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Inhibition of the *M. tuberculosis* 3β-hydroxysteroid dehydrogenase by azasteroids

Suzanne T. Thomas, Xinxin Yang [†], Nicole S. Sampson [*]

Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400, United States

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ABSTRACT

The cholesterol metabolism pathway in *Mycobacterium tuberculosis* (*M. tb*) is a potential source of energy as well as secondary metabolite production that is important for survival of *M. tb* in the host macrophage. Oxidation and isomerization of 3β-hydroxysterols to 4-en-3-ones is requisite for sterol metabolism and the reaction is catalyzed by 3β-hydroxysteroid dehydrogenase (*Rv1106c*). Three series of 6-azasteroids and 4-azasteroids were employed to define the substrate preferences of *M. tb* 3β-hydroxysteroid dehydrogenase. 6-Azasteroids with large, hydrophobic side chains at the C17 position are the most effective inhibitors. Substitutions at C1, C2, C4 and N6 were poorly tolerated. Our structure–activity studies indicate that the 6-aza version of cholesterol is the best and tightest binding competitive inhibitor (K_i = 100 nM) of the steroid substrate and are consistent with cholesterol being the preferred substrate of *M. tb* 3β-hydroxysteroid dehydrogenase.

The cholesterol metabolism pathway of *Mycobacterium tuberculosis* (*M.* tb), the causative agent of tuberculosis, has recently attracted interest because of the dual possibilities that this pathway may represent a major source of energy production as well as secondary metabolite production with complex molecular structures within the macrophage.[1,2] Blocking this pathway with small molecule inhibitors presents an attractive avenue for developing new anti-tubercular therapies that are specific to the survival of the organism in the host. 3β-Hydroxysteroid dehydrogenase (Rv1106c) is proposed to catalyze the first step of the cholesterol metabolism pathway (Scheme 1).[3] However, the physiological substrate specificity has not been fully investigated. Herein, we employ the 6-azasteroid series of inhibitors as a tool set to determine the structure–activity relationship for *M. tb* 3β-hydroxysteroid dehydrogenase.

3β-Hydroxysteroid dehydrogenases (3β-HSD) catalyze the oxidation and isomerization of Δ^5-3β-hydroxysteroids to Δ^4-ketosteroids. In mammals, this enzyme is required for the biosynthesis of steroid hormones, and pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone, and androst-5-ene-3,7-diol are all substrates of mammalian 3β-HSDs.[11] 3β-HSD orthologs have been identified in plants, fish, amphibians, viruses, and actinomycete bacteria.[12–17] Their respective substrate specificities and metabolic functions are not as well established.

Notably, in *M. tb*, the enzyme is proposed to catalyze the first step in cholesterol degradation because the 3β-hydroxysteroid dehydrogenase is required for conversion of cholesterol to cholest-4-en-3-one in cell lysates. However, *M. tb* 3β-HSD also can oxidize and isomerize dehydroepiandrosterone and pregnenolone to their respective α,β unsaturated ketones with equal efficiency.[3] Direct comparison of substrate specificities is difficult in this system because the conditions employed to solubilize the steroids differ, and substrate inhibition by the NAD^+ cofactor is observed at millimolar concentrations. Therefore, the relative binding affinities for the enzyme are not readily derived from the kinetic experiments.

As a consequence, the precise sequence of catalytic events in the *M. tb* cholesterol metabolism pathway is not known. In the predicted pathway for *M. tb* cholesterol metabolism, the steroid skeleton can undergo oxidative degradation simultaneous with side-chain truncation (Scheme 1). The substrate preference of each enzyme in the pathway has been explored to a limited extent with the exception of 3-ketosteroid dehydrogenase (KstD) which is suggested to prefer the 5α-androstane-3,17-dione and 5α-testosterone as substrates,[4] and the *meta*-cleavage product hydrolase HsaD for which X-ray crystal structures suggest a preference for 4,5–9,10-diseco-3-hydroxy-5,9,17-tri-oxoandrosta-1(10),2-diene-4-oic acid as the substrate.[7] In order to develop antibacterial

Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; *M. tb*, *Mycobacterium* tuberculosis; DHEA, dehydroepiandrosterone.

