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More Than Cholesterol Catabolism: Regulatory Vulnerabilities in *Mycobacterium tuberculosis*

Amber C. Bonds¹ and Nicole S. Sampson^{2,*}

¹Molecular and Cellular Pharmacology Program, Stony Brook University, Stony Brook, NY 11794-8651

²Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400

Abstract

Mycobacterium tuberculosis (*Mtb*) is the epitome of persistent. *Mtb* is the pathogen that causes tuberculosis, the leading cause of death by infection worldwide. The success of this pathogen is due in part to its clever ability to adapt to its host environment and its effective manipulation of the host immune system. A major contributing factor to the survival and virulence of *Mtb* is its acquisition and metabolism of host derived lipids including cholesterol. Accumulating evidence suggests that the catabolism of cholesterol during infection is highly regulated by cholesterol catabolites. We review what is known about how regulation interconnects with cholesterol catabolism. This framework provides support for an indirect approach to drug development that targets *Mtb* cholesterol metabolism through dysregulation of nutrient utilization pathways.

Graphical abstract

Keywords

drug discovery; target vulnerability; metabolism; steroid; degradation; cAMP; persistence; tolerance; regulation

*corresponding author: nicole.sampson@stonybrook.edu; +1-631-632-7952.

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Introduction

Mycobacterium tuberculosis (*Mtb*) is transmitted through aerosol droplets. Once *Mtb* finds its way into the airways of a human host, the bacteria are engulfed by resident alveolar macrophages, where *Mtb* employs various mechanisms to avoid destruction and to persist in the host [1,2]. Through mechanisms not fully elucidated, *Mtb* develops a habitable environment for itself within the macrophage by switching metabolism to use host derived lipids such as cholesterol, cholesterol esters and fatty acids. This switch in carbon source is accompanied by induction of a foamy phenotype in macrophages [3]. It has been postulated that *Mtb* co-localizes with lipid droplets formed in foamy macrophages to access these lipids for metabolism [4–7]. However, recent experiments suggest the lipid droplets are formed as a host defense mechanism [8] and that *Mtb* obtains its lipid nutrients from other sources in the host cell. Regardless of source, metabolism and regulation of a major lipid component, cholesterol, has intriguing potential to develop new treatments for tuberculosis disease (TB) caused by both drug-sensitive and drug-resistant *Mtb* (Figure 1). In this review, we focus on how cholesterol metabolism is regulated and the potential for drug discovery efforts that obliquely target cholesterol metabolism.

Importance of Cholesterol during Infection

Mtb's predominant route for cholesterol import is through the ATP-dependent *mce4* transport system [9]. The ability to sustain an infection in mouse models is significantly attenuated in *mce4* mutants suggesting that cholesterol utilization is important for persistence. The complete degradation of cholesterol is an extensive, iterative process, mediated by members of the KstR1 (side chain and A/B ring) and KstR2 (C/D ring) regulons (Figure 1). The pathway of cholesterol catabolism is comprised of many steps to degrade the side chain and cyclic framework to simple metabolites acetyl-CoA, propionyl-CoA, succinyl-CoA, and pyruvate and to generate NADH [10].

Catabolite Metabolism

Cholesterol catabolites acetyl-CoA, propionyl-CoA, succinyl-CoA and pyruvate are utilized by various carbon assimilating pathways. Acetyl-CoA and pyruvate (upon transformation into acetyl-CoA) are used as substrates for the citric acid cycle to generate ATP. Acetyl-CoA also serves as a precursor for fatty acid biosynthesis. *Mtb* utilizes two pathways to process propionyl-CoA: the methylcitrate cycle and the vitamin B₁₂-dependent methylmalonyl-CoA pathway. The methylmalonyl-CoA pathway generates succinyl-CoA for anaplerosis [11–14]. Intermediates of the methylmalonyl-CoA pathway also contribute precursors to the biosynthesis of cell wall lipids and virulence factors, sulfolipid and phthiocerol dimycocerosate [15]. Recent studies suggest that acetyl-CoA, propionyl-CoA, succinyl-CoA, and redox potential may regulate metabolic activity in these pathways.

