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New Targets and Inhibitors of Mycobacterial Sulfur Metabolism[§]

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Abstract

The identification of new antibacterial targets is urgently needed to address multidrug resistant and latent tuberculosis infection. Sulfur metabolic pathways are essential for survival and the expression of virulence in many pathogenic bacteria, including *Mycobacterium tuberculosis*. In addition, microbial sulfur metabolic pathways are largely absent in humans and therefore, represent unique targets for therapeutic intervention. In this review, we summarize our current understanding of the enzymes associated with the production of sulfated and reduced sulfur-containing metabolites in *Mycobacteria*. Small molecule inhibitors of these catalysts represent valuable chemical tools that can be used to investigate the role of sulfur metabolism throughout the *Mycobacterial* lifecycle and may also represent new leads for drug development. In this light, we also summarize recent progress made in the development of inhibitors of sulfur metabolism enzymes.

Keywords

Tuberculosis; mycobacteria; sulfur metabolism; enzymes; thiols; sulfation; drug design and inhibitors

Mycobacterium tuberculosis

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is one of the most lethal infectious agents affecting the humans [1, 2]. In 2011, ~10,521 new TB cases were reported in the United States, an incidence of 3.4 cases per 100,000 population [3]. The majority of people afflicted with TB live in developing countries, where lethal synergy with HIV infection also fuels the TB pandemic. According to World Health Organization (WHO) recent report, in 2011, worldwide there were an estimated 8.7 million new cases of TB (13% co-infected with HIV) and 1.4 million people died from TB [4]. Geographically, the burden of TB is highest in Asia and Africa. India and China together account for almost 40% of the world's TB cases. About 60% of cases are in the South-East Asia and Western Pacific regions. The African region has 24% of the world's cases and the highest rates of cases and

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deaths per capita. Almost 80% of TB cases among people living with HIV reside in Africa. There were an estimated 0.5 million cases and 64,000 deaths among children in 2011 [3–6].

M. tuberculosis is difficult to treat, requiring 6–9 months of chemotherapy with a combination of four frontline antibiotics – isoniazid, rifampin, pyrazinamide, and ethambutol [7, 8]. In large part, the lengthy drug therapy is necessary because mycobacteria exist as metabolically diverse population within the human host [8]. Some bacteria will be actively dividing, rendering them susceptible to antibiotic treatment. However, less active subpopulations of mycobacterium also exist in stationary phase or as dormant bacteria leading to latent TB infection [9, 10]. Since TB drugs target biological processes required for bacterial growth (*e.g.,* cell wall biosynthesis), they are far less effective at killing the persistent population [8, 10, 11]. Nonetheless, the treatment for the individuals with latent *M. tuberculosis* infection begins with diagnosis on the basis of a positive tuberculin skin test or an interferon-γ release assay result and includes use of one of the frontline drugs, isoniazid, rifapentine, or rifampin for 3–9 months [12, 13].

In addition to toxic side effects, the lengthy treatment regime results in poor patient compliance and drug resistant strains are beginning to emerge [5]. According to WHO, worldwide, 3.7% of new cases and 20% of previously treated cases were estimated to have multi-drug resistant TB (MDR-TB). India, China, the Russian Federation and South Africa have almost 60% of the world's cases of MDR-TB [4]. The highest proportions of TB patients with MDR-TB reside in eastern Europe and central Asia [4]. Taken together, the growing problem of MDR-TB and the lack of drugs that effectively target persistent bacteria, stress the urgent need for identification of new antimicrobial targets [6].

Many fundamental aspects of mycobacterial metabolism and pathogenesis are poorly understood, in part because of the technical difficulties inherent to studying *M. tuberculosis*. The organism must be manipulated in a biosafety level 3 laboratory, and the slow growth rate (3 weeks for colonies, up to 1 year for completion of animal models) imposes limitations on apparent research productivity. However, the availability of complete mycobacterial genome sequences [14–17] and the maturation of methods for disrupting mycobacterial genes [18–20] have provided tools that can accelerate the discovery of potential drug targets and elucidate metabolic pathways that are essential for mycobacterial survival.

OVERVIEW OF TB INFECTION

M. tuberculosis infection is a complex process that initiates with aerosol inhalation to the host lung [13, 21]. Alveolar macrophages respond to the inhaled pathogen and phagocytose them. During this process, the infected macrophages release chemokines to recruit the neutrophils, macrophages, NK cells, and γδ-T cells to mount inflammatory response and to wall out the infected macrophages. Activation of the immune system and lung inflammation induced expectoration provides an exit strategy for the bacteria to spread to another host [22, 23]. Thus the macrophages are the building blocks of the granuloma [24–27]. The granuloma is kernel like structure initiated by infected macrophages and surrounded by layers of foamy macrophages, macrophages, lymphocytes and with

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penetrating blood vessels [28]. During maturation, the number of blood vessels passing through granuloma diminishes and a fibrous sheath develops walling out the infected mycobacteria from rest of the host [28]. Within the context of the granuloma, T-cells can proliferate in response to specific mycobacterial antigens and some may leave the granuloma to reenter the circulation thus, the granuloma is a dynamic structure [28–30]. Recent experiments to study the formation of granuloma in Zebra fish models of infection revealed that mycobacterial RD1-dependent signal induces macrophage migration and facilitates random movement within the granuloma. The dying infected macrophages generate another signal that recruits nearby macrophages for phagocytosis. These studies also showed that RD1-deficient bacteria fail to elicit efficient granuloma formation despite their ability to grow inside infected macrophages [13, 22, 27, 31, 32].

However, less than 10% of infected individuals will develop active TB infection. In the rest, mycobacteria residing within granulomas enter into a persistent or "latent" state characterized by a lack of cell division and a change in basic metabolism [33, 34]. These latent mycobacteria are difficult to eradicate since they are not reliant on machinery targeted by conventional antibiotics [8]. However the granuloma which contains infected mycobacterium, may fail when the immune status of the host changes because of old age, malnutrition or co-infection with HIV which impairs the function of T cells. Following such a change in immune status the granuloma decays, ruptures and spills thousands of viable, infectious bacilli into the airways. This results in the development of a productive cough that facilitates aerosol spread of infectious bacilli [13, 28]. Hence, effective treatment of TB will require efficacy against persistent *M. tuberculosis*, or at the least a better understanding of the mechanisms underlying immune cell activation, bacterial adaptation and survival within the granuloma [8, 35, 36].

SULFUR AND MYCOBACTERIAL SURVIVAL

To complete its lifecycle, *M. tuberculosis* must survive within the hostile, nutrient-poor, reactive oxygen/nitrogen rich, di-oxygen deficient environment of the host macrophage [37, 38]. At the same time, *M. tuberculosis* must activate sufficient immune effector functions to induce granuloma formation in the lung [22, 39–42]. The mechanisms by which bacilli survive the hostile environment and transition in to dormancy are not well understood. A Recent study conducted by Forrellad *et al*. showed that a large number of different virulence factors have evolved in *M. tuberculosis* as a response to the host immune reaction [43]. However, genes involved in the metabolism of sulfur have consistently been identified as up-regulated in response to oxidative stress, nutrient starvation and dormancy adaptation (culture conditions that model aspects of mycobacterial life in the granuloma and during macrophage infection [43–53].

Sulfur is an essential element for life and plays a central role in numerous microbial metabolic processes [54]. In its reduced form, sulfur is used in the biosynthesis of the amino acids cysteine and methionine. Cysteine is incorporated into biomolecules such as proteins, coenzymes, and mycothiol (the mycobacterial equivalent of glutathione) [See Fig. 1]. Found in all actinomycetes, mycothiol regulates cellular redox status and is essential for *M. tuberculosis* survival [55–58]. Another reduced sulfur-containing metabolite, coenzyme A

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(CoA), is heavily utilized for lipid metabolism and biosynthesis of mycolic acid, which is an important constituent in mycobacterial cell wall and plays major role in antigenicity of the pathogen [59]. In its oxidized form, sulfur is present as a sulfuryl moiety ($–SO_3^-$) that can modify hydroxyls and amines in proteins, polysaccharides and lipids [See Fig. 2] [49, 50]. Sulfated glycolipids of mycobacterium are very closely related to the virulence of the pathogen. For example, Sulfolipid-1 is present only in virulent species of mycobacterium [60]. On the other hand, sulfated menaquinone, S881, suppresses bacterial virulence [61, 62]. Hence, acquisition and metabolism of sulfur are essential for mycobacterial virulence and survival. The identification of new antibacterial targets is essential to address MDR- and latent-TB infection [63, 64]. Toward this end, mycobacterial sulfur metabolism represents a promising new area for anti-TB therapy [62, 65, 66]. Numerous studies have validated amino acid biosynthetic pathways and downstream metabolites as antimicrobial targets [67–70] and sulfur metabolic pathways are required for the expression of virulence in many pathogenic bacteria [71–74]. In particular, mutants in mycobacterial sulfur metabolism genes are severely impaired in their ability to persist and cause disease [49, 50, 73, 75–77]. Furthermore, most of the microbial sulfur metabolic pathways are absent in humans and therefore, represent unique targets for therapeutic intervention. In this review, we focus on the enzymes associated with the production of sulfated and reduced sulfur-containing metabolites in *Mycobacteria*. Small molecule inhibitors of these catalysts represent valuable chemical tools that can be used to investigate the role of sulfur metabolism in *M. tuberculosis* survival and may also represent new leads for drug development. In this light, we also highlight major efforts devoted towards inhibitor discovery of mycobacterial sulfur metabolic pathways.

