Supplementary Material

cAMP-mediated inhibition of cholesterol catabolism in *Mycobacterium tuberculosis* by a novel drug candidate GSK2556286

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Running title: cAMP-mediated inhibition of *Mycobacterium tuberculosis*

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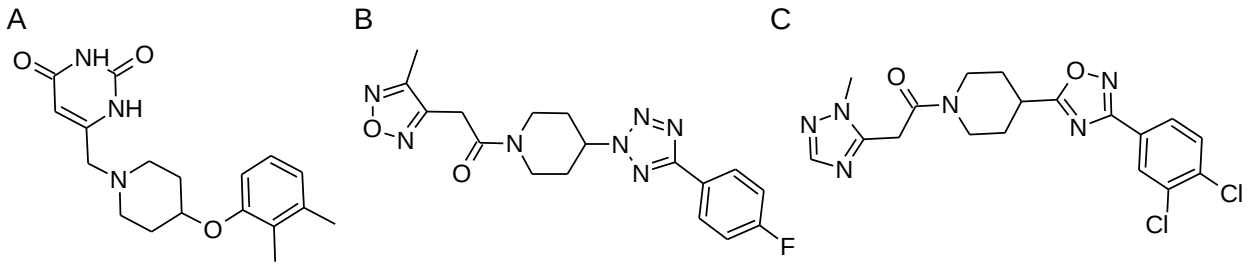
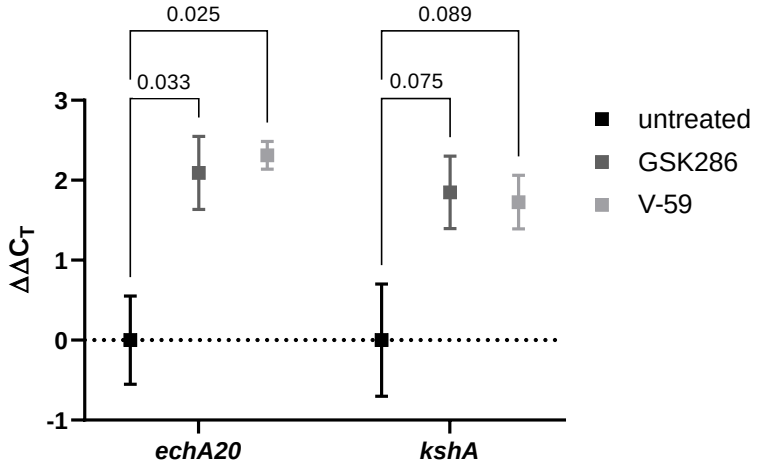
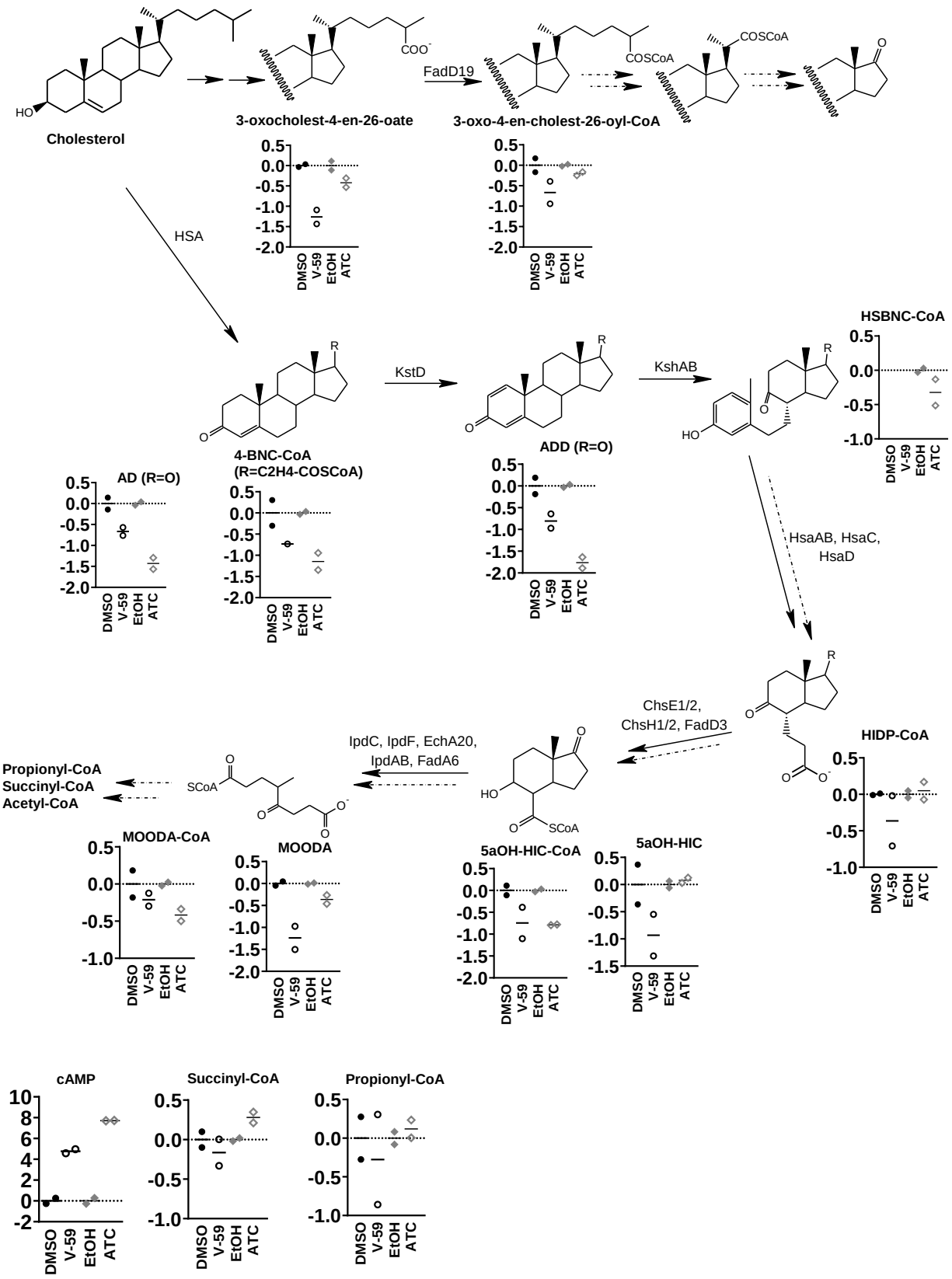