* Corresponding author. Tel.: +1 631 632 7952; fax: +1 631 632 5731.
E-mail address: nicole.sampson@stonybrook.edu (N.S. Sampson).
† Present address: Tuberculosis Research Section, LCID/NIAID/NIH, Bethesda, MD 20892, United States.

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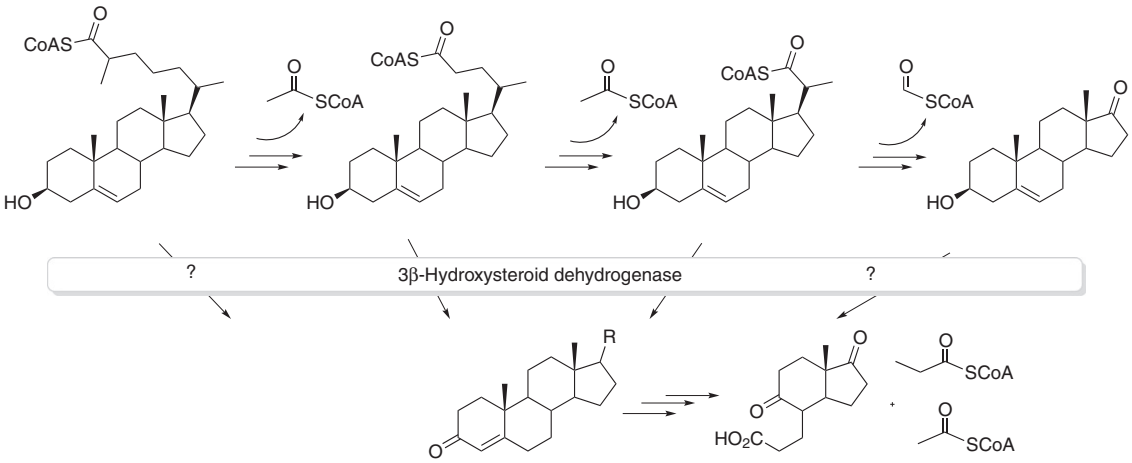


Scheme 1. Partial cholesterol metabolism pathway in *M. tb*.[1,3–10]

therapies that target the pathway and to understand the metabolic consequence of blocking enzymes within the pathway, the preferred sequence of reactions must be elucidated.

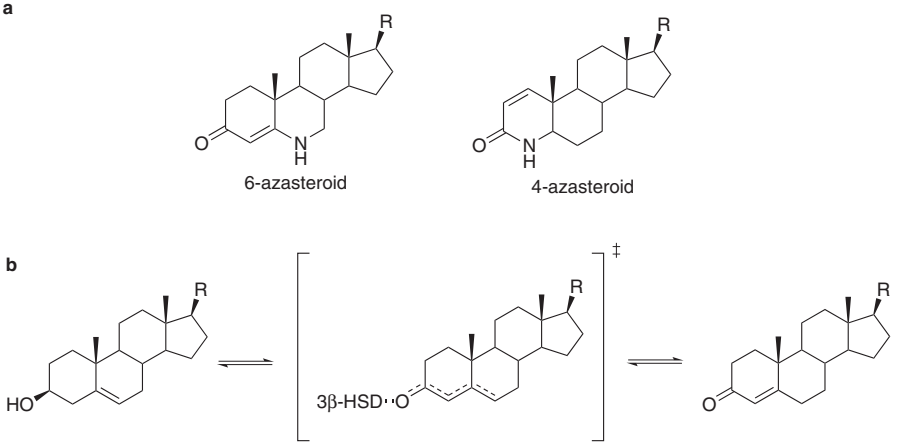
Azasteroids (Scheme 2a) are validated, therapeutically-useful compounds that inhibit enzymes in steroid biosynthetic pathways. For example, finasteride and dutasteride inhibit 5α-reductase-catalyzed production of dihydrotestosterone from testosterone. Inhibition of 5α-reductase is indicated for treatment of benign prostatic hyperplasia and some prostate cancers. Investigation of the 6-azasteroid series was undertaken by Frye et al. at GlaxoWellcome during SAR work to develop enzyme selectivity.[18] However, cross-reactivity with human 3β-HSD could not be eliminated and development efforts were refocused on the 4-azasteroid series.[19–21]

M. tb 3β-HSD shares 29% amino acid sequence identity with type I and type II human 3β-HSD (UniProtKB ID P14060 and P26439) and these enzymes catalyze the same reaction. Both the active site catalytic triad S131, Y158, K162 and Rossman fold motif for NAD^+ cofactor binding are conserved. As the 6-azasteroid moiety is proposed to act as a transition state mimic of the 3β-HSD-catalyzed reaction,[19] we expected that the transition state analogy would apply to the *M. tb* enzyme (Scheme 2b). Given the low amino acid identity of non-catalytic residues between orthologs, we did not expect inhibitor specificity of the *M. tb* enzyme to necessarily parallel that of the human enzyme.