SULFATE ASSIMILATION IN MYCOBACTERIA

Sulfate assimilation begins with the active transport of inorganic sulfate (SO_4^{2-}) across the mycobacterial cell membrane by the *cysTWA SubI* ABC transporter complex [see Fig. 3] [78, 79]. Once sulfate is imported, it gets activated by ATP sulfurylase (encoded by *cysND*) via adenylation to produce adenosine-5’-phosphosulfate (APS) [47, 66, 80]. In mycobacteria, APS lies at a metabolic branch point [66]. For sulfation of biomolecules such as proteins, lipids and polysaccharides, APS is phosphorylated at the 3’-hydroxyl by APS kinase (encoded by *cysC*) to form 3’-phosphoadenosine-5’-phosphosulfate (PAPS), the universal sulfate donor for sulfotransferases (STs) [66, 80–82]. Transfer of $–SO_3^-$ to hydroxyl or amino functionalities of biomolecules plays important roles in regulation of cell-cell communication and metabolism [60]. Alternatively, for production of reduced sulfur-containing metabolites, the sulfate moiety in APS is reduced to sulfite (SO_3^{2-}) by APS reductase (gene product of *cysH*) [66, 73, 83]. Sulfite is further reduced to sulfide (S_2^-) by sulfite reductase (encoded by *nirA*) [84] and results in the form of sulfur that is used for the biosynthesis of sulfur-containing metabolites including cysteine, methionine, coenzymes, and mycothiol [54, 55, 85]. Each branch of sulfate assimilation is discussed in terms of the available genetic and biochemical data below.

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SULFATE IMPORT AND ACTIVATION

Present at 300–500 μM, inorganic sulfate is the fourth most abundant anion in human plasma [86]. Sulfate transporters have been identified in all major human tissues investigated to date, and of particular relevance to the intracellular lifestyle of *M. tuberculosis*, the existence of endosomal-associated transporters has also been demonstrated [86]. The genes encoding the *cysTWA SubI* ABC transporter complex in mycobacteria have been identified by homology to *Escherichia coli* and *Salmonella typhimurium* [78], are essential [49], robustly up-regulated during oxidative stress [45], dormancy adaptation [44], and expressed in macrophages [51]. Consistent with this annotation, *cysA* or *subI* mutants (*cysA* or *subI*, respectively) in *M. bovis* bacillus Calmette-Guérin (BCG) – an attenuated, vaccine strain of *M. bovis* – are compromised in their ability to transport sulfate [78, 87]. When grown in media supplemented with casamino acids, the rate of sulfate transport in *cysA* is ~1.1% relative to wild-type *M. bovis* BCG [78]. The minor amount of transport is not enough to meet bacterial sulfur requirements and hence, these sulfate transport mutants are auxotrophic for reduced sulfur.

Interestingly, no significant difference in the number of viable bacilli was observed in the organs of mice infected with *cysA* and wild-type *M. bovis* BCG up to 63 days post-infection [78]. These data indicate that *M. bovis* BCG may scavenge sufficient amounts of reduced sulfur from the host for survival. However, an important question raised from the findings of this study is whether the sulfur requirements for an attenuated *M. bovis* strain reflect those of *M. tuberculosis* known to elicit a more potent host immune response [22, 29, 40]. It is also possible that the mycobacterial genome encodes for an additional sulfate transporter which is not expressed under culture conditions, but is specifically up-regulated during infection [88, 89]. In support of this hypothesis, mRNA array analysis has shown significant up-regulation of hypothetical proteins from Rv1739c and Rv1707 [51, 78] 24 h post infection of activated macrophages in response to nitric oxide [46] or hypoxia [52]. Rv1739c expression in *E. coli* has been shown to enhance sulfate uptake, though complementation of the M. bovis *cysA* mutant with the Rv1739cgene was not sufficient to restore sulfate prototrophy [90]. In contrast, little is known about the Rv1707 gene product [62]. However these gene products are believed to be associated with inner membrane of *M. tuberculosis* [78, 91, 92]. It has recently been established that mycobacteria have an outer membrane [91], which is the primary permeability barrier to overcome the transport of any nutrient molecule. In line with these findings, recent studies conducted by Song *et al.* demonstrate that specific outer membrane proteins, called porins, are responsible for transport of inorganic anions like sulfate and nitrate in *M. tuberculosis* [93, 94] and in *M. bovis* BCG [92, 95]. From these findings, it can be inferred that the porins and *cysTWA SubI* should align to allow the transport of anions from outside the cell to inside of the cell. However, additional studies will be required to confirm the mechanism for the sulfate transport and its relevance to sulfate acquisition *in vivo*.

Once sulfate is transported to the cytosol, ATP sulfurylase (encoded by *cysD*) catalyzes the first committed step in sulfate assimilation [see Fig. 3] [47, 80]. In this reaction, the adenylyl moiety of adenosine 5’-triphosphate (ATP) is coupled to sulfate. The product that results, APS, contains a unique high-energy phosphoric-sulfuric acid anhydride bond, the

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biologically activated form of sulfate [85]. Formation of APS is energetically unfavorable (K_{eq} of $10^{-7} - 10^{-8}$ near physiological conditions) [85] and in prokaryotes, the hydrolysis of guanosine 5'-triphosphate (GTP) is coupled to sulfurylation of ATP to surmount this energetic hurdle [96]. The GTPase (encoded by *cysN*) forms a heterodimer with ATP sulfurylase (*cysD*) and synthesis of APS is driven 1.1×10^{6}-fold further during GTP hydrolysis [80, 97]. Notably, eukaryotic ATP sulfurylases do not bear any sequence or structural similarity to their prokaryotic counterparts, nor do they employ aGTPase for PAPS biosynthesis [77]. These mechanistic and structural differences, in particular the unique G protein subunit, could be exploited to develop small molecule inhibitors of bacterial sulfate activation [88]. In this direction, Pinto *et al*. showed that vaccination with ATP sulfurylase conferred significant protection against murine TB and boosted BCG-induced protective immunity in the lung [98] thereby demonstrating that components of sulfate assimilation pathway are promising candidates for inclusion in new vaccines to control TB in humans.

The final step in PAPS biosynthesis is catalyzed by APS kinase (encoded by *cysC*) [66, 82]. In this reaction, ATP is utilized to phosphorylate the 3'-hydroxyl of APS. Depending on the organism, APS kinase can be encoded as a separate protein or as a fusion with ATP sulfurylase, without significant variation in catalytic mechanism [66, 99]. Most eukaryotes (including those of humans) encode for ATP sulfurylase (*cysD*) and APS kinase (*cysC*) on a single polypeptide. In *M. tuberculosis*, however, APS kinase (*cysC*) is genetically fused to the GTPase subunit (*cysN*) of ATP sulfurylase [66]. The APS kinase domain of *M. tuberculosis cysNC* was identified through sequence homology and confirmed by genetic complementation [66]. In a subsequent report, a mutant strain of *M. tuberculosis* that removes the APS kinase domain of the bifunctional *cysNC* gene was constructed [82]. As expected, the *cysC* knockout (*cysC*) strain was able to grow on sulfate as a sole sulfur source (indicating a functional ATP sulfurylase), but was unable to synthesize PAPS [82].

Fusion of APS kinase to the GTPase domain of ATP sulfurylase raised the interesting possibility of substrate channeling between subunits [88, 99]. In this scenario, the final product PAPS, and not the APS intermediate, would be released into solution. Leyh *et al*. have recently tested this hypothesis for *M. tuberculosis* ATP sulfurylase [99]. Although PAPS synthesis is 5,800 times more efficient than APS synthesis [80], these studies demonstrate that APS is not channeled from the *M. tuberculosis* adenylyltransferase to the APS kinase domain [99], consistent with the domain arrangement proposed from a recent crystal structure of the *cysNC* complex [97].

Collectively, *cysNC* and *cysD* proteins form a multifunctional enzyme complex ~300 KDa (consistent with a trimer of CysNC•D heterodimers), referred to as the sulfate-activating complex (SAC) [47, 80, 100]. In *M. tuberculosis*, expression of the SAC operon is induced by conditions likely to be encountered by pathogenic mycobacteria within the macrophage, including sulfur limitation, oxidative stress, and is repressed by cysteine [45, 47]. The SAC operon is also up-regulated during stationary phase growth, an *in vitro* model of persistent *M. tuberculosis* infection [44]. *M. tuberculosis* SAC gene expression is also augmented within the intracellular environment of the macrophage [51, 101]. Taken together, these data

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are consistent with increased activity of sulfate activating enzymes and flux through the sulfate assimilation pathway during mycobacterial infection.

REGULATION OF SULFUR METABOLISM

Transcriptional regulation

Upon infection, *M. tuberculosis* undergoes phagocytosis by alveolar macrophages exposing the pathogen to nutrient poor and oxidative environment. This stress induces several genes in *M. tuberculosis* related to sulfate assimilation pathway to counteract against the oxidative stress and transition to dormancy [44, 47, 102, 103]. Genes that coordinate the sulfate transport and the first few steps of sulfate assimilation are over expressed when *M. tuberculosis* is subjected to variety of stress conditions. For example, *cysT* was induced following by stimulus with hydrogen peroxide. Nutrient starvation conditions induced *cysA1*, *cysT*, *cysW*, *subI* genes that encode for sulfate transporter complex. Also *cysD* which encodes ATP sulfurylase, and *cysNC,* which encodes for bifunctional APS kinase, are induced in response to environmental challenges [104]. Hypoxia mediated induction of *cysD*, *cysNC*, *cysK2*, *cysM*, cys*T* and subsequent activation of ATP sulfurylase and cysteine biosynthesis indicate genetic level regulation of sulfate assimilation to defend against reactive species in the granuloma [105].