Supplementary Figure S1. Structures of Mtb inhibitors used in the study. (A) GSK286 (B) V-59 and (C) mCLB073.

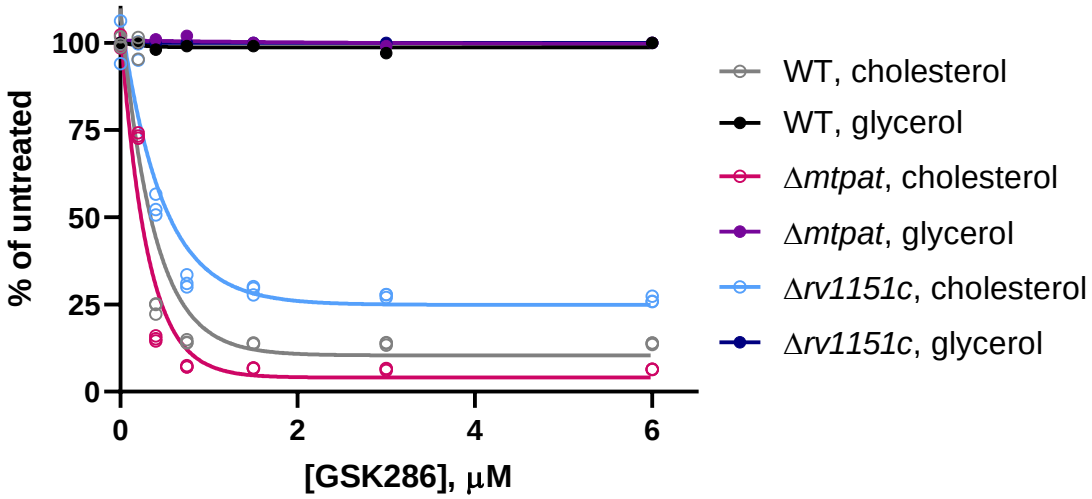


Supplementary Figure S2. Effect of GSK286 on levels of cholesterol gene transcripts. Mtb was grown on the cholesterol media supplemented with 10 µM GSK286 (dark grey), 10 µM V-59 (light grey) or no additional compound (black). Transcript levels were determined using RT-qPCR and duplicate biological replicates were normalized to levels of the *sigA* transcript. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison test with adjusted P-values indicated.



Supplementary Figure S3.

Supplementary Figure S3. LC-MS analysis of cholesterol degradation products. Extracts from cell pellets for DMSO-treated control cells (solid black circle), V-59-treated (dissolved in DMSO, open black circle), ethanol-treated control (solid grey diamond), and Atc-treated TetOn-cAMP (dissolved in ethanol, open grey diamond) after 48 hrs of treatment. Untargeted molecular feature extractions were performed, and data were normalized using total ion abundance from features present in all samples. Cholesterol degradation intermediates were identified in the normalized data by *m/z* (< 15 ppm error) and retention time matches to standards. Identified cholesterol metabolites are indicated by chemical structures and shorthand labels on a simplified cholesterol degradation schematic. Arrows represent enzyme catalyzed reactions, with one or two arrows representing one or multiple steps in the degradation pathway and known enzymes are indicated above the reaction arrow. Relative compound abundance is indicated as Log_2 fold-change in compound abundance relative to their respective DMSO (for V-59) and ethanol (for ATC treated) controls are shown on the Y-axis. Experiments were performed in duplicate with individual samples shown as points and the horizontal line representing the median. The symbol "nd" indicates the compound was not identified in the sample. Additionally, cAMP was identified in these samples indicating a >50-fold change in cAMP levels, as previously determined. Of the final products of cholesterol degradation, only propionyl- and succinyl-CoA were identified, though they are consumed and produced in many other cellular processes. AD: androst-4-ene-3,17-dione, 4-BNC-CoA: 3-oxo-4-pregnene-20-carboxyl-CoA, ADD: androsta-1,4-diene-3,17-dione, HSBNC-CoA: 3-Hydroxy-9-oxo-9,10-seco-23,24-bisnorchola-1,3,5(10)-trien-22-oyl-CoA, HIDP-CoA: 3aα-H-4α(3'-propanoate)-7aβ-methylhexahydro-5-indanone-1-propionyl-CoA, 5aOH-HIC: 3-[(3aS,4S, 5R,7aS)-5-hydroxy-7a-methyl-1-oxo-octahydro-1H-indene-4-carboxylate, 5aOH-HIC-CoA: 3-[(3aS,4S, 5R,7aS)-5-hydroxy-7a-methyl-1-oxo-octahydro-1H-indene-4-carboxyl-CoA, MOODA: 4-methyl-5-oxo-octanedioate, MOODA-CoA: 7-carboxy-4-methyl-5-oxoheptanoyl-CoA.



Supplementary Figure S4. Sensitivity of Δ*mtpat* and Δ*rv1151c* Mtb Erdman to GSK286. WT and mutant strains were grown in 7H9 medium supplemented with 0.2% glycerol or 0.5 mM cholesterol in 96-well plates with increasing concentrations of GSK286 (in DMSO) or DMSO alone. Cells were incubated for 7 days at 37°C, and then the resazurin assay was performed. Resazurin signals for GSK286 treated samples are shown after normalizing to the resazurin signal for the corresponding strain and growth media treated with DMSO alone (untreated). Data represent the mean of biological triplicates for glycerol controls (filled circles) and individual triplicate data points are shown for cholesterol-grown strains (open circles).