Post-Translational Modifications Regulate Metabolic Enzyme Activity

Regulation of cholesterol metabolism in *Mtb* occurs through many mechanisms such as transcriptional regulation, protein degradation, and metabolite feedback inhibition. Here, we

focus specifically on regulation by covalent modification which can function to stimulate or inhibit enzymatic activity. Cholesterol catabolites acetyl-CoA, propionyl-CoA, succinyl-CoA, and NADH are substrates that generate post-translational modifications of metabolic enzymes and these modifications contribute to the complexity of inhibiting *Mtb* metabolism during infection (Figure 2).

Acetylation and Propionylation

Reversible acetylation occurs extensively in *Mtb* and most of the protein targets are involved in central carbon metabolism, lipid biosynthesis and degradation, as well as propionate assimilation pathways (Figure 3) [16]. Acetylation in mycobacteria is catalyzed by protein lysine acetyltransferases (Pat/Rv0998 and Rv2170) and is reversed by an NAD⁺-dependent sirtuin, CobB (Rv1551c). Biochemical and biophysical characterization of Pat has been conducted in several mycobacterial species (*Mtb*, *Mycobacterium bovis* BCG, and *Mycobacterium smegmatis*) [17–20]. Mycobacterial Pat (*MPat*) possesses a distinct protein architecture; a domain fusion with an N-terminal cyclic nucleotide binding domain (cNMP) and a C-terminal (GNAT) acetyltransferase domain; which may provide a pathogenic advantage for *Mtb* [17], functioning as a bridge between external stimuli and metabolic adaptation. The ability to bind cyclic nucleotides is necessary for *MPat* acetyltransferase activity; with the binding of cyclic AMP (cAMP) significantly increasing *MPat*'s catalytic activity [17–23].

cAMP is a universal secondary messenger that helps elicit biological responses to external stimuli. *Mtb*'s intracellular cAMP concentrations increase during the infection of macrophages [24]. cAMP-dependent regulation of *MPat* and its protein substrates provides an interesting mechanism by which *Mtb* can adapt its metabolism to its microenvironment in the host. Excess intracellular cAMP can be secreted into the macrophage cytosol, causing a down-regulation of TNF α production [25,26]. Intracellular cAMP concentrations are dependent on carbon source in *M. bovis* BCG [18]. Thus, cAMP levels likely determine the regulation of catabolic enzymes during *Mtb* infection of the macrophage.

MPat, although originally annotated as an acetyltransferase, catalyzes various acyltransferase activities including acetylation, propionylation, and butyrylation [17,18,22]. *MPat* catalyzes acetylation and propionylation of multiple substrates including acetyl-CoA synthetase (Rv3667), propionyl-CoA synthetase (MSEMEG_5404), a universal stress protein, USP (MSMEG_4207), and several fatty acid-CoA ligases (FadDs), including FadD3, which is used for activation of cholesterol C/D ring catabolism [17,18,20,23]. *MPat*-mediated modification of these enzymes results in a loss of their enzymatic activity. While non-enzymatic acylation can occur in the cell, the acetylation (and propionylation) by *MPat* is site-specific [20,27]. The NAD⁺ dependent deacylase activity of CobB restores enzymes to their unmodified state and restores their catalytic activity [17,18,20]. Deletion of either *MPat* or *CobB* results in significant growth defects on both acetate and propionate [18,19]. The reversible regulation of metabolic enzymes suggests an overarching control mechanism of central carbon metabolism that is dependent on metabolite levels and redox state.

Rv2170 is a protein acetyltransferase with the ability to acetylate, succinylate, and propionylate isocitrate dehydrogenase (Icd1/Rv3339c). As with the modification of other

metabolic enzymes, acetylation of Icd1 reduces isocitrate dehydrogenase activity and shifts fatty acid metabolism toward the glyoxylate cycle and reduces flux through the TCA cycle [28]. Acetylation of isocitrate lyase (Icl) has varying effects on enzymatic activity and protein stability depending on the site of the modification [29]. These data strongly suggest that dysregulation of formation and/or removal of post-translational modifications will alter maintenance of acyl-CoA pools necessary for energy production and subsequent carbon assimilation.