Some frontline antibiotics used to treat TB also induce genes of the sulfate assimilation pathway. For example, menadione (a vitamin K precursor that promotes the production of reactive oxygen species) induces *cysA1, cysT, cysW, subI* genes [106, 107], Vancomycin (peptidoglycan biosynthesis inhibitor) induces *cysK2, cysD, cysNC* [62, 108], and Chloropyrazinamide (fatty acid biosynthesis inhibitor) induces *cysNC* [62, 106]. These data suggest that genes involved in sulfite assimilation respond to antibiotics used in current treatment regimes.

Sigma (σ) factors are bacterial transcription initiation factors, which direct the RNA polymerases to specific promoter sites and regulates the expression of housekeeping genes. It is proposed that one or more σ factors play a role in modulating the various stress adaptive genes to cope with unfavorable conditions in the granuloma [109]. One such factor is σ^H (SigH), which is induced in response to heat shock, oxidative stress, pH variation and phagocytosis [109–113]. It has been shown that σ^H regulates the transcription of several sulfate metabolism genes (*cysA1, cysT, cysW, cysD and cysNC*) following diamide treatment [62, 114] and is required for virulence in animal models of TB infection [115]. The σ^H factor is auto-regulated by its own promoter at the transcriptional level by anti-sigma factor RshA [116–118]. An analogous type of regulation was identified in *Streptomyces coelicolor* [119]. Site directed mutagenesis studies and deuterium exchange mass spectrometry conducted by Kumar *et al.* showed that these opposing sigma factors communicate through salt bridges [117]. Disruption of such communication creates a positive feedback loop that leads to rapid and strong induction of SigH-regulated gene expression [116]. It has been shown that partial inhibition of the SigH-RshA interaction is also possible when RshA is phosphorylated by protein kinase B, and is shown to result in partial activation of SigH [115]. It is not clear why mycobacterium utilizes partial activation of SigH, compared to

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complete activation. It is possible that regulatory mechanism is used to fine-tune the response to adverse environmental conditions.

Biochemical regulation

In addition to regulation at the transcriptional level, several additional proteins are involved in the sulfate import and activation. Recent studies demonstrated that mycobacterium possesses an outer membrane, which is the primary barrier for transport of molecules across the membrane [91]. The outer membrane was shown to contain specific channels called porins which allow the passage of specific anions through the inner membrane [92–95]. The primary sulfate transporter is an ABC transporter encoded by *subI-cysTWA1* operon [78] located at the inner membrane and is expressed in response to oxidative stress and phagocytosis. Genetic disruption of *cysA1* gene in *M. bovis* results in complete inhibition of sulfate uptake and renders the mutant auxotrophic to methionine characteristics. However, this mutation does not result in bacterial death [78]. This observation indicates the presence of alternate sulfate transporters that are activated during infection, or implies the existence of other biochemical pathways that feed into the cysteine biosynthetic pathway. Interestingly, *M. tuberculosis* is shown to be expressing two additional putative sulfate transporters, Rv1739c and Rv1707 [51, 90]. In *E. coli,* Rv1739c is shown to be associated with increased up take of sulfate. However, when the *M. boviscysA1* mutant was complemented with the *Rv1739c* gene, sulfate prototrophy was not restored [90]. This suggests that the pathogen relies on alternative biochemical pathway to generate cysteine during critical times. In agreement with this hypothesis, recent biochemical studies established an alternative route for cysteine biosynthesis in *M. tuberculosis*, which utilizes the *O*-phosphoserine as carbon skeleton [120, 121]. It is possible that this pathway may be contributing to the survival of the mutant *cysA1* strain of *M. bovis*.

Adenosine-5'-phosphosulfate (APS) reducatse, *cysH*, catalyzes the first committed step in sulfate assimilation pathway. CysH catalyzes the formation sulfite and adenosine-5'-phosphate (AMP) from APS [83]. The resulting sulfite is further converted into sulfide, which is the required form of sulfur for biosynthesis of cysteine, mycothiol and other sulfur-containing metabolites. It has been shown that AMP is a potent inhibitor of APS reductase activity [122] which may indicate that this reaction byproduct negatively regulates cysH to control the downstream biosynthesis of sulfur-containing metabolites. The 3'-phosphoadenosine-5'-phosphatase, *cysQ*, is another regulator of *M. tuberculosis* sulfur metabolism. CysQ dephosphorylates both 3'-phosphoadenosine-5'-phosphate (PAP), and its counterpart3'-phosphoadenosine-5'-phosphosulfate (PAPS) which is utilized in the sulfation of biomolecules [62, 123]. It has also been shown that PAP is an inhibitor of at least one sulfotransferase [124] and that PAPS accumulation being cytotoxic [125]. Recent studies demonstrate that cysQ activity is inhibited by alkali metal cations *in vitro* at physiological concentrations in *Streptococcus mutans* [126]. Taken together, these studies demonstrate that the dephosphorylation of PAP and PAPS by cysQ regulates sulfation and balances sulfur utilization in defense mechanisms.

Sulfotransferases and sulfatases are the major enzymes responsible for sulfate transfer and removal processes. It has been reported that the sulfotransferases, stf0 and stf3, from *M.*

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tuberculosis are involved in the biosynthesis of outer envelope molecules termed SL-1 and S881 [127, 128]. Sulfatases hydrolyze sulfate esters from sulfated proteins, peptides and arylsulfates [129]. The *M. tuberculosis* genome encodes six type 1 sulfatases which are characterized as having a unique active site formylglycine residue used for catalysis. The formylglycine is either co- or post-translationally installed in the active site by the formylglycine activating enzyme. Recent studies identified a sulfatase, atsG, which possesses aryl sulfatase activity. However, the precise role of this sulfatase is not yet established [130]. In the absence of formylglycine activating enzyme, *M. tuberculosis* retains sulfatase activity indicating that *M. tuberculosis* possesses formylglycine independent sulfatases [62, 131]. Given the importance of sulfur compounds under stress conditions, it is possible that the mycobacterial sulfatases may play a role in scavenging the residual sulfate from the nonessential metabolites.

SULFOTRANSFERASES AND SULFATION

Sulfotransferases (STs), are the enzymes that install sulfate esters, transfer sulfate from PAPS (produced by the SAC) to a hydroxyl or, less frequently, to an amide moiety on glycoproteins, glycolipids and metabolites [see Fig. 3] [132]. Sulfated metabolites are abundant in higher eukaryotes, particularly mammals, where they function primarily in cell-cell communication. For example, sulfated glycoproteins mediate interactions of leukocytes with endothelial cells at sites of chronic inflammation, sulfated peptides such as hirudin and cholecystokinin act as hormones, and sulfated glycolipids are involved in neuronal development [133, 134]. In contrast, reports of sulfated metabolites in prokaryotes have been rare. In 1992, Long *et al*. reported the first functionally characterized sulfated metabolite from the prokaryotic world – the nodulation factor NodRm-1 from *Sinorhizobium meliloti* [135]. This sulfated glycolipid is secreted from the bacterium and acts on host plant cell receptors thereby initiating symbiotic infection [136].

Among pathogenic bacteria, only one family has been reported to produce sulfated metabolites – the *Mycobacteria*. More than 40 years ago, Goren and coworkers isolated an abundant sulfated glycolipid from the *M. tuberculosis* cell wall and characterized its structure as shown in Fig. 2 [137, 138]. Termed as sulfolipid-1 or SL-1, this compound has only been observed in the tuberculosis complex; it is absent in non-pathogenic mycobacteria such as *M. smegmatis*. Comprising a trehalose-2-sulfate (T2S) core modified with four fatty acyl groups, SL-1 accounts for almost 1% of the dry weight of *M. tuberculosis*. Early studies found a correlation between the abundance of SL-1 and the virulence of different clinical *M. tuberculosis* isolates [139, 140] and its location in the outer envelope has prompted speculation that it may be involved in host-pathogen interactions [141]. The possible link between SL-1 and *M. tuberculosis* virulence led to the search for the exact functions of SL-1. Indeed. SL-1 has been attributed to altering phagosome-lysosome fusion, disrupting oxidative phosphorylation and controlling cytokine and reactive oxygen species produced by human leukocytes in cell culture models [142–147]. However, the biosynthetic pathway for SL-1 has recently been elucidated [88, 147–150] and knockout studies of SL-1 biosynthesis (stf0) have revealed that *M. tuberculosis* strains lacking SL-1 exhibited enhanced intracellular survival in human but not in murine macrophages suggesting a role for SL-1 in *M. tuberculosis* virulence in host specificity [151, 152].

Recent genetic and biochemical studies identified two integral membrane proteins, Chp1 and Sap (corresponding to gene loci Rv3822 and Rv3821) associated with transfer of acyl group regioselectively to SL-1278 in two successive reactions to yield tetra acetylated product, SL-1 (see Fig 2)[147]. These data indicate that Chp1 aids in the biosynthesis of SL-1 within the cytosolic compartment. Then Sap, together with MmpL8 (sulfolipid transporter) transports SL-1 across the membrane. The mechanism Chp1 localization and the coupling of biosynthesis/transport *via* MmpL-8 indicate formation of complex macromolecular protein complex to facilitate the function. However, the precise mechanism by which MmpL-8 and Sap transport SL-1 is unknown [61, 153].

