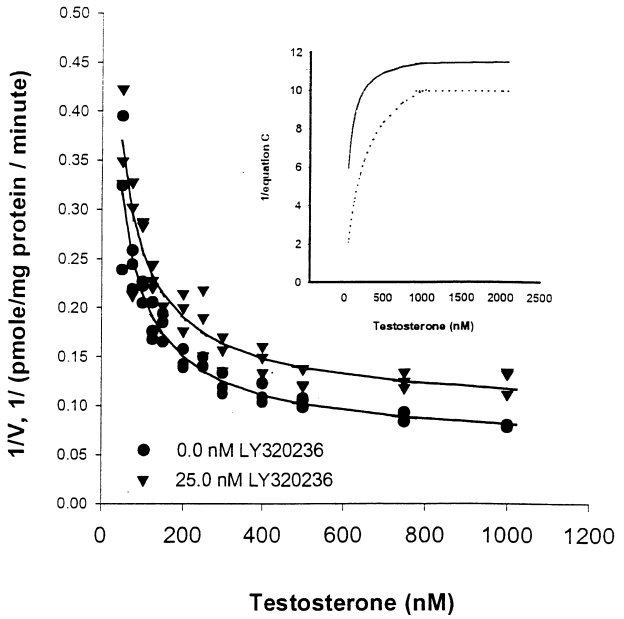

Fig. 9. LY320236 is a non-competitive inhibitor of type II 5α-R. The plot of the data indicated that V_{max} values in the presence or absence of LY320236 are different. All experimental data points that relate to multiple (0–1000 nM) testosterone concentrations combined with two concentrations of LY320236 are represented in this figure. Modeling of the data to a modification of the Dixon reciprocal form of the velocity equation (1/Eq. (3)) evaluated both competitive and non-competitive inhibition (figure insert). The insert in this figure represents the best-fit line as determined by 1/Eq. (3) using JMP™ statistical software. The root mean square error (RMSE) terms for competitive (0.06345) and non-competitive (0.05920) inhibition provided conformation of the fit of the data to the model and provided further evidence for non-competitive inhibition.

prostate. Published experimental results using the two BQ type I specific inhibitors LY306089 and LY300502 demonstrated limited inhibitory activity (30% reduction at 1.0 μM) in human prostatic homogenates [20,21]. Further characterization of prostatic tissues using reverse transcription-polymerase chain reaction (RT-PCR), and Northern Blot analyses confirmed type II 5α-R mRNA expression in BPH stroma, and types I and II mRNA expression in epithelial tissues [7]. The pharmacological data presented herein are in agreement with these observations. Recent results from clinical trials, RT-PCR, and Northern Blot analyses, combined with limited type I activity (LY306089) in prostatic homogenates suggest that a dual inhibitor of 5α-R could be more efficacious in the treatment of androgen-related disorders than single isoform-specific agents.

Compounds derived from the BQ nucleus including LY191704, LY300502, LY306089 are potent inhibitors of type I 5α-R [19–21,30]. LY320236 contains the BQ ring nucleus similar to the inhibitors of type I 5α-R described in our previous studies. Structural modifications of LY320236 have retained type I activity and increased the potency of inhibitory activity against the type II isoform. LY191704 and LY306089 are non-competitive inhibitors of type II 5α-R. These findings support the idea that modifications of the BQ ring nucleus can produce compounds with varying 5α-R inhibitory activities.

The inhibitory activity of LY320236 is being evaluated for cross-reactivity against mixed function oxidase enzymes involved in steroid and xenobiotic metabolism. LY320236 does not exhibit in vitro or in vivo toxicology that would preclude study in humans. Chronic administrations of high doses of LY320236 were well tolerated by rodents and dogs. In these studies, minimal testicular and adrenal effects were observed that would be suggestive of significant activity against steroidogenic enzymes at expected therapeutic dose levels (data not shown). LY320236 is a potent inhibitor of LNCaP tumor growth in a thymic mice. Administration of the compound, for four weeks, produced significant inhibition of the androgen-dependent LNCaP tumor growth without any overt host toxicity (data not shown).