Succinylation

Succinylation has been recently identified as a post-translational modification that is unique, but shows extensive overlap with acetylation in prokaryotes and eukaryotes [16,30,31]. Whole cell proteomic analyses have characterized the *Mtb* succinylation proteome [32]. In *Mtb*, the majority of modified proteins are involved in central carbon metabolism and fatty acid metabolism. For example, succinylation of Icl reduces enzymatic activity and increases *Mtb* sensitivity to rifampicin and streptomycin [33] (Figure 3).

Nineteen KstR-regulated proteins are succinylated (Table 1), and their sites of succinylation have potential enzymatic and structural consequences. Succinylation of ChsE4–ChsE5, a cholesterol side-chain acyl-CoA dehydrogenase, occurs near the active site suggesting modification regulates enzymatic activity, as seen with the acetylation of acetyl-CoA synthetase. Succinylation of ChsH1–ChsH2, a cholesterol side-chain hydratase, occurs on the DUF35 domain of ChsH2. This domain interacts with the aldolase, Ltp2, which catalyzes the subsequent step in cholesterol side chain β -oxidation [34]. Thus, it is possible that succinylation of ChsH1–ChsH2 may alter its interaction with Ltp2 and therefore affect Ltp2 stability and/or catalytic activity.

A succinyltransferase has not been definitively identified. *MtPat* and Rv2170 [28] may catalyze succinylation in addition to acetylation and/or propionylation. Rv0802c, a GCN5-related N-acetyltransferase (GNAT) family member, co-crystallizes with succinyl-CoA suggesting that Rv0802c catalyzes succinylation of lysines [35]. Alternatively, an enzyme may not be required for protein succinylation due to the high acylation potential of succinyl-CoA compared to other thioesters [27].

Disulfides

FadA5 is a β -keto-acyl CoA thiolase involved in the β -oxidation of the cholesterol side chain. The catalytic activity of FadA5 is controlled by the reversible formation of two disulfide bonds (Cys93–Cys377 and Cys59–Cys91). The midpoint potential of FadA5 is –223 mV, which is close to the redox potential of *Mtb* mycothiol (–240 mV) in activated macrophages [36]. This redox matching suggests that during infection, *Mtb* can sense environmental changes, reduce the catalytic activity of FadA5 and thus modulate the overall production of propionyl-CoA from cholesterol.

NAD⁺/NADH

Various enzymes within lipid and central carbon metabolism utilize NAD⁺ as a co-factor and the alterations in the NAD⁺/NADH ratio act as an indicator of the metabolic state of the

cell [37]. Thus, the NAD⁺ dependent activity of CobB provides another level of molecular sensing to regulate metabolic activity.

Compounds Targeting Cholesterol Metabolism

There is an urgent need for drugs with new mechanisms of action that can help combat the rising rates of multi-drug resistant TB disease. The data presented above in combination with other studies suggest that cholesterol metabolism plays a significant role in the virulence and survival of one of the world's most successful persisters, *Mtb*. Therefore, there is an impetus to develop small molecule inhibitors against cholesterol metabolism as a new therapeutic strategy to combat drug-resistant TB. Early efforts to inhibit cholesterol catabolism include azoles to target cytochrome P450 mono-oxygenases [38] and 6-azasteroids that inhibit 3 β -hydroxysteroid dehydrogenase (Hsd/Rv1106c) [39]. Although the latter catalyzes the first enzymatic step in *Mtb* cholesterol catabolism, conversion of cholesterol to cholestenone, its function was found to be non-essential in infection [40]. One possible interpretation of non-essentiality is that cholestenone formation may not be necessary to form a cholesterol metabolite that is regulatory.

Stress resistance

Recently, we discovered that several 6-azasteroids improve the efficacy of approved anti-TB therapies isoniazid and bedaquiline against *Mtb* under both aerobic and anaerobic conditions (<https://doi.org/10.26434/chemrxiv.5901226.v1>). The lead 6-azasteroids are effective regardless of carbon source and do not require cholesterol catabolism genes for activity, suggesting that pathways other than cholesterol catabolism are targeted. Intriguingly, genes in the Mce3R regulon are required for 6-azasteroid activity. Mce3R is a Tet-like repressor derepressed by cholesterol or a metabolite (similar to KstR1 and KstR2) and Mce3R controls the transcription of 22 genes, which have not been biochemically characterized to date [41–43].