In addition to SL-1, other novel sulfated metabolites have also been identified in *M. tuberculosis* using an innovative metabolomic approach that combines genetic engineering, metabolic labeling with a stable sulfur isotope ($^{34}SO_4^{2-}$) together with mass spectrometry analysis [154] [see Fig. 2]. Structurally distinct sulfated metabolites have also been identified in several other mycobacterial species, including *M. smegmatis*, *M. fortuitum*, and the HIV-associated opportunistic pathogen *M. avium* [see Fig. 2] [154–157]. Interestingly, in *M. avium* a sulfated cell wall glycopeptidolipid was recently found to be up-regulated in HIV patients with acquired drug resistance [155]. Significant work remains to fully characterize and elucidate the biological significance of sulfated metabolites found in mycobacteria. A major step toward this objective is to define the biosynthetic pathways of mycobacterial sulfated metabolites, including the STs responsible for installing the sulfuryl moiety.

In 2002, an analysis of mycobacterial genomes reported by Mougous *et al*. revealed a large family of open reading frames with homology to human carbohydrate sulfotransferases [132]. The predicted proteins shared regions of sequence homology associated with binding to their common substrate, PAPS. Presently, four such genes have been identified in *M. tuberculosis* (annotated as *stf0–3*) and the *M. avium* genome encodes nine putative STs (stf0, 1, 4–10) [88]. To date, of the 11 predicted STs found in mycobacterial genomes, genetic and biochemical studies have only been reported for stf0, stf3 and stf9.

Stf0 is present in a number of other pathogenic bacteria and initiates the biosynthesis of SL-1 by sulfating the disaccharide, trehalose, to form T2S [see Fig. 2 **and** 3] [150]. The structure of stf0 in complex with trehalose has recently been reported and has revealed several interesting features [150]. In the presence of trehalose, stf0 forms a dimer both in solution and in the crystal structure. Moreover, stf0-bound trehalose participates in the dimer interface, with hydroxyl groups from a glucose residue bound in one monomer forming interactions with the other monomer. Residues involved in substrate binding and dimerization have been identified, along with a possible general base (*i.e.,* Glu36) that may facilitate nucleophilic attack of the 2'-hydroxyl group on PAPS. A panel of synthetic glucose and trehalose analogs has also been tested for binding and it was found that any modification to the parent disaccharide compromises substrate sulfation [150]. A kinetic study of the enzyme using MS revealed the order of substrates binding which is consistent with a random sequential mechanism involving a ternary complex with both PAPS [or 3'-phosphoadenosine-5'-phosphate, (PAP)] and trehalose (or T2S) bound in the active site [158].

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Stf3 may play a regulatory role in *M. tuberculosis* virulence [159]. In a mouse model of TB infection, a mutant strain in which stf3 was disrupted (stf3) was unable to produce a sulfated molecule termed, as “S881”. Interestingly, when compared to wild-type *M. tuberculosis*, stf3 exhibited a hyper virulent phenotype indicating that stf3 may negatively regulate virulence through the synthesis and cell surface localization of S881 [128].

Stf9 shows higher similarity to human heparan sulfate 3-*O*-sulfotransferase isoforms compared to bacterial STs [160]. Stf9 possesses the characteristic of PAPS binding motif inherent to sulfotransferases and can transfer a sulfate group from *p*-nitrophenolsulfate onto 3'-phosphoadenosine-5'-phosphate. Stf9 is also capable of transferring a sulfate group from PAPS onto certain acceptor substrates in *E. coli*. [161]. Recently the crystal structure of stf9 in complex with a sulfate ion was solved and a possible mechanism for sulfation was proposed [160]. Despite this advance, the actual substrate for stf9 remains unknown. No other relatives of the remaining Stf family members are found in any other prokaryotic genomes, suggesting that they are unique to mycobacteria. Substrates for the majority of mycobacterial STs remain to be elucidated.

Historically, sulfotransferase assays have often been conducted using ^{35}S labeled substrate in combination with chromatography, electrophoresis, or filter binding [162–165]. Non-radioactive assays using spectrophotometry and mass spectrometry have also been reported [166–168]. Recently, Prather *et al.* developed a universal phosphatase-coupled sulfotransferase assay. In this method, Golgi-resident PAP-specific 3-phosphatase (gPAPP) is coupled to a sulfotransferase reaction by release of 3′-phosphate from PAP. The released phosphate is then detected by malachite green [169]. The enzyme kinetics of gPAPP allowed them to calculate coupling rate (i.e. the ratio of product-to-signal conversion) of the coupled reaction. Using this method, Michaelis–Menten constants were obtained for human carbohydrate sulfotransferase (CHST10) and cytosolic sulfotransferase (SULT1C4) with the substrates phenolphthalein glucuronic acid and α-naphthol, respectively. The activities obtained with the method were also validated by performing simultaneous radioisotope assays [169]. Thus this assay eliminates the requirement for radio-labeled substrates and should accelerate drug discovery campaigns for sulfotransferase targets.

ST Inhibitor Discovery

Although the roles of sulfated metabolites in the mycobacterial lifecycle remain under investigation ([88] and references therein), the analogy to sulfation in higher eukaryotes is compelling. The challenges to defining their role in mycobacterial infection and survival are two-fold: (1) the collection of sulfated metabolites must be identified and structurally characterized; and, (2) the biosynthetic pathway of the sulfated metabolites must be elucidated. In addition to traditional genetic approaches, small molecule inhibitors of STs in mycobacteria would also be useful tools to dissect their physiological roles. In addition, since STs play critical biological roles in higher eukaryotes and are implicated in several disease states, they also represent promising therapeutic targets [133, 170]. Since prokaryotic STs have not been discovered until relatively recently, the majority of research and inhibitor discovery has focused primarily on eukaryotic STs. Nonetheless, these studies

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can serve as a platform for mycobacterial ST inhibitor design and the most fruitful efforts to date have been highlighted.

There are two classes of STs - cytosolic and Golgi-resident enzymes [132, 133, 171]. In general, cytosolic STs sulfonate small molecules such as hormones and bioamines while membrane-bound STs prefer larger substrates such as proteins and carbohydrates. STs have also been further classified according to their functional role into estrogen STs (EST), heparin STs, tyrosyl protein STs (TPST), N-Acetyl glucosamine 6-*O*-ST and carbohydrate STs. The first crystal structure to be elucidated was that of murine estrogen sulfotransferase (mEST) in 1997 [172] and since then, structures of nine other STs have been characterized. These include cytosolic STs such as Phenol ST (SULT1A1) [173], catecholamine ST (SULT1A3) [174], mycobacterial stf0 [150] and Golgi-resident STs (GSTs) such as heparan *N*-deacetylase-*N*-ST-1 (NDST-1) [175]. Structures of STs in complex with PAPS or PAP reveal a conserved nature of the cofactor binding site, suggesting that STs share a similar mechanisms of sulfuryl transfer. The catalytic site of each ST must also accommodate diverse substrates and these differences in specificity are reflected in the substrate-binding site of each ST [170].

Bisubstrate analogs

To investigate molecules that inhibit both the PAPS- and substrate–binding domains of STs simultaneously, synthetic bisubstrate analogs have been employed [176, 177]. Compounds were designed to incorporate elements from the cofactor, PAPS and the substrate, providing specificity via critical interactions within both binding pockets of the enzyme [178]. Inhibitor potency is achieved from the entropic advantage of linking structures that mimic each substrate. On screening a 447 member 3’-phosphoadenosine library, several bisubstrate-based compounds were identified (**1**) [176], (**2**) [177] as inhibitors of EST (see Fig. 4). The activities of these compounds were comparable to some of the other compounds known to be inhibitors of EST including polychlorinated biphenols (**3**), discovered by testing a large number of hydroxylated polychlorinated biphenyl metabolites [179] and dietary agents like Quercetin (4), identified from a study investigating the inhibitory effects of natural flavonoids on EST activity (see Fig. 4) [180].

Similar substrate-emulating approaches have also been used to design inhibitors for E-, P- and L-selectins, all prime targets for anti-inflammatory drug discovery [181]. GSTs are involved in biosynthesis of the L-selectin ligand, 6’-sulfo sialyl Lewis X [182]. The sulfonation of sialyl Lewis X motif by GST leads to a strong interaction with receptors on L-selectin cell adhesion molecules resulting in a potent anti-inflammatory response. A “glycomimetic” strategy was used to design inhibitors for these STs. In this approach, the inhibitors retained structural and functional aspects of the natural ligands, but were designed to be synthetically more feasible [183]. One selectin antagonist (**5**), was identified using this strategy and it is currently under clinical trials (see Fig.4) [184].

Kinase-Derived Inhibitors

The “kinase inhibitor” approach exploits the similarity between reactions catalyzed by STs and kinases. Since STs and kinases use adenosine-based donor nucleotides to transfer an

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anionic moiety onto their respective substrates (PAPS for STs and ATP for kinases), it was proposed that ATP derivatives might also function as ST inhibitors [133, 185]. Furthermore, the hydrophobic adenine binding pockets of EST [172, 186] and heparin N-sulfotransferases [175] are similar to those of several kinases. A 2, 6, 9-trisubstituted purine library [187], originally designed to target cyclin dependent kinase 2, was tested for inhibitory activity with carbohydrate STs. Of the 139 compounds screened, the six most potent purines exhibited half maximal inhibitory concentrations (IC_{50s}) that ranged from 20 – 40 μM (**6**) [185], with five of them having a common benzyl substituent at N6 (see Fig. 4). Though these inhibitors showed selectivity for carbohydrate STs, achieving selectivity over kinases still remains a challenge. A high throughput screen of 35,000 purine and pyrimidine analogs has also identified a potent inhibitor of β–arylsulfotransferases (β-AST-IV) (**7**) [188] (see Fig. 4).