These studies provide definitive kinetic evaluation of the inhibitory activity of LY320236 in tissue preparations enriched with type I and type II 5α-R activities. LY320236 is a potent inhibitor of both type I and type II 5α-R, which contrasts to selective 5α-R inhibitors MK906, SKF105657 (type II) [31] and MK383 [15], LY191704, LY300502, LY306089 (type-I) [18]. Data have been published describing four dual inhibitors of type I and type II 5α-R (FCE 28260 [32], FK143 [29], GG745 [33], and UK-117025 [34]). LY320236 is structurally dissimilar from the 4-azasteroids and carboxy-steroids described previously, and only FCE 28260 possess similar balanced potency

Table 1
Determination of the fit of experimental data to Eq. (3)

	Type I 5α-R mode of inhibition		Type II 5α-R mode of inhibition	
	Competitive	Non-competitive	Competitive	Non-competitive
In0c	1	0	1	0
Ki	3.8 ± 0.47	3.3 ± 0.38	29.7 ± 3.4	8.62 ± 0.8
RMSE	1.35883	1.30000	0.05920	0.06345

against type I and type II 5α-R. Limited kinetic characterization of other dual 5α-R inhibitors have been published. LY320236 competitive (type I) and non-competitive (type II) 5α-R inhibitions may have profound effects on intra-tumor metabolism of T and androgen dynamics in the circulation.

Non-linear curve fitting (JMP™) of experimental kinetic data to mathematical models (Eqs. (1)–(3)) allows estimates of individual nonlinear parameters (*Ki*, *Km*, V_{max}) instead of the graphically generated slope and intercept analyses (Lineweaver–Burk, Dixon). We obtained consistent results using both linear and non-linear modeling techniques, however, *Ki* and *Km* values proved to be somewhat variable between analytic techniques. The type of analysis (linear versus non-linear), heterogeneity of the enzyme preparations, goodness-of-fit of the data to the model equations and the equations themselves likely contribute to the observed variability. Using a recombinant source of the 5α-R enzymes would likely reduce experimental variability. The non-linear results obtained for LY320236 were in agreement with the mode of inhibitory activity from other experiments.

In conclusion, LY320236 is a potent, unique, dual inhibitor of human type I (scalp) and type II (prostate) 5α-R. Our analyses of LY320236 activities are supported by confirming the 5α-R activities in human scalp (type I) and prostatic (type II) tissue homogenates. The enzyme kinetic experiments conducted with LY320236 assessed the mode of inhibitory action and graphical and non-linear modeling techniques confirmed the results.

References

[1] A.E. Thigpen, R.I. Silver, J.M. Guileyardo, M.L. Casey, J.D. McConnell, D.W. Russell, Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression, J. Clin. Invest. 92 (1993) 903–910.

[2] S. Andersson, R.W. Bishop, D.W. Russell, Expression cloning and regulation of steroid 5 alpha-reductase, an enzyme essential for male sexual differentiation, J. Biol. Chem. 264 (1989) 16249–16255.

[3] B.W. Metcalf, M.A. Levy, D.A. Holt, Inhibitors of steroid 5 alpha-reductase in benign prostatic hyperplasia, male pattern baldness and acne, Trends Pharmacol. Sci. 10 (1989) 491–495.

[4] S. Andersson, D.M. Berman, E.P. Jenkins, D.W. Russell, Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism, Nature 354 (1991) 159–161.

[5] S. Andersson, D.W. Russell, Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases, Proc. Natl. Acad. Sci. USA 87 (1990) 3640–3644.

[6] D.W. Russell, J.D. Wilson, Steroid 5 alpha-reductase: two genes/two enzymes, Annu. Rev. Biochem. 63 (1994) 25–61.

[7] N. Bruchovsky, D. Marianne, K. Akakura, S.L. Goldenberg, D. Russell, K. Matsuoka, P. Rennie, Characterization of 5-alpha-reductase gene expression in stroma and epithelium of human prostate, J. Steroid Biochem. Mol. Biol. 59 (1996) 397–404.

[8] N. Bruchovsky, P.S. Rennie, F.H. Batzold, S.L. Goldenberg, T. Fletcher, M.G. McLoughlin, Kinetic parameters of 5 alpha-reductase activity in stroma and epithelium of normal, hyperplastic, and carcinomatous human prostates, J. Clin. Endocrinol. Metab. 67 (1988) 806–816.

