



Structure/function of the inhibition of human 3 β -hydroxysteroid dehydrogenase type 1 and type 2 by trilostane

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ABSTRACT

The human type 1 (placenta, breast tumors) and type 2 (gonads, adrenals) isoforms of 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD) are key enzymes in biosynthesis of all active steroid hormones. Human 3 β -HSD1 is a critical enzyme in the conversion of DHEA to estradiol in breast tumors and may be a major target enzyme for the treatment of breast cancer. 3 β -HSD2 participates in the production of cortisol and aldosterone in the human adrenal gland. The goals of this project are to evaluate the role of the 2 α -cyano group on trilostane (2 α -cyano-4 α ,5 α -epoxy-17 β -ol-androstane-3-one) and determine which amino acids may be critical for 3 β -HSD1 specificity. Trilostane without the 2 α -cyano group, 4 α ,5 α -epoxy-testosterone, was synthesized. Using our structural model of 3 β -HSD1, trilostane or 4 α ,5 α -epoxy-testosterone was docked in the active site using Autodock 3.0, and the potentially critical residues (Met187 and Ser124) were identified. The M187T and S124T mutants of 3 β -HSD1 were created, expressed and purified. Dixon analyses of the inhibition of wild-type 3 β -HSD1, 3 β -HSD2, M187T and S124T by trilostane and 4 α ,5 α -epoxy-testosterone suggest that the 2 α -cyano group of trilostane is anchored by Ser124 in both isoenzymes. Kinetic analyses of cofactor and substrate utilization as well as the inhibition kinetics of M187T and the wild-type enzymes suggest that the 16-fold higher-affinity inhibition of 3 β -HSD1 by trilostane may be related to the presence of Met187 in 3 β -HSD1 and Thr187 in 3 β -HSD2. This structure/function information may lead to the production of more highly specific inhibitors of 3 β -HSD1 to block the hormone-dependent growth of breast tumors.

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1. Introduction

The human type 1 (placenta, mammary gland, breast tumors) and type 2 (gonads, adrenals) isoforms of 3 β -hydroxysteroid dehydrogenase (EC 1.1.1.145)/steroid Δ^5 - Δ^4 -isomerase (EC 5.3.3.1) (3 β -HSD/isomerase) are encoded by two distinct genes which are expressed in a tissue-specific pattern [1]. 3 β -HSD/isomerase catalyzes the conversion of 3 β -hydroxy-5-ene-steroids (dehydroepiandrosterone, pregnenolone) to 3-oxo-4-ene-steroids (androstenedione, progesterone) on a single, dimeric protein containing both enzyme activities [2]. In addition to placenta and other human peripheral tissues, type 1 3 β -HSD/isomerase (3 β -HSD1) is selectively expressed in mammary glands and breast

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tumors [3], where it catalyzes the first step in the conversion of dehydroepiandrosterone (DHEA) to estradiol to promote tumor growth. In human adrenals, type 2 3 β -HSD/isomerase (3 β -HSD2) is required for the production of cortisol and aldosterone [4]. The selective inhibition of human 3 β -HSD1 in breast tumors represents a potential new treatment for hormone-sensitive breast cancer. Our studies of the structure/function of human 3 β -HSD took a dramatic turn in 2002 when we discovered a 14- to 16-fold higher affinity of purified human 3 β -HSD1 for substrate (DHEA) and inhibitor steroids compared to human 3 β -HSD2 [5]. A key difference in the structure of the two isoforms was determined to be His156 in 3 β -HSD1 and Tyr156 in 3 β -HSD2, which is in the catalytic domain, Y154-X-H156/Y156-X-K158, of the isoenzymes [5]. However, His156/Tyr156 was shown to reside in the α -helical subunit interface of the dimeric enzyme. This residue does not interact directly with bound substrate or inhibitor steroids but apparently influences subunit interactions that modify the topography of the active site [6]. Identifying fingerprint residues that interact with key groups on inhibitors, substrates and cofactors may determine

the structural basis of the higher affinity of 3 β -HSD1 for ligands relative to 3 β -HSD2.

The aims of this study are to identify the residue that interacts with the 2 α -cyano group of trilostane (Ser124) and to determine the functional significance of a non-identical amino acid in the active sites of the isoenzymes—Met187 in 3 β -HSD1 and Thr187 in 3 β -HSD2. Docking studies of trilostane with our structural model of human 3 β -HSD1 predicts that the 2 α -cyano side chain of trilostane (2 α -cyano-4 α ,5 α -epoxy-17 β -ol-androstane-3-one) may interact with the Ser124 residue of 3 β -HSD1. Because Ser124 was reported to be a critical residue for substrate binding in human 3 β -HSD1 [7], the S124T mutation of 3 β -HSD has been produced, expressed and purified to determine how steric hindrance by the methyl group of Thr124 affects the kinetics of 3 β -HSD inhibition by trilostane. In addition, an analog of trilostane that lacks the 2 α -cyano group, 4 α ,5 α -epoxy-testosterone, has been synthesized, docking of this analog with 3 β -HSD1 has been performed and the inhibition kinetics of 4 α ,5 α -epoxy-testosterone have been compared to those of trilostane. Our structural model also predicts that the Thr187 of 3 β -HSD2 is positioned to interact with the carbonyl group of the nicotinamide moiety of enzyme-bound NAD⁺, while Met187 of 3 β -HSD1 is unlikely to interact with this functional group on cofactor. Met187/Thr187 is the only different amino acid in 3 β -HSD1 and 3 β -HSD2 in the region of the enzyme active-site that approximates the nicotinamide group of NAD⁺ and the A-ring of bound steroid [5,6], suggesting that this may be a key structural basis for the high affinity of 3 β -HSD1 for the inhibitors and substrates relative to 3 β -HSD2. To test this prediction, the M187T mutation of 3 β -HSD1 has been created, expressed and purified for kinetic analyses of enzyme inhibition by trilostane and 4 α ,5 α -epoxy-testosterone as well as for analyses of substrate and cofactor kinetics. Correlation of the structure/function relationships of the inhibition of 3 β -HSD1 by trilostane characterized by these novel S124T and M187T mutations may lead to the development of new, more specific inhibitors of 3 β -HSD1 that can slow the growth of breast tumors without compromising steroidogenesis by 3 β -HSD2 in the adrenal enzyme.