A second class of kinase inhibitors, isoquinoline sulfonamides, has also been tested for inhibitory activity against a panel of STs consisting of EST, NodH, GST-2 [189]. Isoquinoline sulfonamide inhibitors were developed after a crystal structure of cyclic adenosine-5'-phosphate (cAMP) dependent protein kinase in complex with isoquinoline showed that the heterocycle moiety was bound in the subsite occupied by the adenine ring of ATP. Among 100 isoquinoline and quinoline derivatives screened, the most active compounds inhibited single enzyme selectively with modest IC_{50} values in the range of 30 – 100 μM (**8, 9**) [170, 190] (see Fig. 4).

Combinatorial Target-Guided Ligand Assembly

In this strategy, a library of ligands or 'monomers' carry a common chemical handle to facilitate their combinatorial assembly [190]. In the first round, monomers were screened against the ST target at concentrations of 1 mM or higher. Compounds that demonstrated inhibitory activity were then used to construct a library of 'dimers' via an oxime linkage, and were screened for inhibitory activities. This approach resulted in the identification of two of the first known inhibitors of Golgi-resident tyrosyl protein ST-2 (TPST-2) (**10, 11**) [190] (see Fig. 4).

ST inhibitors identified in the studies above are a promising start in drug discovery efforts. However, to date the majority of ST inhibitor compounds possess fairly modest IC_{50}s, are neither "drug-like", nor suffer from a lack of specificity. Recent advances in structure-based drug design and high-throughput screening should greatly facilitate the discovery of new inhibitors for STs and other sulfonucleotide-binding enzymes.

OXIDATIVE MACROPHAGE ANTIMICROBIAL ACTIVITY

In order to replicate and persist in its human host, *M. tuberculosis* must survive within the hostile environment of the macrophage, where bactericidal oxidants – superoxide ($O_2\cdot^-$) and nitric oxide (NO·) – are generated in response to infection [191]. Two enzymes, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and inducible nitric oxide synthase (NOS2), are largely responsible for production of these reactive oxygen and nitrogen intermediates (termed as ROI and RNI, respectively) [192, 193].

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NADPH oxidase is a membrane protein that generates $O_2\cdot^-$ by transferring electrons from NADPH inside the cell across the phagosomal membrane; the electrons are coupled to molecular oxygen to produce $O_2\cdot^-$ [194]. Subsequently, $O_2\cdot^-$ can accept an electron spontaneously or can be reduced by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2) [195]. In turn, H_2O_2 can oxidize cellular targets or can be converted into the highly damaging hydroxyl radical (OH·) through the iron-catalyzed Fenton-Haber-Weiss reaction in which solvent accessible ferrous iron (Fe^{+2}) is oxidized by H_2O_2 to yield (OH·) [196]. Because Fe^{+2} is capable of localizing to DNA, proteins and lipids, generation of OH· may occur in the immediate vicinity of these biomolecules. Thusly generated hydroxyl radicals indiscriminately attack nearby proteins, lipids, iron-sulfur clusters and DNA in a cytotoxic or mutagenic manner to induce cell death. Unfortunately, there does not exist any known enzyme to detoxify the cellular hydroxyl radicals [38]. It has been shown that Fe^{+2} can specifically bind to sequences that exist in the operator sites and promoter regions of DNA where the iron regulatory transcription factor, Fur, binds and has been implicated in the iron homeostasis [197, 198]. Thus, these regions are at high risk for oxidative modifications through hydroxyl radicals.

In the NOS2 reaction, the guanidino nitrogen of arginine undergoes a five-electron oxidation via a *N*-ω-hydroxy-L-arginine (NOHLA) intermediate to yield ·NO [199]. The reaction of ·NO with biological thiols can lead to *S*-nitrosylation, or to sulfenic acid, or to disulfide formation. However all of these forms are potentially reversible changes [200]. The combination of the two oxidant-generating systems can also exert a synergistic effect in bacterial killing as macrophages can generate $O_2\cdot^-$ simultaneously with ·NO, yielding the more reactive peroxynitrite ($ONOO^-$) [201]. Peroxynitrite has pK_a of 6.8 at 37 °C and peroxynitrous acid (OONOH) is unstable with half life of under 1.0 second and readily decomposes to give OH· and nitrogen dioxide ($\cdot NO_2$) [200, 202]. Peroxynitrite is a highly reactive oxidant capable of oxidizing deoxyribose and dimethylsulfoxide with high yields at acidic pH [200]. It has been shown that peroxynitrite anion oxidizes sulfhydryls 10^3 times faster than H_2O_2 and proposed that this reaction might be the important mechanism of oxygen radical mediated toxicity [202]. The other product of peroxynitrite decomposition is nitrogen dioxide which is also a strong oxidant with significant cytotoxic potential [200]. A consequence of NADPH oxidase and NOS2 enzymatic activities and the resulting "oxidative burst" is that phagocytosed bacteria are killed by oxidative damage to a range of protein and DNA targets [191, 199, 203].

In mice, activation of macrophages induces production of NOS2 and phagosomal NADPH oxidase, via ligation of toll-like receptors (TLRs), or via stimulation by the cytokines IFN-γ or TNF-α [204, 205]. In mouse models of TB, numerous studies have demonstrated that NOS2 plays an essential role in controlling persistent infection. Macrophages can inhibit mycobacterial growth via NOS2-generated RNI, inhibition of NOS2 during persistent infection leads to reactivation of disease, and NOS2 gene-disrupted mice are extremely susceptible to TB infection [204, 205]. More recently, a proteomics study has identified proteins in *M. tuberculosis* that are targeted by RNI stress [206]. Notably, many essential metabolic and antioxidant defense enzymes are among those proteins found modified for RNI.

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While good evidence exists for ROI-mediated bacterial killing of other bacterial, fungal and parasitic pathogens, their bactericidal effect on mycobacteria has been less clear. Studies demonstrate that *M. tuberculosis* resists killing by ROI *in vitro* and that mice with defects in p47 or gp91 subunits of phagocyte NADPH oxidase (Phox) are also relatively resistant to TB infection [204, 207]. However, NADPH oxidase is highly active during the persistent phase of *M. tuberculosis* infection in mice [208]. This observation suggests that *M. tuberculosis* must possess extremely effective detoxification pathways to counter ROI stress. Consistent with this hypothesis, mice deficient in the KatG catalase-peroxidase survived longer than *pg91phox*-deficient mice [208]. More recently, it was shown that macrophages deficient in early stages of Phox assembly exhibited reduced bacterial killing, correlating with decreased production of ROI [209]. Taken together, these observations indicate that survival of *M. tuberculosis* within macrophages depends upon the ability of the bacteria to counter oxidative assault.

Mycobacteria produce enzymes such as SOD, peroxidases, catalases, and nitrosothiol reductases to help counteract the effect of ROI/RNI and promote intracellular survival and persistence in the host [191, 210–212]. The analysis of the *M. tuberculosis* genome has revealed that *M. tuberculosis* lacks classical redox sensors such as fumarate/nitrate reduction regulator (FNR), oxygen binding heme protein, FixL, and peroxide stress induced transcriptional regulatory protein OxyR [213]. However, recent studies have established that *M. tuberculosis* possesses some unique redox sensors, such as heme-based DosS and DosT. These sensors can detect different types of redox stress, including hypoxia, nitric oxide, carbon monoxide, and the intracellular redox environment [213]. In addition to enzymatic detoxification of ROI and RNI, reduced sulfur-containing metabolites are an essential component of bacterial antioxidant defense systems [214–218]. Specifically in mycobacteria, low molecular-weight thiols such as mycothiol [see Fig. 1], play a central role in maintaining a reduced cellular environment [214, 219]. Proper redox homeostasis is essential for normal cellular function and to mitigate the effects of oxidative stress. Hence, the metabolic route used for the production of reduced sulfur-containing metabolites [see Fig. 3] is predicted to be important for mycobacterial survival [88, 220, 221]. Consistent with this hypothesis, expression of mycobacterial genes involved in reductive sulfate assimilation is induced by oxidative stress within the environment of the macrophage [47, 222–230].

THE ROLE OF ROI/RNI IN MODULATING CIDAL ACTIVITY OF ANTIMYCOBACTERIAL DRUGS

Bacteria growing aerobically generate ROS/RNS as metabolic byproducts, which are detoxified by specific intracellular mechanisms. Recent studies with *E. coli* showed that amplification of endogenous ROS/RNS production and inhibition of detoxification/repair by manipulating metabolism increase the sensitivity of the pathogens to antibiotics [231]. During *in vivo* infection, *M. tuberculosis* is also exposed to varying amounts of oxygen at different stages ranging from 100 mm Hg in the alveolus to 60 mm Hg in normal lung and 3mm Hg in the center of granuloma [232]. The survival of mycobacteria at 3 mm Hg in granuloma indicates that the pathogen balances the severe hypoxia imposed by the host

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immune system with its own defense mechanisms. Any shift in the pathogen's redox balance towards higher intracellular ROS/RNS could exceed the threshold level for ROS/RNS tolerance. Thus, there is a possibility to improve the bactericidal activity of the antibacterial agents by selectively modulating pathogen ROS/RNS metabolism.