[9] R.J. Moore, J.D. Wilson, Steroid 5alpha-reductase in cultured human fibroblasts. Biochemical and genetic evidence for two distinct enzyme activities, J. Biol. Chem. 251 (1976) 5895–5900.

[10] V. Luu-The, Y. Sugimoto, L. Puy, Y. Labrie, I. Lopez Solache, M. Singh, F. Labrie, Characterization, expression, and immunohistochemical localization of 5 alpha-reductase in human skin, J. Invest. Dermatol. 102 (1994) 221–226.

[11] R.I. Silver, E.L. Wiley, A.E. Thigpen, J.M. Guileyardo, J.D. McConnell, D.W. Russell, Cell type specific expression of steroid 5 alpha-reductase 2, J. Urol. 152 (1994) 438–442.

[12] H. Bonkhoff, U. Stein, G. Aumuller, K. Remberger, Differential expression of 5 alpha-reductase isoenzymes in the human prostate and prostatic carcinomas, Prostate 29 (1996) 261–267.

[13] G. Aumuller, W. Eicheler, H. Renneberg, K. Adermann, P. Vilja, W.G. Forssmann, Immunocytochemical evidence for differential subcellular localization of 5 alpha-reductase isoenzymes in human tissues, Acta Anat. (Basel) 156 (1996) 241–252.

[14] P. Bonnet, E. Reiter, M. Bruyninx, B. Sente, D. Dombrowicz, J. de Leval, J. Closset, G. Hennen, Benign prostatic hyperplasia and normal prostate aging: differences in types I and II 5 alpha-reductase and steroid hormone receptor messenger ribonucleic acid (mRNA) levels, but not in insulin-like growth factor mRNA levels, J. Clin. Endocrinol. Metab. 77 (1993) 1203–1208.

[15] K. Ellsworth, B. Azzolina, W. Baginsky, H. Bull, B. Chang, G. Cimis, S. Mitra, J. Toney, R.K. Bakshi, G.R. Rasmusson, R.L. Tolman, G.S. Harris, MK386: a potent, selective inhibitor of the human type 1 5 alpha-reductase, J. Steroid Biochem. Mol. Biol. 58 (1996) 377–384.

[16] S. Delos, C. Iehle, P.M. Martin, J.P. Raynaud, Inhibition of the activity of 'basic' 5 alpha-reductase (type 1) detected in DU 145 cells and expressed in insect cells, J. Steroid Biochem. Mol. Biol. 48 (1994) 347–352.

[17] M.A. Levy, M. Brandt, K.M. Sheedy, J.T. Dinh, D.A. Holt, L.M. Garrison, D.J. Bergsma, B.W. Metcalf, Epristeride is a selective and specific uncompetitive inhibitor of human steroid 5 alpha-reductase isoform 2, J. Steroid Biochem. Mol. Biol. 48 (1994) 197–206.

[18] P.N. Span, J. Benraad Th, C.G. Sweep, A.G. Smals, Kinetic analysis of steroid 5alpha-reductase activity at neutral pH in benign prostatic hyperplastic tissue: evidence for type I isozyme activity in the human prostate, J. Steroid Biochem. Mol. Biol. 57 (1996) 103–108.

[19] K.S. Hirsch, C.D. Jones, J.E. Audia, S. Andersson, L. McQuaid, N.B. Stamm, B.L. Neubauer, P. Pennington, R.E. Toomey, D.W. Russell, LY191704: a selective, nonsteroidal inhibitor of human steroid 5 alpha-reductase type 1, Proc. Natl. Acad. Sci. USA 90 (1993) 5277–5281.

[20] M. Kaefer, J. Audia, N. Bruchovsky, R. Goode, K. Hsiao, I. Leibovitch, J. Krushiski, C. Lee, C. Steidle, D. Sutkowski, B. Neubauer, Characterization of type I 5 alpha-reductase activity in DU145 human prostatic adenocarcinoma cells, J. Steroid Biochem. Mol. Biol. 58 (1996) 195–205.