2. Methods and materials

2.1. Chemicals

Dehydroepiandrosterone (DHEA), testosterone and pyridine nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO); 5-androstene-3,17-dione from Steraloids Inc. (Newport, RI); reagent grade salts, chemicals and analytical grade solvents from Fisher Scientific Co. (Pittsburgh, PA). Glass distilled, deionized water was used for all aqueous solutions.

2.2. Bioinformatics/computational biochemistry/graphics

As described previously [8], a three-dimensional model of human 3 β -HSD1 has been developed based upon X-ray structures of two related enzymes: the ternary complex of *E. coli* UDP-galactose 4-epimerase (UDPGE) with an NAD cofactor and substrate (PDB AC: 1NAH) [9] and the ternary complex of human 17 β -hydroxysteroid dehydrogenase (17 β -HSD1) with NADP and androstenedione (PDB AC: 1QYX) [10]. Amino acid sequence alignments were performed using CLUSTAL W (1.81) multiple sequence alignment [11].

Using this PDB file for 3 β -HSD1 in Autodock 3.0 (The Scripps Research Institute, <http://autodock.scripps.edu>) [12], the steroid ligand was removed, leaving the NAD⁺ co-factor in the binding site. All docking experiments were carried out on Autodock 3.0

using the Genetic Algorithm with Local Searching. Independent runs (256) were carried out and the docking results were then analyzed by a ranked cluster analysis. Compounds were identified that had the lowest overall binding energy. The three-dimensional graphics of the enzyme docked with trilostane were created using DeepView/Swiss-PdbViewer (<http://www.exspasy.org/spdbv/>).

2.3. Site-directed mutagenesis

Using the Advantage cDNA PCR kit (BD Biosciences Clontech, Palo Alto, CA) and pGEM-3 β HSD1 as template [13], double-stranded PCR-based mutagenesis was performed with the primers listed below to create the cDNA encoding the S124T and M187T mutants of 3 β -HSD1. The forward and reverse primers (mutated codons italicized) used to produce the S124T mutant cDNA were: 5'-CTACACCAGTACCATAGAGGTAGCC-3'; 5'-CCTCTATGGT-*ACTGGT*TAGATGAAGAC-3', respectively. The forward and reverse primers used to produce the M187T mutant cDNA were: 5'-ACGACCCACGTATATCTATGGGGAAG-3'; 5'-AGATATACGTGGGTCGTAAGGCACAAG-3', respectively. The presence of the mutated codon and integrity of the entire mutant 3 β -HSD cDNA were verified by automated dideoxynucleotide DNA sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA). Chou-Fasman and Garnier-Osguthorpe-Robson analysis of each mutant enzyme was used to choose amino acid substitutions that produced no apparent changes in the secondary structure of the protein (Protolize program, Scientific and Educational Software, State Line, PA).

2.4. Expression and purification of the mutant and wild-type enzymes

The mutant 3 β -HSD1 cDNA was introduced into baculovirus as previously described [13]. Recombinant baculovirus was added to 1.5×10^9 Sf9 cells (1 L) at a multiplicity of infection of 10 for expression of each mutant enzyme. The expressed mutant and wild-type enzymes were separated by SDS-polyacrylamide (12%) gel electrophoresis, probed with our anti-3 β -HSD polyclonal antibody and detected using the ECL western blotting system with antigoat, peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). The goat anti-3 β -HSD polyclonal primary antibody and rabbit polyclonal anti-Goat IgG (heavy and light cross adsorbed, conjugated to HRP) were obtained from Novus Biologicals (Littleton, CO). Each expressed enzyme was purified from the 100,000 g pellet of the Sf9 cells (2 L) by our published method [2] using Igepal CO 720 (Rhodia Inc., Cranbury, NJ) instead of the discontinued Emulgen 913 detergent (Kao Corp., Tokyo). Each expressed, purified mutant and wild-type enzyme produced a single major protein band (42.0 kDa) on SDS-polyacrylamide (12%) gel electrophoresis that co-migrated with the purified human 3 β -HSD1 control enzyme. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard [14].

2.5. Synthesis of the trilostane analog

4 α ,5 α -Epoxy-testosterone (trilostane without the 2 α -cyano group) was synthesized by reacting testosterone with hydrogen peroxide in chloroform and methanol. Sodium hydroxide was added to alkalize the mixture as previously described [15]. NMR verified the loss of the 4,5-double bond on testosterone and the addition of the 4 α ,5 α -epoxide group (data not shown). Trilostane (2 α -cyano-4 α ,5 α -epoxy-17 β -ol-androstane-3-one) [16]

was obtained as gift from Gavin P. Vinson, DSc PhD, School of Biological Sciences, Queen Mary University of London.