Recent studies conducted by Bulatovic *et al.* support the hypothesis that increased oxidative stress may augment the susceptibility of *M. tuberculosis* to Isoniazid (INH) [233]. INH is the first line antibiotic used to treat TB. Notably, INH is a pro-drug and requires oxidative activation by the catalase-peroxidase hemoprotein, KatG [234]. When INH is combined with clofazimine and/or plumbagin (compounds capable of generating super oxide), it lowers the MIC of INH for *M. tuberculosis* H37Rv [233]. Biochemical studies indicate that mutations in the KatG result in impaired activation of INH and, thereby, the pathogen becomes resistant to INH. Interestingly, however, clofazimine and plumbagin, in combination with INH are able to inhibit an S315T KatG mutant of *M. tuberculosis*. Since clofazimine alone has antimycobacterial activity, these studies raise the possibility of using both drugs in combination to treat TB [233].

Like INH, Pyrazinamide (PZA), is also a frontline antitubercular drug and requires activation by nicotinamidase/pyrazinamidase of *M. tuberculosis for conversion into the* active pyrazinoic acid (POA) [235]. Recent studies indicate that pyrazinamide treatment induces the production of hydroxyl radicals and other ROS in bacteria under slightly acidic pH (~6.0–6.5), analogous to that observed in macrophages of the granuloma [236]. Thus Kim *et al.* have shown that up-regulation of hydroxyl radicals by INH and PZA may initiate autophagy for effective antimycobacterial action [236]. ROS release during chemotherapy has been observed in both TB patients and experimental studies. A combination of antibiotics, INH, PZA and rifampin significantly increased plasma allantoin, an ROS marker, in TB patients [237]. Another study of TB infection in animal models has shown that INH and rifampin induce a significant respiratory burst involving NADPH oxidase and NOS [238] and the generated ROS are implicated in bactericidal activity *in vivo* [236, 239–242]. Thus, ROS generation may be induced by antimycobacterial drugs and that ROS production can be augmented using different drug combinations.

One challenge in TB treatment is to eradicate the persistent population. Recent biochemical studies conducted by Grant *et al.* have demonstrated that persistent cells constitute a distinct subpopulation within the larger culture population. They found that survival of persistent cells requires a small (i.e. ~20%) drop in the dissolved oxygen. If the dissolved oxygen is maintained at high levels this population is killed over time. Higher dissolved oxygen may increase ROS production during infection, leading to the killing persisters [239]. Supporting this hypothesis, they found that the hydroxyl radical scavenger, thiourea, protects persisters at high levels of dissolved oxygen. Conversely, clofazimine, which produces ROS, successfully eradicates the persistent population [239]. These data suggest that persistent population has differential susceptibility to antibiotic-induced hydroxyl radicals compared with the larger antibiotic susceptible population.

There is a growing evidence for the critical role of ROS and oxidative damage in bactericidal action of antibiotics. This understanding of how bactericidal antibiotics result in

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cell death raises the hypothesis that drug tolerance may be mediated by pathogens own abilities to detoxify the ROS and the surrounding oxygen levels [239]. Thus, next generation approaches to antibiotic discovery may focus on identifying small molecules that potentiate hydroxyl radical formation or inhibit the molecular mechanisms that detoxify the intracellular oxidative stress. Such drugs might play a major role in eradicating the persistent population of *M. tuberculosis* from the host.

SULFATE REDUCTION

APS reductase (encoded by *cysH*) catalyzes the first committed step in the biosynthesis of reduced sulfur compounds [see Fig. 3]. In this reaction, APS is reduced to SO_3^{2-} and adenosine-5'-phosphate (AMP) [243]. Thioredoxin (Trx), a 12.7 kDa protein with a redox active disulfide bond, supplies the reducing potential necessary for this two-electron reduction [244]. The SO_3^{2-} product of this reaction is reduced further to S^{2-}, which is used for the biosynthesis of reduced sulfur-containing metabolites, such as cysteine, methionine, CoA, iron-sulfur clusters and mycothiol [245, 246] [see Fig. 1]. Consistent with its important metabolic role, APS reductase was identified in a screen for essential genes in *M. bovis* BCG [226] and *cysH* was actively expressed during the dormant phase of *M. tuberculosis* and in the environment of the macrophage [222, 229].

Humans do not reduce sulfate for *de novo* cysteine biosynthesis and therefore, do not have a CysH equivalent. Thus, APS reductase may be an attractive drug target if the enzyme is required for bacterial survival or virulence *in vivo* [88, 220, 221, 247]. To test this hypothesis, Senaratne *et al*. generated an *M. tuberculosis* mutant strain lacking *cysH* (*cysH*) [220]. As predicted, the mutant strain was auxotrophic for cysteine and could only be grown in media supplemented with this amino acid, methionine or glutathione (from which cysteine can be generated catabolically). The *cysH* mutant exhibited attenuated virulence in BALB/c and C57BL/6 immunocompetent mice. Growth kinetics in the lungs, spleen and liver of mice infected with *cysH* or wild-type *M. tuberculosis* were also quantified. Strikingly, the number of colony-forming units recovered from the *cysH* mutant mirrored those of wild-type *M. tuberculosis* during the acute stage of infection [up to 16 days post-infection (pi)]. However, the number of viable bacteria in the mutant became significantly less (*i.e.,* by 3 orders of magnitude) coincident with the emergence of adaptive T_H1-mediated immunity and the induction of persistence in the mouse (between 16 and 42 days pi) [248]. In addition, *cysH* was highly compromised in the liver, where the host's oxidative antimicrobial response is thought to play an especially important role in antimicrobial defense. Since the replication of *cysH* in mouse tissues during the first 16 days persistent infection was identical to that of wild-type, these data suggest that mouse tissues can provide *M. tuberculosis* with sufficient reduced sulfur-containing amino acids (*e.g.,* cysteine and methionine), for initial growth (see discussion below) [88, 220, 249, 250]. Hence, APS reductase activity appears to be dispensable during the acute phase of infection, but indispensable in the later, the persistence phase where access to or supply of reduced sulfur-containing nutrients becomes limiting [220].

As discussed above, NOS2 plays a vital role in controlling persistent *M. tuberculosis* infection in mice [251–253]. In order to determine and test the role of APS reductase in

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protecting the bacteria against the effects of NOS2, NOS2$^{-/-}$ mice were infected with wild-type and *cysH M tuberculosis* [220]. In contrast to the observation made in wild-type mice, *cysH* did not lose viability after the first 21 days pi in NOS$^{-/-}$ mice; all mice succumbed to infection within 26 to 31 days. Thus, *cysH* is significantly more virulent in the absence of NOS2. Taken together, these studies indicate that APS reductase plays a central role in protecting *M. tuberculosis* against the effects of reactive nitrogen species produced by NOS2 and is critical for bacterial survival in the persistence phase of infection in mice [220]. Furthermore, a follow-up study demonstrates that immunization of mice with *cysH* generates protection equivalent to that of the BCG vaccine in mice infected with *M. tuberculosis* [254].

Attenuation of *cysH* in a mouse model of *M. tuberculosis* infection and the importance of APS reductase in mycobacterial persistence further motivated investigation of the molecular details of the reaction catalyzed by APS reductase [220]. Biochemical, spectroscopic, mass spectrometry and structural investigation of APS reductase support a two-step mechanism, in which APS undergoes nucleophilic attack by an absolutely conserved cysteine to form an enzyme *S*-sulfocysteine intermediate, E-Cys-Sγ–SO_3^- [220, 247, 255–257]. Positively charged amino acids in the active site, including His252, Lys145, Arg237, and Arg240, are likely candidates for stabilization of the thiolate in the active site [258]. In a subsequent step, SO_3^{2-} is released in a Trx-dependent reaction. During the catalytic cycle, nucleophilic attack at Sγ atom of the *S*-sulfocysteine intermediate results in the transient formation of a mixed disulfide between Trx and APS reductase, with concomitant release of sulfite. The structure of this complex has recently been reported and reveals a unique protein-protein interface as a potential candidate for disruption for small molecules or peptide inhibitors [259].

In addition to the conserved catalytic cysteine, the primary sequence of APS reductase is also distinguished by the presence of a conserved iron-sulfur cluster motif, -CysCys-$X_{\sim 80}$-CysXXCys- [221, 255]. Biochemical studies demonstrate that the four cysteines in this motif coordinate a [4Fe-4S] cluster, and that this cofactor is essential for catalysis [247, 255]. The first structure of an assimilatory APS reductase was recently reported, with its [4Fe-4S] cluster intact and APS bound in the active site [256]. Consistent with prior biochemical observations, the structure revealed that APS binds in close proximity to the iron-sulfur center. Progress in this area has been hampered by the failure to generate a paramagnetic state of the [4Fe-4S] cluster that can be studied by electron paramagnetic resonance (EPR) spectroscopy. Recently Bhave *et al.* overcame this bottleneck and reported the EPR characterization of *M. tuberculosis* APR in the [4Fe-4S$^+$] state and identified an essential role for the active site residue Lys-144, whose side chain interacts with both the iron-sulfur cluster and the sulfate group of adenosine 5'-phosphosulfate. On the basis of the data, the co-factor is believed to play a role in pre-organizing active site residues and in substrate activation [260, 261]. Thus compounds that target the metal site and/or nucleotide-binding site may represent promising approaches toward rational inhibitor design. This approach is actively being explored, as well as inhibitors that target the Trx-APS reductase interface and will be reported in due course [262].