[21] B. Neubauer, H. Gray, W. Hanke, K. Hirsch, K. Hasio, D. Jones, V. Kumar, L. De, J. Lindzey, L. Mcquaid, D. Tindall, R. Toomey, R. Yao, J. Audia, LY191704 inhibits type I steroid 5alpha-reductase in human scalp, J. Clinical Endocrinology and Metabolism 81 (1996) 2055–2060.

[22] E. Moore, B. Bracken, W. Bremner, J. Geller, J. Imperato-McGinley, J. McConnell, J. Roy, L. Tenover, D. Vaughan, F.

Pappas, Proscar: five-year experience, Eur. Urol. 28 (1995) 304–309.

[23] D.A. Holt, M.A. Levy, H.J. Oh, J.M. Erb, J.I. Heaslip, M. Brandt, H.Y. Lan-Hargest, B.W. Metcalf, Inhibition of steroid 5 alpha-reductase by unsaturated 3-carboxysteroids, J. Med. Chem. 33 (1990) 943–950.

[24] C. Jones, J. Audia, D. Lawhorn, L. McQuaid, B. Neubauer, A. Pike, P. Pennington, N. Stamm, R. Toomey, K. Hirsch, Nonsteroidal inhibitors of human type I steroid 5-alpha-reductase, J. Med. Chem. 36 (1993) 421–423.

[25] G.H. Rasmusson, G.F. Reynolds, N.G. Steinberg, E. Walton, G.F. Patel, T. Liang, M.A. Cascieri, A.H. Cheung, J.R. Brooks, C. Berman, Azasteroids: structure-activity relationships for inhibition of 5 alpha-reductase and of androgen receptor binding, J. Med. Chem. 29 (1986) 2298–2315.

[26] T. Liang, M.A. Cascieri, A.H. Cheung, G.F. Reynolds, G.H. Rasmusson, Species differences in prostatic steroid 5 alpha-reductases of rat, dog, and human, Endocrinology 117 (1985) 571–579.

[27] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[28] R.E. Toomey, R.L. Goode, V. Petrow, B.L. Neubauer, In vivo assay for conversion of testosterone to dihydrotestosterone by rat prostatic steroid 5 alpha-reductase and comparison of two inhibitors, Prostate 19 (1991) 63–72.

[29] J. Sall, JMP Statistics and Graphics Guide Version 3, SAS Institute, Cary, NC, 1994.

[30] D.M. Sutkowski, J.E. Audia, R.L. Goode, K.C. Hsiao, I.Y. Leibovitch, A.M. McNulty, B.L. Neubauer, Responses of LNCaP prostatic adenocarcinoma cell cultures to LY300502, a benzoquinolinone human type I 5 alpha-reductase inhibitor, Prostate Suppl. 6 (1996) 62–66.

[31] J. Hirosumi, O. Nakayama, T. Fagan, K. Sawada, N. Chida, M. Inami, S. Takahashi, H. Kojo, Y. Notsu, M. Okuhara, FK143, a novel nonsteroidal inhibitor of steroid 5 alpha-reductase. Part I: In vitro effects on human and animal prostatic enzymes, J. Steroid Biochem. Mol. Biol. 52 (1995) 357–363.

[32] D. Giudici, G. Briatico, C. Cominato, T. Zaccheo, C. Iehle, M. Nesi, A. Panzeri, E. di Salle, FCE 28260, a new 5 alpha-reductase inhibitor: in vitro and in vivo effects, J. Steroid Biochem. Mol. Biol. 58 (1996) 299–305.

[33] H.N. Bramson, D. Hermann, K.W. Batchelor, F.W. Lee, M.K. James, S.V. Frye, Unique preclinical characteristics of GG745, a potent dual inhibitor of 5AR, J. Pharmacol. Exp. Ther. 282 (1997) 1496–1502.

[34] B.S. Blagg J, K. Cooper, P.W. Finn, P.S. Johnson, F. MacIntyre, G.N. Maw, P.L. Spargo, The development of non-steroidal dual inhibitors of both human 5α-reductase isozymes, Bioorganic Med. Chem. Lett. 6 (1996) 1517–1522.