We reasoned that a comprehensive study of 6-azasteroids would provide rapid entry into the structure–activity relationship (SAR) of *M. tb* 3β-HSD and insight into the true substrate for the enzyme in vivo. Moreover, 6-azasteroids have excellent biodistribution and pharmacokinetic properties in humans.[20] Inhibitors of *M. tb* 3β-HSD are important for targeting the cholesterol metabolic pathway and would require little development before in vivo analysis of enzyme inhibition could be undertaken. Here we report the in vitro inhibition SAR for *M. tb* 3β-HSD using a family of azasteroids to explore the enzyme specificity.

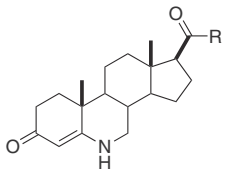
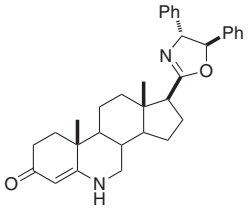
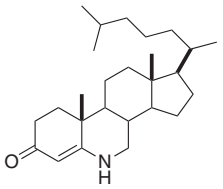
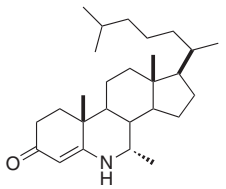
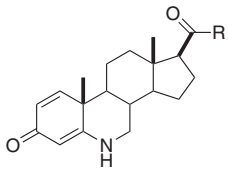
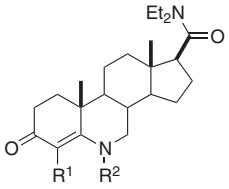
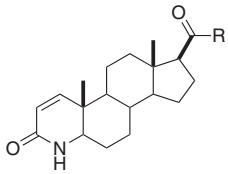
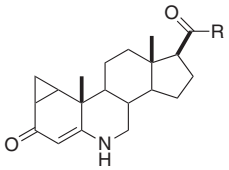
Three series of azasteroids were tested to survey the importance and tolerance of substituents at (a) the 17-position of the D-ring, (b) the 4–7-positions of the A- and B-rings, and (c) the 1,2 positions of the A-ring (Table 1). In order to identify the most potent compounds, the IC_{50}'s for 20 different azasteroids were measured at the K_M of dehydroepiandrosterone (120 μM) and at $2 \times K_M$ of NAD^+ (400 μM). Previously, we had demonstrated that *M. tb* 3β-HSD follows a compulsory order mechanism in which NAD^+ binds first.[3] Therefore, we expected that competitive inhibitors of steroid binding would bind to the E-NAD^+ complex. However, inhibitors were tested with a less than fully saturating concentration of NAD^+ because substrate inhibition occurs at millimolar levels of the cofactor.[3] The IC_{50}'s were determined at eight different inhibitor concentrations ranging from 6 nM to 400 μM. The maximum concentration that was used ranged from 50 to 400 μM due to the limited solubility of some of the azasteroids.

The mechanism of inhibition was determined for azasteroids **3**, **7**, and **17**, which had IC_{50}'s that varied over 2 orders of magnitude. We measured steady-state rates as a function of both DHEA and



Scheme 2. Reagents and conditions: (a) azasteroid frameworks; (b) reaction catalyzed by 3β-HSD.