2.6. Kinetic studies

Michaelis–Menten kinetic constants for the 3β -HSD substrate were determined for the purified mutant and wild-type enzymes in incubations containing dehydroepiandrosterone (DHEA, 2–100 μ M) plus NAD⁺ (0.2 mM) and purified enzyme (0.03 mg) at 27 °C in 0.02 M potassium phosphate, pH 7.4. The slope of the initial linear increase in absorbance at 340 nm/min (due to NADH production) was used to determine 3β -HSD1 activity. Kinetic constants for the isomerase substrate were determined at 27 °C in incubations of 5-androstene-3,17-dione (20–100 μ M), with or without NADH (0.05 mM) and purified enzyme (0.02 mg) in 0.02 M potassium phosphate buffer, pH 7.4. Isomerase activity was measured by the initial absorbance increase at 241 nm (due to androstenedione formation) as a function of time. Blank assays (zero-enzyme, zero-substrate) assured that specific isomerase activity was measured as opposed to non-enzymatic, “spontaneous” isomerization [17]. Changes in absorbance were measured with a Varian (Sugar Land, TX) Cary 300 recording spectrophotometer. The Michaelis–Menten constants (K_m , V_{max}) were calculated from Lineweaver–Burke (1/S vs. 1/V) plots and verified by Hanes–Woolf (S vs. S/V) plots. The k_{cat} values (min^{-1}) were calculated from the V_{max} values (nmol/(min mg)) and represent the maximal turnover rate (nmol product formed min/nmol enzyme dimer).

Kinetic constants for the 3β -HSD cofactor were determined for the purified mutant and wild-type enzymes in incubations containing NAD⁺ (10–200 μ M), DHEA (100 μ M) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4, at 27 °C using the spectrophotometric assay at 340 nm. Kinetic constants for the isomerase cofactor as an allosteric activator were determined in incubations of NADH (0–50 μ M), 5-androstene-3,17-dione (100 μ M) and purified enzyme (0.02 mg) in 0.02 M potassium phosphate buffer, pH 7.4 at 27 °C using the spectrophotometric assay at 241 nm. Zero-coenzyme blanks were used as described above for the substrate kinetics.

Inhibition constants (K_i) were determined for the inhibition of the 3β -HSD1, 3β -HSD2, M187T and S124T activities by trilostane and $4\alpha,5\alpha$ -epoxy-testosterone using conditions that were appropriate for each enzyme species based on substrate K_m values. For 3β -HSD1, the incubations at 27 °C contained sub-saturating concentrations of DHEA (4.0 μ M or 8.0 μ M), NAD⁺ (0.2 mM), purified human type 1 enzyme (0.03–0.04 mg) and trilostane (0–0.75 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–10.0 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. For 3β -HSD2, similar incubations contained DHEA (15.0 μ M or 40.0 μ M) and trilostane (0–7.5 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–10.0 μ M). For S124T, similar incubations contained DHEA (15.0 μ M or 40.0 μ M) and trilostane (0–1.0 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–17.5 μ M). For M187T, similar incubations contained DHEA (8.0 μ M or 20.0 μ M) and trilostane (0–5.0 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–17.5 μ M). Dixon analysis (I vs. $1/V$) was used to determine the type or mode of inhibition (competitive, non-competitive) and calculate the inhibition constant (K_i) values [18,19]. The Dixon plot is widely used to characterize enzyme inhibition kinetics and is preferable to direct binding analysis, which only determines a dissociation constant for the inhibitor as a ligand and does not determine the type of inhibition. The K_i value represents the inhibitor concentration that reduces maximal enzyme activity by 50% and is considered a measure of the affinity of the enzyme for the inhibitor. A decrease in K_i indicates an increase in affinity [18].

Our triplicate determinations of the kinetic constants used highly purified mutant and wild-type enzymes to produce very reliable results with little variation between the values. The standard deviations reported for the mean values in the kinetic tables show that the variation between triplicates is 5–9%. With such low variation in the kinetic determinations, the observed differences of 2- to 65-fold between the kinetic constants of mutant and wild-type enzymes for substrates, cofactors and inhibitor analogs are interpreted to represent real differences.

3. Results

3.1. Predictions of the functions of the Ser124 and Met187/Thr187 residues by docking analysis

Predictions of the functions of Ser124 and Met187/Thr187 of 3β -HSD1/ 3β -HSD2 are based on the docking results obtained with our structural model of human 3β -HSD1, which has 51% homology of the Rossmann-fold domain and 40% identity of the key fingerprint residues that interact with bound substrate and cofactor compared to the crystallographic structures of *E. coli* UDP-galactose-4-epimerase and human 17β -hydroxysteroid dehydrogenase (17β -HSD1) [8]. Site-directed mutagenesis has confirmed the function of the catalytic residues and key substrate and cofactor binding residues as predicted by the structural model [5,7,8,19,20]. In the current study, trilostane was docked in the active site of our structural model of human 3β -HSD1 using Autodock 3.0. The docking result shown in Fig. 1A had the lowest binding energy of -9.81 kcal/mol and a predicted K_i of 0.072 μ M, which is similar to the measured K_i of 0.10 μ M for trilostane. As shown in Fig. 1A, the 2α -cyano group of trilostane is positioned to interact with the hydroxyl group of Ser124 (3.4 Å) wild-type 3β -HSD1. Ser124 was previously identified as a key recognition residue of the 3-oxo group of steroid substrate by human 3β -HSD [7]. To test the prediction that Ser124 also functions as a key recognition residue for inhibition by trilostane, the S124T mutant of 3β -HSD1 was created, expressed and purified. In docking studies using the S124T mutant enzyme, the presence of Thr124 produced a substantial shift in the orientation of trilostane (Fig. 1B). In the S124T mutant, the 2α -cyano group of trilostane is at a 2.2-fold greater distance from the hydroxyl group of Thr124 (7.4 Å, Fig. 1B) compared to Ser124 (3.0 Å, Fig. 1A) in wild-type 3β -HSD1. As shown in Fig. 2, when $4\alpha,5\alpha$ -epoxy-testosterone (trilostane without the 2α -cyano group) is docked with wild-type 3β -HSD1 containing Ser124, this steroid (yellow) superimposes with trilostane docked using the S124T mutant enzyme (with steric hindrance by Thr124 as indicated in Fig. 1B).