TABLES

Supplementary Table 1. Bacterial strains used in this study.

Species	Strain	Modification	Antibiotic resistance[a]	Source
M. tuberculosis	Erdman	-	-	
M. tuberculosis	Erdman	*Δrv1625c*	Hyg	This study
M. tuberculosis	Erdman	*Δmtpat*	Kan, Hyg	This study
M. tuberculosis	Erdman	*Δrv1151c*	Kan, Hyg	This study
M. tuberculosis	CDC1551	-	-	
M. tuberculosis	CDC1551	Tn::*rv1625c*	Kan	(1)
R. jostii	RHA1	-		

[a]Kan, kanamycin; Hyg, hygromycin

Supplementary Table 2. Plasmids used in this study.

Name	Description[a]	Reference
pTipQC2	*Rhodococcus* expression vector, Thiostrepton-inducible promoter, Cam^R, Amp^R	(2)
pTip1625	*rv1625c* cloned into pTipQC2 with a C-terminal His-tag, Cam^R, Amp^R	This study
pYUB854	Plasmid for generating recombineering constructs. Hyg^R	(3)
pJV53	Plasmid for recombineering in Mtb, Kan^R	(3)
pKO0998	Up- and downstream regions of *rv0998* cloned on either side of the Hyg^R cassette in pYUB854 for deleting *rv0998* in Mtb. Hyg^R	This study
pKO1151	Up- and downstream regions of *rv1151* cloned on either side of the Hyg^R cassette in pYUB854 for the deletion of *rv1151* in Mtb. Hyg^R	This study
2×Rv1625c	*rv1625c* expressed from the hsp60 promoter in pMV306, Apr^R	(1)
prpD'::GFP	Mycobacterial reporter vector, GFP expressed by the *prpD* promoter and mCherry expressed from the smyc promoter, Kan^R	(4)
tetON-cAMP	Catalytic domain of Rv1264 with C-terminal His-tag expressed from the Atc inducible promoter p606, Hyg^R	(1)

[a]Amp, ampicillin; Cam, chloramphenicol; Apr, apramycin; Kan, kanamycin; Hyg, hygromycin

Supplementary Table 3. Oligonucleotides used in this study

Name	Sequence	Restriction
Rv1625-F	GCTGAACCATATGGCGGCAAGAAAATGCGGC	*Nde*I
Rv1625-R	GGAATTCGTGATGGTGATGGTGATGACTAGTAGCGACCCCTGCCGTG CGGGGTTCGACCCCTGCCGTGCGGGG	*EcoR*I
Rv0998up-F	AAAGCTTCACGTGGTCGACGGATCCGTTGGTAGCGCGACTCGTTCGC	*Hin*dIII
Rv0998up-R	CGCCAATGACACCAGACCCTCG	-
Rv0998do-F	CGAGGGTCTGGTGTCATTGGCGACCATGATCGATGTGCCGGGTC	-
Rv0998do-R	TGACACTATAGAATACATAGGATCCGCGTCAGATGTACGACCGGGTG	*Bam*HI
Rv1151up-F	GTGATAAACTACCGCATTAAAGCTTCAACCTGCGCACGCTGGCTAAG	*Hin*dIII
Rv1151up-R	GTCGAATCGGGCCCACAATCC	-
Rv1151do-F	GGATTGTGGGCCCGATTCGACAATCCCGAGCCCACGCCGTTG	-
Rv1151do-R	CTCACTATAGGGAGACCGGAAGCTTGCGTGTCCGACCTCATGCCTC	*Hin*dIII

References

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2. Nakashima N, Tamura T. 2004. Isolation and characterization of a rolling-circle-type plasmid from *Rhodococcus erythropolis* and application of the plasmid to multiple-recombinant-protein expression. Appl Environ Microbiol 70:5557-68.
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