The *mel*₂ locus (*Rv1936–Rv1941*) resides in the Mce3R regulon and is only found in pathogenic mycobacteria (*M. tuberculosis* and *Mycobacterium marinum*) [44]. *mel*₂ mutants show defects in persistence and reduced dissemination to the spleen of infected mice [45]. The proteins encoded in the *mel*₂ locus share homology with known fatty aldehyde synthesis pathways, a known mechanism for protection against ROS damage [46]. Consistent with their bioinformatic annotation, *mel*₂ mutants show increased susceptibility to reactive oxygen and nitrogen species [47], suggesting that the *mel*₂ operon may be another clever mechanism by which *Mtb* resists destruction by the host immune system [48]. The *echA13-fadE17-fadE18* operon also resides in the Mce3R regulon, and *fadE17–fadE18* encodes a $\alpha_2\beta_2$ heterotetrameric structure unique to *Mtb* cholesterol catabolic enzymes [49,50]. The existence of the Mce3R regulon suggests that cholesterol is more than a nutrient for *Mtb*, and cholesterol catabolites may function in other pathways contributing to *Mtb* virulence such as stress resistance. The regulatory mechanisms described above may serve to switch use of cholesterol between catabolism and stress control.

cAMP regulation

Using the macrophage infection model to recapitulate *Mtb*'s microenvironment in the host is a recent approach to increase the likelihood of finding lead compounds with promising *in vivo* anti-*Mtb* activity [51]. Using the macrophage model and cholesterol-rich media to focus on cholesterol-dependent pathways, VanderVen and colleagues identified compounds that inhibit HsaAB (cholesterol CD ring degradation) and prpC (methylcitrate cycle) [52]. Surprisingly, they also found inhibitors of *Mtb* growth that do not directly target cholesterol catabolism.

Recently, VanderVen and colleagues determined these compounds target an adenylyl cyclase (Rv1625c). Small molecule V-58 specifically stimulates Rv1625c activity and increases intracellular cAMP levels [26]. V-58 activity is carbon-source specific, reducing bacterial growth on cholesterol and propionate, but not acetate. The exact mechanism of growth inhibition is not fully understood. One possibility is that increases in cAMP (via V-58 stimulation of Rv1625c) activate acetyltransferase activity, i.e., *MPat*. These recent studies demonstrate that there are cholesterol-related targets outside of the cholesterol catabolic network which are vulnerable and druggable.

Conclusion

Cholesterol metabolism impacts myriad aspects of *Mtb* survival *in vivo*. Efforts to focus inhibitor discovery on the cholesterol catabolism pathway revealed vulnerable targets in metabolic pathways networked with cholesterol catabolism. Moreover, the cholesterol metabolic network is highly regulated. Therefore, targeting regulation of the cholesterol pathway and its interconnected pathways is an enticing approach to disrupting *Mtb*'s survival in the human host.

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Highlights

- Cholesterol is important for survival of *Mycobacterium tuberculosis* (*Mtb*) *in vivo*
- *Mtb* cholesterol catabolism is highly regulated
- Small molecule screening focused on cholesterol metabolism identified non-catabolic targets
- Regulatory vulnerabilities are an enticing prospect for future *Mtb* drug discovery

Figure 1. The regulatory network of cholesterol catabolism and utilization is a vulnerable drug target

Cholesterol metabolites induce the expression of genes in the KstR1, KstR2, and Mce3R regulons. Enzymes encoded in the KstR1 and KstR2 regulons catabolize cholesterol and are regulated by PTMs, cAMP, and mycothiol potential. The Mce3R regulon influences stress resistance and is targeted by 6-azasteroids. cAMP regulates protein acyltransferases and cAMP production is increased by small molecule V-58.

Figure 2. Post-translational Regulation of Cholesterol Catabolism

Cholesterol catabolic enzymes are post-translationally regulated by acetylation, succinylation, and redox potential. **(A)** The cholesterol catabolic pathway and the enzymes involved that are post-translationally regulated. **(B)** The different post-translational regulatory mechanisms for catabolic enzymes. The cAMP-dependent *MtPat* acetylates lysine and this modification is removed by NAD⁺-dependent sirtuin, CobB. CobB also functions as a desuccinylase. Disulfide formation in FadA5 regulates catalytic activity and is reversible.