Also shown in Fig. 1B, the nicotinamide carbonyl of NAD⁺ is positioned near the hydroxyl group of Thr187 of the M187T 3β -HSD1 mutant (6.3 Å). However, Met187 in wild-type 3β -HSD1 is unlikely to interact with nicotinamide carbonyl on NAD⁺ according to our docking analysis (Fig. 1A). Because Met187/Thr187 is the only non-identical residue of human 3β -HSD1 and 3β -HSD2 in this enzyme region near the intersection of bound cofactor, inhibitor and substrate (residues 177–194), the M187T mutation of 3β -HSD1 was created, expressed and purified. Kinetic analyses of the expressed, purified mutant enzymes, S124T and M187T, have been performed to test the predictions of these docking results.

3.2. Site-directed mutagenesis, expression and purification of the mutant enzymes

The cDNA encoding the S124T and M187T mutants of 3β -HSD1 were produced by double-stranded, PCR-based mutagenesis and inserted into baculovirus as previously described [13]. As shown

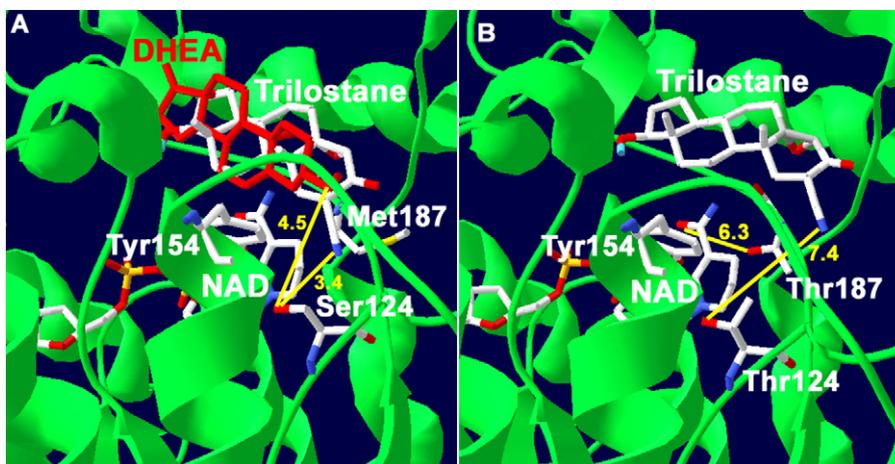


Fig. 1. (A) Docking of trilostane with our structural model of human wild-type 3β -HSD1 shows the predicted interaction between the 2α -cyanogroup of trilostane and Ser124 residue of the wild-type enzyme (3.4 Å in yellow). The interaction of the 3β -hydroxyl group of substrate, DHEA, with Ser124 is also shown (4.5 Å yellow). The overlapping of DHEA (red) and trilostane is consistent with a competitive mode of inhibition. Met187 of 3β -HSD1 is shown to illustrate its lack of interaction with the nicotinamide group of NAD. (B) Docking of trilostane with the S124T mutant of 3β -HSD1 reveals a binding shift so that the 2α -cyanogroup of the inhibitor is 7.4 Å (yellow) from Thr124. In addition, the predicted interaction between the nicotinamide carbonyl of NAD and Thr187 of the M187T mutant enzyme (6.3 Å yellow) is shown. The position of the catalytic Tyr154 is labeled to identify the active site of the enzyme. The protein backbone (green), carbon (white), oxygen (red) and nitrogen (blue) atoms are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

by the immunoblots in Fig. 3A, the baculovirus system successfully expressed a single S124T or M187T mutant enzyme protein as well as wild-type human 3β -HSD1, 3β -HSD2 in Sf9 cells. Each expressed 3β -HSD enzyme (42 kDa monomer) was highly purified (90–95%) according to protein bands visible in SDS-PAGE (Fig. 3B) using our published method [2].

3.3. Kinetic analyses of the inhibition of the wild-type and mutant enzymes by the trilostane analogs

Dixon analyses of the inhibition of wild-type 3β -HSD1, 3β -HSD2, M187T and S124T by trilostane and $4\alpha,5\alpha$ -epoxy-testosterone produced a profile of K_i values and inhibition modes that clarifies the structural basis for the inhibition of 3β -HSD1 by trilostane with a 16-fold higher-affinity than 3β -HSD2 (Table 1). As illustrated by the Dixon plots, trilostane inhibits 3β -HSD1

($K_i = 0.10$ M) in a competitive manner (Fig. 4A) but inhibits 3β -HSD2 ($K_i = 1.60$ μ M) non-competitively (Fig. 4B).

The prediction by the docking study that Ser124 may interact with the 2α -cyano group of trilostane is supported by a 4.1-fold increase in K_i value and the shift to a non-competitive mode of inhibition for the S124T mutant of 3β -HSD1 by trilostane (Fig. 4A, Table 1). The methyl group on Thr124 may sterically hinder the binding of the 2α -cyano group of trilostane (Fig. 1B). In further support, when the inhibitor lacks the 2α -cyano group as in $4\alpha,5\alpha$ -epoxy-testosterone, wild-type 3β -HSD1 (with Ser124) is inhibited with a 41-fold higher K_i value than trilostane and the mode of inhibition is switched to non-competitive (Fig. 5A, Table 1, Fig. 2). The effect of the absence of the 2α -cyano group is also seen for the inhibition of 3β -HSD2 by $4\alpha,5\alpha$ -epoxy-testosterone with an increase in K_i of 2.3-fold with the mode of inhibition being shifted to competitive (Fig. 5B, Table 1).