The final step in sulfate reduction, the six electron reduction of SO_3^{2-} to S^{2-}, is catalyzed by sulfite reductase (encoded by *nirA*) [see Fig. 3] [263]. Like *cysH*, *nirA* is an essential gene

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[226] and is active during the dormant phase of *M. tuberculosis* [222, 229]. The sulfite reductase in *M. tuberculosis* belongs to the family of ferredoxin-dependent sulfite/nitrite reductases [263]. These enzymes contain a [4Fe-4S] center and a siroheme. In this reaction, the external electron donor (likely ferredoxin) binds transiently to sulfite reductase and transfers electrons to the [4Fe-4S] center, one by one. Subsequently, sulfite reduction is accomplished by transferring electrons from the cluster to the siroheme, which coordinates the sulfite substrate. In 2005, Schnell and coworkers reported the structure of *M. tuberculosis nirA* [263]. Interestingly, the structure depicts a covalent bond between the side chains of residues Tyr69 and Cys161 adjacent to the siroheme in the active site of sulfite reductase. Site-directed mutagenesis of either residue impairs catalytic activity, though their involvement in the mechanism of sulfite reduction is presently unknown [263]. However, recent site directed mutagenesis studies by Smith *et al*. indicate that the first three protons come from solvent, either as part of the HSO_3^- anion or from ordered active site waters. While the last three come from Lys215, Arg153 and Lys217, whereas Asn149 and Arg153 play a role in the structure of the flexible loop that controls anion binding and release. Arg83 is primarily responsible for siroheme binding. Together, the study revealed specific roles for each active site residue in anion binding and in coupled proton transfer that facilitates electron transfer for reduction of sulfite to sulfide [264].

CYSTEINE BIOSYNTHESIS

De novo cysteine biosynthesis in mycobacterium occurs via condensation of S^{2-} with *O*-acetyl-L-serine (cysE, a serine acetyl transferase, catalyzes the condensation of serine with acetyl group to form *O*-acetyl-L-serine which acts as the source of the carbon skeleton for biosynthesis of cysteine) by *O*-acetylserine sulfhydrylase [245, 246] [see Fig. 3]. The *M. tuberculosis* genome contains three *O*-acetylserine sulfhydrylase genes, *cysM*, *cysK* and *cysM3* that can catalyze this reaction. Notably, *cysE* and *cysM* are essential for survival in a mouse model of *M. tuberculosis* infection or in primary macrophages, respectively [225, 228]; *cysM* is also up-regulated under oxidative stress conditions [223]. Cysteine is an important intermediate in biosynthesis of many important sulfur containing metabolites such as methionine, mycothiol, iron-sulfur clusters and other co-factors. Perhaps to avoid the toxicity as a result of accumulation of high levels of cysteine, *M. tuberculosis* might be evolved to convert and store the excessive cysteine into less reactive methionine and non-toxic mycothiol.

Alternative cysteine biosynthesis in M. tuberculosis

In 2005, Burns *et al*. presented an *in vitro* evidence for an additional pathway to make cysteine from sulfide [see Fig. 5] [265]. The *cysM* (Rv1336)-dependent pathway utilizes *O*-phospho-L-serine (OPS) and a sulfide carrier protein, cysO (Rv1335)-thiocarboxylate (cysO-SH), resulting in a cysO-cysteine adduct that is hydrolyzed by the carboxypeptidase *mec*, (Rv1334) releasing L-cysteine and regenerating CysO (15). OPS is synthesized from 3-phosphoglycerate (3PG) in two steps by serA (*serA1*, Rv2996c; *serA2*, Rv0728c) and serC (Rv0884c) [121, 266]. However, the precise sulfur source for the sulfur carrier protein remains unclear. Recent mechanistic and kinetic studies revealed that *cysM* proceeds through a stable α-aminoacrylate intermediate and showed that the *cysM* has 500-fold

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greater specificity for *O*-phospho-L-serine than for *O*-acetyl-L-serine, suggesting that *O*-phospho-L-serine is the likely substrate *in vivo* and the carbon skeleton donor in this cysteine biosynthetic pathway [267].

The presence of this pathway in *M. tuberculosis* is also supported by the analysis of the genome of this pathogen, which reveals the necessary genes for biosynthesis of phosphoserine, such as D-3-phosphoglycerate dehydrogenase *SerA1*, (Rv2996c) [268] and phosphoserine aminotransferase *serC* (Rv0884c) present in the genome. A transposon mutagenesis study further suggested *that serA1* and *serC*, are essential for *M. tuberculosis* [50]. The proteins cysM (Rv1336), cysO (Rv1335), and mec (Rv1334) operating in the OPS-dependent cysteine biosynthesis pathway are encoded within the same transcriptional unit in the H37Rv genome [120]. This operon organization is a common feature of several species in the Actinomycetales group comprising Corynebacteria, Streptomyces, and Mycobacteria, again suggesting that this pathway is also operational in species other than *M. tuberculosis* [121].

An appealing feature of this pathway is that a protein-bound thiocarboxylate would be much more stable to oxidative species in the macrophage, relative to free sulfide [265]. Analysis of mRNA expression demonstrates that each of these genes is upregulated during exposure to toxic oxidants [223]. The existence of an alternative pathway for cysteine biosynthesis, independent of *O*-acetyl serine as carbon skeleton, has implications for attempts to inhibit biosynthesis of this metabolite as a means of pathogen control. Inhibition of only one of these pathways may not be sufficient to kill this pathogen, as already indicated by *cysM* mutants that are attenuated in macrophages but still survive [48]. Complete inhibition of this pathway thus may require at least two inhibitors for the two different branches of biosynthesis of this amino acid [121].

Like most organisms, mycobacteria do not have large pools of free cysteines [249]. Once cysteine is produced it is rapidly utilized in protein synthesis, or for the biosynthesis of methionine and reduced sulfur containing Fe-S cofactors [See Fig. 1]. The most abundant thiol metabolite in mycobacteria (present in millimolar concentrations) is mycothiol [269]. Found in all actinomycetes, mycothiol is essential for *M. tuberculosis* survival and intracellular levels of this thiol are associated with changes in resistance to antibiotics and oxidative stress [219].

BIOSYNTHESIS OF IRON-SULFUR CLUSTER

Fe-S cofactors are involved in the electron transfer, enzymatic catalysis, maintenance of protein structure, and regulation of gene expression. Eukaryotic iron-sulfur clusters biosynthesis necessitates mitochondrial components. *In vivo* [Fe-S] proteins depend on a dedicated machinery to assemble the [Fe-S] cluster and transfer it to the apoprotein. Bacteria possess at least three different pathways for iron-sulfur clusters biosynthesis [270–275]. All pathways utilize a cysteine desulfurase which cleaves the sulfur atom from cysteine for donation to the scaffold protein. In turn, the scaffold protein receives iron atoms from iron donors and assembles the different conformations of the [Fe-S] clusters. Thusly prepared [Fe-S] clusters are then transferred to the apoprotein target. Depending on the organism,

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type of cluster and transfer mechanism, additional components are utilized to aid in the [Fe-S] cluster installation processes [266, 272].

Among the three systems, the so-called ISC (iron-sulfur-cluster-formation) system encoded by *isc* operon *iscR-SUA-hsc BA-fdx* may be the most common route under physiological conditions [276]. Another system, NIF, encoded by *nif* operon is tailored for nitrogenase maturation. Relatively little is known about the systems involved in [Fe-S] cluster formation in actinobacteria. However, the SUF (suppressors mobilization of sulfur) system encoded by *suf ABCDSE* operon functions under oxidative stress or iron starvation conditions, which are frequently encountered by *M. tuberculosis* [76]. *M. tuberculosis* SUF system is encoded by an operon comprised of Rv1460–Rv1466. This operon includes seven genes and SufB, the most conserved protein encoded by Rv1461, contains an intein, which self excises and rejoins the remaining fragments to convert into its active form [277, 278]. Unspliced SufB cannot interact with other components in the SUF system and is inactive during iron-sulfur cluster protein assembly. Thus, SufB maturation is an interesting target for inhibitors to block iron sulfur cluster biosynthesis in *M. tuberculosis* [279–281].

The ISC system consists of cysteine desulfurization enzymes iscS (sulfur donor), frataxin (iron donor), iscU and iscA (scaffold proteins), thioredoxin (reduced oxidant), and chaperones. In addition to the scaffold and sulfur donor proteins, the *isc* gene encodes two heat shock chaperones and contains the gene *cysE*, needed for cysteine biosynthesis [282]. However, the iron donor of NIF and SUF system remains unclear. Despite the complexities, NIF, ISC, and SUF share important components such as, sulfur donor (cysteine desulfurization enzyme), iron-sulfur cluster scaffold protein and catalyze the biosynthesis of iron-sulfur clusters in a very similar fashion [277, 283].

MYCOTHIOL

Mycothiol (MSH) or 1D-myo-inositosyl 2-(N-acetyl-L-cysteinyl)amido-2-deoxy-α-D-glucopyranoside, is an unusual conjugate of *N*-acetylcysteine (AcCys) with 1D-myo-inositol 2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins) [see Fig. 6], and is the major low-molecular mass thiol in most actinomycetes, including mycobacteria [269]. MSH is the functional equivalent of glutathione (GSH) in mycobacteria [219, 284] and is associated with the protection of *M. tuberculosis* from toxic oxidants and antibiotics [214]. Interestingly, the thiol in MSH undergoes copper-ion catalyzed auto oxidation 30-fold more slowly than cysteine and 7-fold more slowly than glutathione [285]. Thus, high concentrations of cellular MSH may increase the capacity of actinomycetes to mitigate the negative effects of oxidative stress.