Table 1
Inhibition of *M. tb* 3β-HSD by azasteroids[a]

Structure	No	R	IC_{50} *M. tb* 3β-HSD (μM)	Structure	No	IC_{50} *M. tb* 3β-HSD (μM)
	**1**	OH	>200		**6**	9 ± 1
	2	NH-phenyl-4-morph-olino	>100			
	3	NH[(2-*t*-butyl, 5-CF3) phenyl]	27 ± 3		**7**	0.5 ± 0.1
	4	NH-2-benzo-phenone	27 ± 2			
	5	NH[(2,5-bis-*t*-butyl) phenyl]	23 ± 2			
		R				**R_1, R_2**
	**8**		8 ± 2		**10**	Me, H >400
					11	H, Me >400
					12	Et, H >260
					13	H, Hex 156 ± 15
	**9**	$NHC(Me)_3$	140 ± 15		**14**	H, Pr >50
					15	H, Bu >50
					16	$-(CH_2)_4-$ 43 ± 3
		R				**R**
	**17**	$NHC(Me)_3$	312 ± 18		**19**	$NHC(Me)_3$ >200
	18	NH[(2,5-bis-CF_3) phenyl	>150		**20**	$N(Et)_2$ 540 ± 390

[a] Experimental conditions: 100 mM TAPS pH 8.5, 150 mM $NaCl_2$, 30 mM $MgCl_2$, 2% DMSO at 30 °C. Fixed concentrations of DHEA and NAD^+ were 120 μM and 400 μM, respectively. Azasteroids were tested up to 400 μM or their maximum solubility, whichever was less.

inhibitor concentrations and globally fit the data to Eq. 2. All three inhibitors were found to be competitive inhibitors of DHEA. We concluded that modifications of the steroid ring framework that reduced the efficacy of inhibition did not alter the mechanism of inhibition. Kinetic competition with DHEA is consistent with the proposal that the 6-azasteroid binds in the steroid binding site and that the IC_{50}'s serve as a valid indicator of relative binding to the steroid binding site.

Functional groups of varying polarity were employed to interrogate the importance of the 6-azasteroid side chain at C17. Carboxylic acid **1**, did not inhibit *M. tb* 3β-HSD at concentrations below 200 μM. In contrast, incorporation of an amide at the 17-carbon was tolerated. Aromatic amide substituents, for example, **3–5**, were effective inhibitors, whereas, a polar heterocycle **2** obviated efficacy. Further constraint of the amide as oxazole **6** improved inhibition a further three-fold. Elimination of heteroatoms at C17 and substitution of the 6-azasteroid with the native cholesterol 8-carbon side chain to provide azasteroid **7** increased potency an order of magnitude compared to the oxazole. The binding preference for large hydrophobic substituents indicates that C27 sterols are most likely the preferred substrate for *M. tb* 3β-HSD.

Addition of a methyl group at C7 to the sterol framework of **7** to provide **8** reduced potency 10-fold. Next, we explored structure–activity space around the A,B ring system using a simple amide substituent at the C17 position. When the C17 was modified as the *t*-butyl amide, **9**, the inhibition potency was poorer than the aromatic substituents. The diethyl amide substituent was selected for further analysis due to its facile synthesis and to compare inhibition constants in a readily measured concentration range. Substitution of the 6-azasteroid framework with linear alkyl moieties at either C4 or N6, **10–15**, reduced potency, and in most cases also reduced solubility. Alkylation at both C4 and N6 in a bridged fashion, **16**, restored potency. Interestingly, swapping N6 and C4 to provide the 4-azasteroids **17** (finasteride) and **18** (dutasteride) had a deleterious effect on potency, which is consistent with the 6-azasteroid framework mimicking the intermediate in the isomerization half-reaction (Scheme 2).

Introduction of unsaturation at C1–C2 in azasteroid **9** was tolerated. However, cyclopropanation across C1–C2 in azasteroids **19** and **20** reduced efficacy at least five-fold. Therefore, the steric requirements about the A,B ring system are quite stringent for the *M. tb* 3β-HSD.

Table 2
Inhibition characteristics of azasteroid **7**[a]

Substrate	Pattern type	K_{ic}	K_{iu}
DHEA	C	124 ± 6 nM	n.o.[b]
NAD^+	Mixed	870 ± 150 nM	98 ± 12 nM

[a] Experimental conditions: 100 mM TAPS pH 8.5, 150 mM $NaCl_2$, 30 mM $MgCl_2$, 2% DMSO at 30 °C. Fixed concentrations of DHEA and NAD^+ were 120 μM and 400 μM, respectively. C: competitive inhibition; Mixed: mixed competitive and uncompetitive inhibition. K_{ic} and K_{iu}: competitive and uncompetitive inhibition constants, respectively.
[b] Not observed.