According to our docking results, Thr187 is positioned to interact with the nicotinamide carbonyl on NAD⁺ in the M187T mutant of 3β -HSD1 (Fig. 1B) and 3β -HSD2 (with Thr187). The low-affinity (high K_i) inhibition profiles of 3β -HSD2 and M187T for both

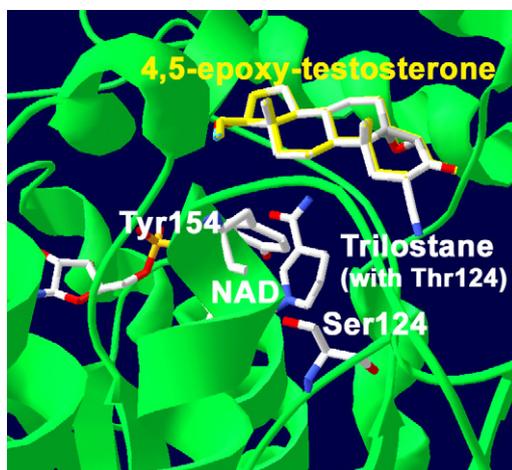


Fig. 2. Docking of the trilostane analog lacking the 2α -cyano group, $4\alpha,5\alpha$ -epoxy-testosterone (yellow), with wild-type 3β -HSD1 (containing Ser124) reveals that it is superimposed over trilostane docked with the S124T mutant enzyme (containing Thr124). The position of the catalytic Tyr154 is labeled to identify the active site of the enzyme. The protein backbone (green), carbon (white), oxygen (red) and nitrogen (blue) atoms are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Comparison of inhibition constants of trilostane and $4\alpha,5\alpha$ -epoxy-testosterone for purified human 3β -HSD1, 3β -HSD2, M187T and S124T

Enzyme	Inhibitor steroid K_i (μ M) ^a	
	Trilostane	$4\alpha,5\alpha$ -Epoxy-testosterone
3β -HSD1	0.10 ± 0.01 (C)	4.08 ± 0.26
3β -HSD2	1.60 ± 0.10	3.75 ± 0.31 (C)
M187T	0.83 ± 0.06	2.80 ± 0.19 (C)
S124T	0.41 ± 0.03	4.34 ± 0.32 (C)

^a For 3β -HSD1, the incubations at 27 °C contained sub-saturating concentrations of DHEA (4.0 μ M or 8.0 μ M), NAD⁺ (0.2 mM), purified human type 1 enzyme (0.03–0.04 mg) and trilostane (0–0.75 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–10.0 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. For 3β -HSD2, DHEA (15.0 μ M or 40.0 μ M) and trilostane (0–7.5 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–10.0 μ M). For S124T, DHEA (15.0 μ M or 40.0 μ M) and trilostane (0–1.0 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–17.5 μ M). For M187T, DHEA (8.0 μ M or 20.0 μ M) and trilostane (0–5.0 μ M) or $4\alpha,5\alpha$ -epoxy-testosterone (0–12.5 μ M). Dixon analysis (I vs. $1/I$) was used to determine the type of inhibition and calculate the K_i values. K_i values are means of triplicate determinations \pm standard deviations. (C) denotes a competitive mode of inhibition, and no notation indicates a non-competitive mode.

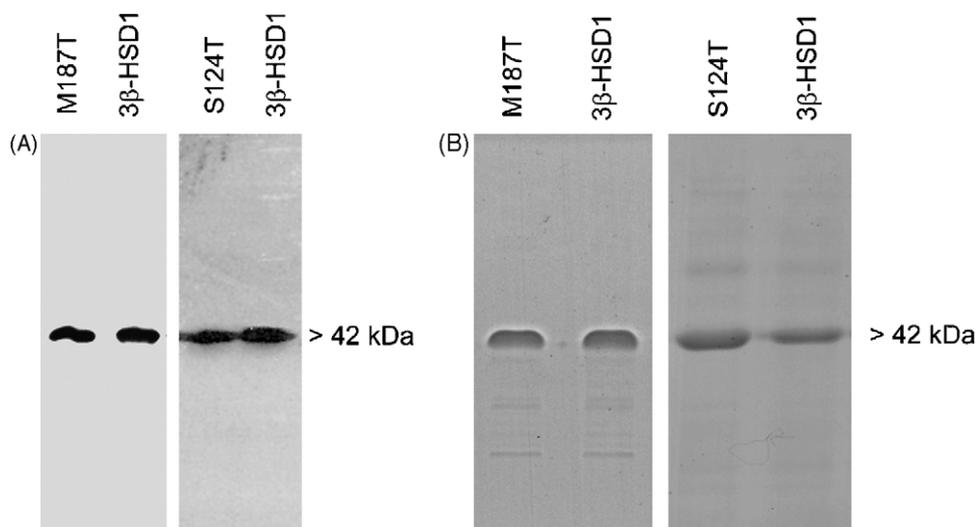


Fig. 3. (A) Western immunoblots of the expressed mutant and wild-type enzymes. The Sf9 cell homogenate (4.0 μg) containing S124T or M187T plus the purified control wild-type 3 β -HSD1 (0.05 μg) were separated by SDS-polyacrylamide (7.5%) gel electrophoresis. The 42.0-kDa band of the enzyme monomer was detected using anti-3 β -HSD antibody as described in the text. (B) SDS-polyacrylamide (7.5%) gel electrophoresis of the purified mutant and wild-type enzymes. Each lane was overloaded with 4.0 μg of purified protein, and the bands were visualized by Coomassie Blue staining.