Figure 3. Post-translational Regulation of Cholesterol Catabolite Metabolic Network

The thioester catabolites of cholesterol degradation are utilized in various metabolic pathways. Propionyl-CoA is consumed by the methylcitrate cycle and methylmalonyl-CoA pathway. Acetyl-CoA is a substrate for the TCA cycle and the glyoxylate cycle. Pyruvate is transformed into acetyl-CoA and succinyl-CoA is converted to succinate. Various proteins in this metabolic network have been identified as carrying marks for acetylation and succinylation.

Table 1

Post-translationally modified proteins utilized for cholesterol metabolism.

Rv Number	Protein	Pathway	Modification ^I	Function
<i>Rv0066c</i>	Icd2	TCA Cycle		Isocitrate dehydrogenase
<i>Rv0467</i>	Icl	Glyoxylate Cycle & Methylcitrate Cycle		Isocitrate lyase (2-methylcitrate lyase)
<i>Rv0889c</i>	CitA	TCA Cycle		Citrate synthase
<i>Rv0951</i>	SucC	Succinyl-CoA Transformation		Succinyl-CoA synthetase β -subunit
<i>Rv0952</i>	SucD	Succinyl-CoA Transformation		Succinyl-CoA synthetase α -subunit
<i>Rv1143</i>	Mcr	Cholesterol Side Chain Degradation		α -methyl-acyl-CoA racemase
<i>Rv1240</i>	Mdh	TCA Cycle & Glyoxylate Cycle		Malate dehydrogenase
<i>Rv1492</i>	MutA	Methylmalonyl-CoA Pathway		Methylmalonyl-CoA mutase (small subunit)
<i>Rv1493</i>	MutB	Methylmalonyl-CoA Pathway		Methylmalonyl-CoA mutase (large subunit)
<i>Rv1837c</i>	GlcB	Glyoxylate Cycle		Malate synthase G
<i>Rv2215</i>	DlaT	Pyruvate Transformation		Pyruvate dehydrogenase E2 component
<i>Rv2241</i>	AceE	Pyruvate Transformation		Pyruvate dehydrogenase E1 component
<i>Rv3280</i>	AccD5	Methylmalonyl-CoA Pathway		Propionyl-CoA carboxylase
<i>Rv3281</i>	AccE5	Methylmalonyl-CoA Pathway		Propionyl-CoA carboxylase

Rv Number	Protein	Pathway	Modification ^I	Function
<i>Rv3285</i>	AccA3	Methylmalonyl-CoA Pathway		Propionyl-CoA carboxylase
<i>Rv3339c</i>	Icd1	TCA Cycle		Isocitrate dehydrogenase
<i>RV3504</i>	ChsE4	Cholesterol Side Chain Degradation		Acyl-CoA dehydrogenase (α-subunit)
<i>Rv3516</i>	EchA19	Cholesterol Side Chain Degradation		Enoyl-CoA hydratase
<i>Rv3542c</i>	ChsH2	Cholesterol Side Chain Degradation		MaoC-like enoyl-CoA hydratase (β-subunit)
<i>Rv3546</i>	FadA5	Cholesterol Side Chain Degradation	S-S	3-Keto-acyl-CoA thiolase
<i>Rv3556c</i>	FadA6	Cholesterol CD Ring Degradation		3-Keto-acyl-CoA thiolase
<i>Rv3561</i>	FadD3	Cholesterol CD Ring Degradation		Fatty acid-CoA ligase
<i>Rv3563</i>	FadE32	Cholesterol CD Ring Degradation		Acyl-CoA dehydrogenase (β-subunit)
<i>Rv3568c</i>	HsaC	Cholesterol AB Ring Degradation		3,4-DHSA dioxygenase
<i>Rv3569c</i>	HsaD	Cholesterol AB Ring Degradation		4,9-DHSA hydrolase
<i>RV3570c</i>	HsaA	Cholesterol AB Ring Degradation		3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione hydroxylase

^I Red circle: acetylation; yellow star: succinylation; S-S: disulfide formation.