This 6-azasteroid series are potent inhibitors against human adrenal 3β-HSD. Increased bulk at C17, for example, trifluoromethyl aryl substituents like in azasteroid **3**, decreased potency.[21] Moreover, the human adrenal 3β-HSD tolerated methylation at N6 and cyclopropanation at C1–C2.[19] However, larger N6 substituents, for example, butyl and hexyl reduced potency against the human enzyme 1000-fold.[19] Therefore, future work to develop *M. tb* specific inhibitors should focus on elaboration of the C17 structure–activity space and introduction of constrained substituents between N6 and C4.

In our screen of 20 compounds, azasteroid **7**, which most closely mimics cholestenone, was the most effective inhibitor. Further inhibition analyses were carried out with azasteroid **7** in which the concentrations of both DHEA and NAD^+ were varied. The azasteroid competitively inhibits DHEA binding ten times more effectively than NAD^+ binding (Table 2, Figs. S1 and S2). Moreover, the azasteroid shows strong uncompetitive inhibition with respect to NAD^+. Previously, we demonstrated that catalysis by *M. tb* 3β-HSD follows a compulsory order binding mechanism in which NAD^+ binds first.[3] The competitive behavior versus DHEA and uncompetitive behavior versus NAD^+ suggests that the azasteroid binds most effectively to the E-NAD^+ complex. Therefore, increasing levels of NAD^+ cofactor cannot overcome inhibition of 3β-HSD by azasteroids.

In summary, three series of azasteroids were evaluated for binding and inhibition of *M. tb* 3β-hydroxysteroid dehydrogenase. 6-Azasteroids with large, hydrophobic side chains at the C17 position are the most effective inhibitors. Substitutions at C1, C2, C4 and N6 were poorly tolerated. Our structure–activity studies indicate that the 6-aza version of cholesterol is the best and tightest binding inhibitor and are consistent with cholesterol being the preferred substrate of *M. tb* 3β-hydroxysteroid dehydrogenase.

Materials and methods: 6-Azasteroids were provided by GlaxoSmithKline (Research Triangle Park, NC). Frye et al. describe the synthesis and purity confirmation by elemental analysis of the 6-azasteroids.[19,20,22] 4-Azasteroids were purchased from Sigma–Aldrich. The purity (>98%) and identity of azasteroids were confirmed by LC/UV/MS (Waters UPLC/diode array/SQD) for use in these assays. The LC was performed with a 1.7 μM C18 (2.1 × 100 mm) column and the gradient was from 100% H_2O to 100% methanol over 10 min. *Hazard warning*: *these 6-azasteroids are potential teratogens to the developing male fetus and appropriate precautions should be taken when handling these compounds.*

Purification and assay of 3β-HSD was carried out as previously described.[3] Briefly, reactions were initiated by the addition of 3β-HSD (125 nM) to substrates DHEA (120 μM) and NAD^+ (400 μM), with or without inhibitor, in 100 mM TAPS pH 8.5 buffer with 150 mM $NaCl_2$ and 30 mM $MgCl_2$. Inhibitor stock solutions were prepared in DMSO. The final DMSO concentration was held fixed at 2% which does not affect enzyme activity.[3] The formation of NADH was monitored at 340 nm for the first 150 s of reaction at 30 °C. Assays were performed in duplicate and IC_{50} values were determined from 8 concentrations by fitting to Eq. 1. Mechanism of inhibition was determined by fitting initial velocity data to Eq. 2 using Grafit 4.0 (Erithacus software, Sussex UK).

$$v = V_m/(1 + [I]/IC_{50}) \quad (1)$$

$$v = V_m[S]/\{K_m(1 + [I]/K_{ic}) + [S](1 + [I]/K_{iu})\} \quad (2)$$

where v is the initial velocity, V_m is the maximum velocity, S is the varied substrate, K_M is the Michaelis–Menten constant for the varied substrate, and K_{ic} is the competitive inhibition constant and K_{iu} is the uncompetitive inhibition constant.

Acknowledgments

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Supplementary data

Supplementary data (additional kinetic plots) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.004.

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