Table 2
Substrate kinetics for the 3 β -HSD and isomerase activities of the purified mutant and wild-type enzymes

Purified enzyme	3 β -HSD ^a			Isomerase ^b		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{uM}^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{uM}^{-1}$)
M187T	11.0 \pm 0.7	5.6 \pm 0.4	0.51 \pm 0.04	78.1 \pm 5.5	87.7 \pm 5.3	1.12 \pm 0.07
S124T	42.1 \pm 2.7	7.1 \pm 0.5	0.17 \pm 0.01	83.7 \pm 5.0	93.8 \pm 9.8	1.68 \pm 0.09
3 β -HSD1	3.7 \pm 0.2	3.3 \pm 0.2	0.89 \pm 0.04	27.9 \pm 1.1	50.2 \pm 2.0	1.80 \pm 0.08
3 β -HSD2	47.3 \pm 2.9	6.9 \pm 0.5	0.15 \pm 0.01	88.4 \pm 5.7	81.4 \pm 6.0	0.92 \pm 0.06

^a Kinetic constants for the 3 β -HSD substrate were determined in incubations containing DHEA (2–100 μM), NAD⁺ (0.2 mM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4.

^b Kinetic constants for the isomerase substrate were determined in incubations of 5-androstene-3,17-dione (17–150 μM), NADH (0.05 mM) and purified enzyme (0.02 mg) in 0.02 M potassium phosphate buffer, pH 7.4. All values are the means of triplicate determinations \pm standard deviations.

trilostane and 4 α ,5 α -epoxy-testosterone may be related to the effect of Thr187 on cofactor alignment and its influence on inhibitor orientation (Fig. 1B, Table 1), even though direct contact of Met187 or Thr187 with the inhibitors is not predicted by the docking results. Further analysis of the function of Thr187 is provided by the kinetic studies of substrate and cofactor utilization described below.

3.4. Kinetic analyses of substrate and cofactor utilization of the wild-type and mutant enzymes

As shown in Table 2, the S124T mutation shifts the high-affinity kinetic profile for the substrate (DHEA) of wild-type 3 β -HSD1 (K_m = 3.7 μM , k_{cat} = 3.3 min^{-1}) to an 11-fold lower affinity substrate profile (S124T K_m = 42.1 μM , k_{cat} = 7.1 min^{-1} for DHEA),

which is similar to that of wild-type 3 β -HSD2 (K_m = 47.3 μM , k_{cat} = 6.9 min^{-1}). The S124T mutant enzyme has 1.6- to 3.2-fold higher K_m and k_{cat} values for isomerase substrates compared to wild-type 3 β -HSD1. However, the K_m values for the coenzymes of 3 β -HSD1 and isomerase measured for the S124T mutant are very similar to those for wild-type 3 β -HSD1 (Table 3). These data with S124T support a critical role for Ser124 as a key residue that interacts with the 3-oxo group of the substrate steroids and are consistent with our previous report in which the S124A mutation abolished 3 β -HSD activity [7]. Although Thr124 of the S124T mutant can function as a substrate recognition residue for human 3 β -HSD1, the presence of the methyl group on Thr124 may sterically hinder the interaction of the amino acid hydroxyl group with the 3-oxo group of the substrate to increase the substrate K_m value

Table 3
Cofactor kinetics for the 3 β -HSD and isomerase activities of the purified mutant and wild-type enzymes

Purified enzyme	3 β -HSD ^a			Isomerase ^b		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{uM}^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{uM}^{-1}$)
M187T	15.3 \pm 1.0	6.0 \pm 0.4	0.32 \pm 0.02	0.5 \pm 0.03	90.8 \pm 5.3	181 \pm 0.07
S124T	37.4 \pm 2.2	5.7 \pm 0.3	0.15 \pm 0.01	2.6 \pm 0.2	86.1 \pm 5.8	33 \pm 0.09
3 β -HSD1	34.1 \pm 1.7	3.5 \pm 0.2	0.10 \pm 0.005	4.6 \pm 0.2	45.0 \pm 1.8	9.8 \pm 0.4
3 β -HSD2	86.3 \pm 5.6	7.1 \pm 0.6	0.08 \pm 0.005	12.6 \pm 0.9	99.1 \pm 6.4	7.9 \pm 0.5

^a Kinetic constants for the 3 β -HSD cofactor were determined in incubations containing NAD⁺ (10–200 μM), dehydroepiandrosterone (100 μM) and purified enzyme (0.03 mg) in 0.02 M potassium phosphate, pH 7.4.

^b Kinetic constants for the isomerase cofactor were determined in incubations of NADH (0–50 μM), 5-androstene-3,17-dione (100 μM) and purified enzyme (0.02 mg) in 0.02 M potassium phosphate buffer, pH 7.4. All values are the means of triplicate determinations \pm standard deviations.

as well as hinder interaction with the 2α -cyano group of trilostane, as discussed above.

The M187T mutant of 3β -HSD1 produced a 2.2-fold decrease in the K_m value for the NAD^+ utilization by of 3β -HSD1 and a 9.2-fold decrease in the K_m measured for NADH as an allosteric activator of isomerase type 1 with 2- to 3-fold higher k_{cat} values for both activities (Table 3). These higher affinity kinetic profiles of the M187T mutant for cofactor utilization is predicted by the interac-

tion between Thr187 and the nicotinamide carbonyl of NAD^+ shown by the docking results (Fig. 1B). The M187T mutant also produced a 3.0-fold increase in the K_m for DHEA and a 2.8-fold increase in the K_m for the isomerase substrate, 5-androstene-3,17-dione with parallel shifts in the k_{cat} and k_{cat}/V_{max} values for both the 3β -HSD and isomerase activities (Table 2). This shift to a low-affinity pro-

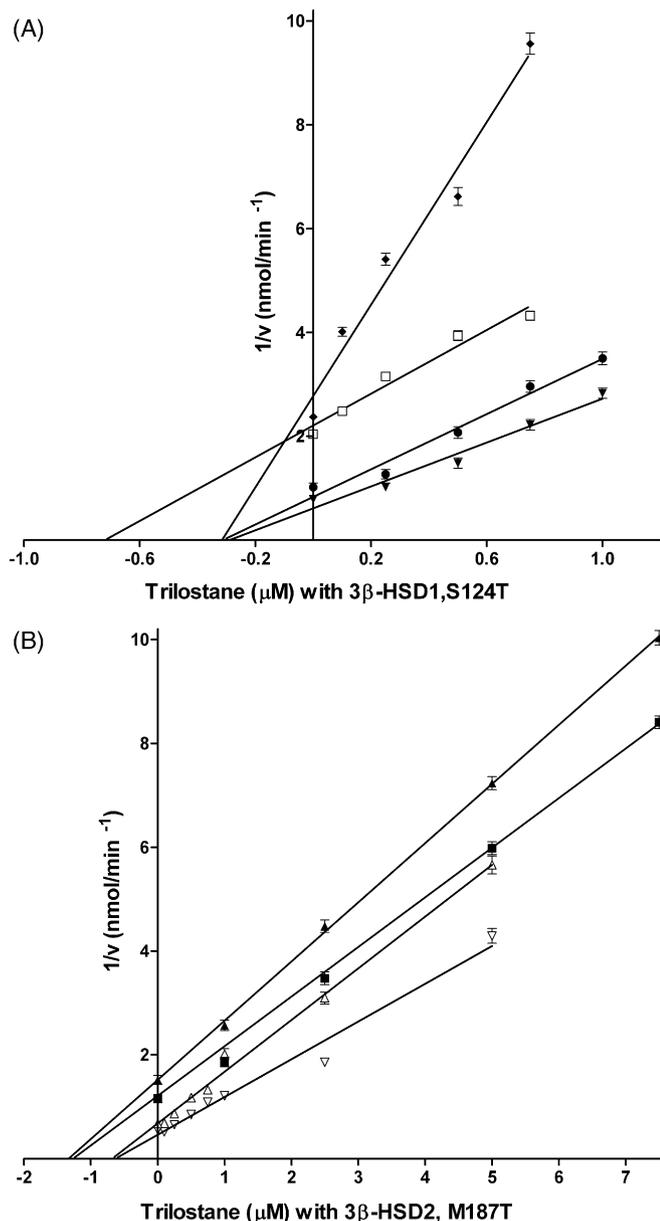


Fig. 4. (A) Inhibition of the 3β -HSD activities of the 3β -HSD1 and S124T enzymes by trilostane. For 3β -HSD1, the incubations at 27 °C contained sub-saturating concentrations (based on K_m values in Table 2) of substrate DHEA, 4.0 μ M (\blacklozenge) or 8.0 μ M (\square), NAD^+ (0.2 mM), purified human 3β -HSD1 enzyme (0.04 mg) and trilostane (0–0.75 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. For S124T, similar incubations contained DHEA, 15.0 μ M (\bullet) or 40.0 μ M (\blacktriangledown) and trilostane (0–1.0 μ M). (B) Inhibition of the 3β -HSD activities of the 3β -HSD2 and M187T enzymes by trilostane. For 3β -HSD2, incubations as described above contained DHEA, 15.0 μ M (\blacktriangle) or 40.0 μ M (\blacksquare) and trilostane (0–7.5 μ M). For M187T, similar incubations contained DHEA, 8.0 μ M (\triangle) or 20.0 μ M (∇) and trilostane (0–5.0 μ M). Each point on the Dixon plot ($1/v$ vs. I) represents the mean of triplicate determinations, and the error bars represent standard deviations. K_i values were calculated from the intersection of the Dixon plots obtained for each enzyme preparation.

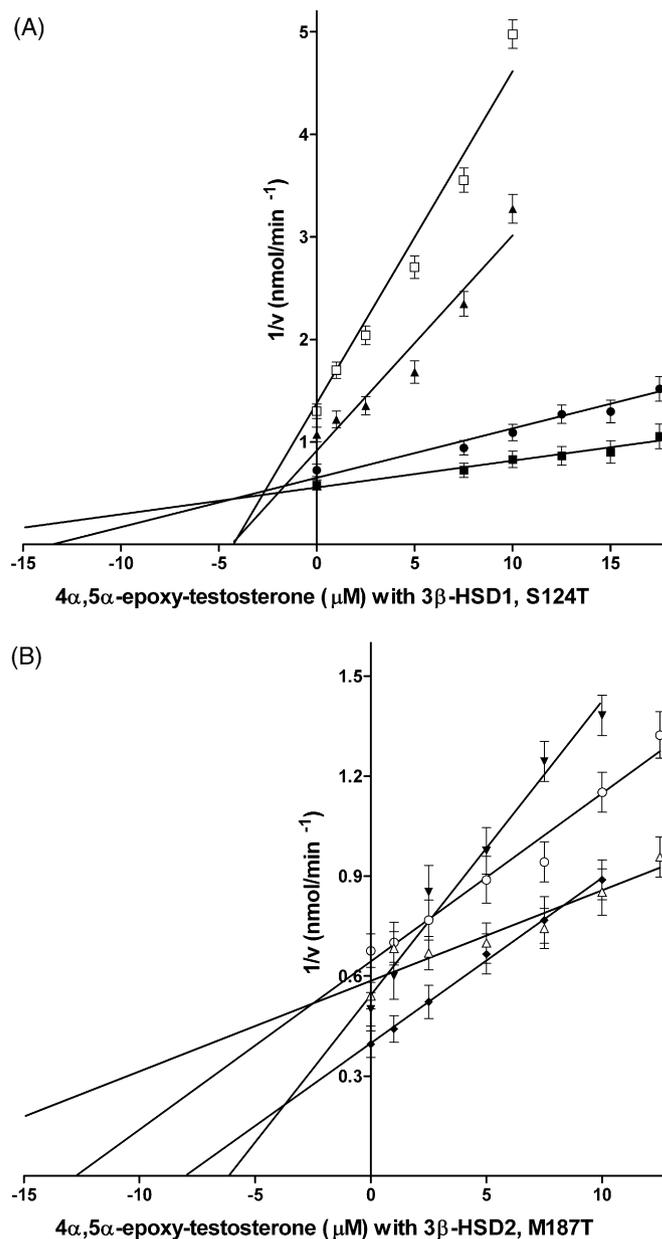


Fig. 5. (A) Inhibition of the 3β -HSD activities of the 3β -HSD1 and S124T enzymes by 4 α ,5 α -epoxy-testosterone. For 3β -HSD1, the incubations at 27 °C contained sub-saturating concentrations of substrate DHEA, 4.0 μ M (\square) or 8.0 μ M (\blacktriangle), NAD^+ (0.2 mM), purified human 3β -HSD1 enzyme (0.04 mg) and 4 α ,5 α -epoxy-testosterone (0–10.0 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. For S124T, similar incubations contained DHEA, 15.0 μ M (\bullet) or 40.0 μ M (\blacksquare) and 4 α ,5 α -epoxy-testosterone (0–17.5 μ M). (B) Inhibition of the 3β -HSD activities of the 3β -HSD2 and M187T enzymes by 4 α ,5 α -epoxy-testosterone. For 3β -HSD2, similar incubations contained DHEA, 15.0 μ M (\blacktriangledown) or 40.0 μ M (\blacklozenge) and 4 α ,5 α -epoxy-testosterone (0–10.0 μ M). For M187T, similar incubations contained DHEA, 8.0 μ M (\circ) or 20.0 μ M (\triangle) and 4 α ,5 α -epoxy-testosterone (0–17.5 μ M). Each point on the Dixon plot ($1/v$ vs. I) represents the mean of triplicate determinations, and the error bars represent standard deviations. K_i values were calculated from the intersection of the Dixon plots obtained for each enzyme preparation.

file of substrate utilization by the M187T mutation is similar to the inhibition kinetics of trilostane for M187T relative to β -HSD1, as discussed above.

4. Discussion

These studies of enzyme-ligand docking, modification of inhibitor structure, enzyme mutagenesis and kinetic analyses of the mutant enzyme strongly support a key interaction of Ser124 of human β -HSD with the 2α -cyano group of trilostane. The docking results of our β -HSD structural model with trilostane and $4\alpha,5\alpha$ -epoxy-testosterone correctly predicted this interaction based on kinetic analyses of the S124T mutant enzyme. The competitive mode of inhibition of β -HSD1 by trilostane may be due to the overlapping of substrate steroid (DHEA) and trilostane as shown by docking results in Fig. 1A. If that overlap is disrupted by a change in the binding orientation of the inhibitor steroid by the S124T mutation (steric hindrance by the methyl group in Thr124, Fig. 1B), the K_i is increased fourfold and mode of inhibition is shifted to non-competitive. When the interaction between Ser124 and the 2α -cyano group is abolished by removal of the 2α -cyano group from trilostane ($4\alpha,5\alpha$ -epoxy-testosterone), the K_i value increases dramatically, and $4\alpha,5\alpha$ -epoxy-testosterone binds in the same orientation as trilostane that is sterically hindered by Thr124 in the S124T mutant of β -HSD1 (Fig. 2). A key role for the 2α -cyano group in the inhibition of β -HSD1 and β -HSD2 by trilostane is strongly supported by these results.

Met187 in β -HSD1 or Thr187 in β -HSD2 is the only difference in the amino acid sequences of the two isoenzymes in the area of the protein that interacts with NAD^+ and the A-rings of trilostane and the substrate steroid. Thr187 in β -HSD2 is a strong hydrogen-bonding donor that is capable of interacting with the carbonyl group on the nicotinamide moiety of enzyme-bound NAD^+ and NADH (Fig. 1), while Met187 in β -HSD1 is a weak acceptor of hydrogen-bonding that most likely does not interact with the nicotinamide group of the cofactor. The significance of this Met187/Thr187 difference in β -HSD1/ β -HSD2 is elucidated by kinetic analyses of the M187T mutation of β -HSD1. The M187T mutant may have shifted cofactor binding, which produced the two to ninefold lower K_m values for NAD^+ and NADH (Table 3). This shift in cofactor binding may have interfered with the utilization of the substrate and the binding of inhibitor steroid to produce threefold higher substrate K_m values (Table 2) and an eightfold higher K_i value for trilostane (Table 1). These lower affinity kinetic profiles of M187T for substrate and trilostane are similar to those measured for human β -HSD2, which contains Thr187. Because other amino acids participate in the binding of the adenosyl and phosphoribosyl moieties of NAD^+ in β -HSD and other members of the short-chain dehydrogenase/reductase family of enzymes [20,21], this single mutation (M187T) that affects only the nicotinamide moiety is not sufficient to shift the kinetic profile for overall cofactor utilization of β -HSD1 to the lower affinity profile of β -HSD2.

Understanding the structure/function relationships of the high-affinity inhibition of β -HSD1 by trilostane may lead to the development of new, more specific inhibitors of β -HSD1 that may be used to block the production of estradiol from DHEA in breast tumors without compromising steroidogenesis mediated by β -HSD2 in the human adrenal enzyme. With the success of the aromatase inhibitors in treating hormone-sensitive breast tumors [22,23], the characterization of a new target enzyme in this biosynthetic pathway enhances our ability to develop new treatments for breast cancer.

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