Apart from protection against toxic oxidants, *M. tuberculosis* relies upon MSH for growth in an oxygen-rich environment and for establishing the pattern of resistance to isoniazid and rifampin [214]. While previous reviews on MSH give a detailed overview of the MSH biochemistry [219] and MSH-dependent proteins [284], the purpose of this section is to highlight research avenues that would help clarify the functional role of MSH in the mycobacterial lifecycle and highlight promising drug targets in MSH metabolism.

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Overview of Mycothiol Biosynthesis

Over a series of seminal papers, R. C. Fahey, G. L. Newton and Y. Av-Gay have elucidated the biosynthetic pathway of MSH [see Fig. 6]. Production of MSH begins from the biosynthesis of 1L-*myo*-inositol 1-phosphate (1L-Ins-1-P), produced from glucose-6-phosphate in a reaction catalyzed by inositol-1-phosphate synthase (*Ino1*) [286]. From this precursor, five enzymes catalyze the conversion of 1L-Ins-1-P to MSH. In the first step, a glycosyltransferase, *mshA*, catalyzes the reaction between a UDP-*N*-acetylglucosamine (UDP-GlcNAc) and 1L-Ins-1-P, generating UDP and 1-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol 3-phosphate (GlcNAc-Ins-P) [286]. A phosphatase, as yet uncharacterized, but designated *mshA2*, dephosphorylates GlcNAc-Ins-P to produce 1-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol (GlcNAc-Ins), the substrate for *MshB* [286]. In the next step, GlcNAc-Ins is deacetylated by *mshB* to yield 1-*O*-(2-amino-1-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol (GlcN-Ins) [287]. Subsequently, *mshC* catalyzes the ATP-dependent ligation of L-cysteine to GlcN-Ins to produce 1-*O*-[[(2R)-2-amino-3-mercapto-1-oxopropyl]amino]-2-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol (Cys-GlcN-Ins) [288]. In the final step, *N*-acetylation of Cys-GlcN-Ins with acetyl-CoA is catalyzed by *mshD* to afford MSH [289]. The total chemical synthesis of MSH has also been reported [290, 291].

The genes encoding the enzymes responsible for MSH biosynthesis have been identified using a variety of methods including transposon [292] and chemical mutagenesis [287, 293, 294]. In turn, these mutants have been utilized to determine the indispensability of the respective genes in the biosynthesis of MSH and their consequence on the viability of mycobacteria [295–297]. Significant progress in the biochemical characterization of these enzymes has also been made [287, 289, 292, 293, 296, 298].

Mycothiol Biosynthetic Enzymes

The gene encoding the glycosyltransferase, *mshA* was first identified as a transposon mutant in *M. smegmatis* that did not produce measurable amounts of GlcNAc-Ins and MSH [292]. By virtue of homology, *mshA* belongs to the known CAZy family 4 glycosyltransferases, [292, 299] which include a number of sucrose synthases, mannosyl transferases and GlcNAc transferases. This classification strongly suggested that *mshA* is the glucosyltransferase required for the biosynthesis of GlcNAc-Ins. *M. smegmatis* and *M. tuberculosis mshA* sequences were shown to be 75% identical over a 446-residue overlap. The *M. tuberculosis mshA* ortholog, *Rv0486,* complemented the mutant phenotype in *M. smegmatis*, thereby confirming its function. In *M. smegmatis* [292] and *M. tuberculosis* [77], *mshA* is essential for production of GlcNAc-Ins and therefore, for MSH synthesis. Interestingly, transposon mutants in *mshA* are viable in *M. smegmatis* [292], whereas in *M. tuberculosis mshA* is essential for growth [77]. The gene encoding the phosphatase, *mshA2*, remains to be identified.

MshB was the first gene identified in the MSH biosynthetic pathway [287]. The deacetylase is encoded by the *M. tuberculosis* open reading frame *Rv1170* and was first discovered as a homolog of *Rv1082,* a mycothiol S-conjugate amidase (*Mca*). Although *mshB* does exhibit some amidase activity, deacetylation of GlcNAc-Ins is the preferred reaction [287].

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Characterization and crystallographic studies have revealed that MshB is a Zn^{2+} metalloprotein and that deacetylase activity is dependent on the presence of a divalent metal cation [300, 301].

Disruption of *mshB* results in decreased production of MSH (limited to about 5–10% of the parental *M. smegmatis* strain [302] and up to 20% that of the parental *M. tuberculosis* strain during log-phase growth, increasing to 100% of the wild-type MSH levels during the stationery phase [214]). Hence, MSH synthesis is not abolished in *mshB* mutants and, in the absence of *mshB*, MSH biosynthesis is accomplished via an alternative deacetylase activity that produces modest levels of GlcN-Ins [214, 302]. Under culture conditions, the amount of MSH produced in *mshB* mutants during log phase growth is sufficient to provide MSH-dependent resistance to moderate oxidative stress. In addition, since normal quantities of MSH are produced in *MshB* mutants during stationary phase, it was not possible to examine the role of MSH during dormancy-like conditions in these studies.

The role of *mshC* involving ATP-dependent ligation of L-cysteine with GlcN-Ins was first elucidated by Bornemann and coworkers [288]. First identified in *M. smegmatis* [298], homologs of *mshC* have been identified in *Streptomyces coelicolor* A3, *Corynebacterium striatum* [269] and orthologs of *M. tuberculosis MshC* (Rv2130c) were also found in *M. leprae* [15], *M. bovis* [17], and in *M. avium* [303]. Interestingly, the enzyme encoded by *mshC* appears to have evolved by gene duplication of the cysteinyl-tRNA synthetase, *cysS* (Rv3580c) as evidenced by their similar mechanism of action [298]. In the reaction catalyzed by *mshC*, the 2’ amine of GlcN-Ins carries out nucleophilic attack of an activated cysteinyl-AMP intermediate to produce Cys-GlcN-Ins. Presumably, a general base removes a proton from the amino group leading to the formation of a tetrahedral intermediate, which decomposes to form the amide [298].

In *M. smegmatis*, chemical and transposon mutants lacking *mshC* activity do not produce detectible amounts of MSH [218]. In the chemical mutants, *mshC* was sequenced and a point mutation (Leu205Pro) was identified. This region in *mshC* is largely conserved among actinomycetes and hence, the Leu205Pro substitution was concluded to be responsible for the lack of *mshC* activity in the mutant [218]. In contrast to *M. smegmatis* that does not require MSH for growth, a targeted disruption of *mshC* in *M. tuberculosis* Erdman produced no viable clones possessing either the disrupted *mshC* gene or reduced levels of MSH. Thus, the *mshC* gene is required for MSH production and is essential for *M. tuberculosis* Erdman survival [304]. The differences in the responses of the mutants between the two strains of mycobacteria could be attributed to the fact that *M. smegmatis* has a larger genome (7 vs. 4.4 Mb) relative to *M. tuberculosis* and therefore, includes genes that facilitate its growth in the absence of MSH [305].

MshD catalyzes the final step in MSH biosynthesis. In this reaction, Cys-GlcN-Ins is acetylated using acetyl-CoA [288]. *MshD* was identified during the characterization of an *M. smegmatis* transposon mutant lacking the transacetylase activity required for MSH biosynthesis. Sequencing from the site of insertion identified the gene that encodes for mycothiol synthase or *mshD*. A homology search revealed an *mshD* ortholog in *M. tuberculosis* as *Rv0819* which exhibits MSH synthase activity when expressed in *E. coli*

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[289]. A crystal structure of *mshD* from *M. tuberculosis* showed structural homology to the GNAT family of N-acetyltransferases [306–308].

MshD mutants in *M. smegmatis* produce high levels of Cys-GlcN-Ins along with two other thiols, *N*-formyl-Cys-GlcN-Ins (fCys-GlcN-Ins) and *N*-succinyl-Cys-GlcN-Ins (succ-Cys-GlcN-Ins) and ~1% the amount of MSH found in the wild-type strain [305, 309]. These data suggest that in the absence of *mshD*, mycobacteria can utilize closely related analogs of MSH such as fCys-GlcN-Ins to maintain a reducing environment in the cells [305, 309]. This hypothesis is further supported by the observation that *mshD* transposon mutants in *M. smegmatis* are as resistant to peroxide-induced oxidative stress as their parental strain [309]. On the other hand, *M. tuberculosis mshD* mutants appear to grow poorly under other stress conditions such as low-pH media or in the absence of catalase and oleic acid [305].

MSH and Antibiotic Resistance

The formation of MSH-adducts of various anti-mycobacterial agents like cerulenin and rifamycin S [310] suggests that *M. tuberculosis* can use MSH in detoxification reactions [see Fig. 7] [293]. As yet an unidentified MSH-*S*-transferase is believed to catalyze the formation of the MSH-drug adduct [311]. MSH-*S*-conjugate amidase (discussed below) then catalyzes the hydrolysis of the MSH-drug adduct to produce a mercapturic acid containing the drug moiety, which is excreted from the cell [293]. Alternatively, since the oxidation state of cell wall components could alter cell wall permeability, MSH may also confer antibiotic resistance by influencing the overall cellular redox state [214]. Experiments with *mshC* mutants demonstrate that MSH, and not any biosynthetic intermediate *en route* to MSH, is critical for antibiotic and peroxide resistance [218]. Mutants lacking various MSH biosynthetic enzymes support the idea of MSH-related resistance to antibiotics [214, 289, 292, 294]. Independent null deletion mutants of all four genes involved in mycothiol biosynthesis pathway (*mshA, mshB, mshC, and mshD*) were generated in *M. smegmatis* and analyzed for MSH levels and isoniazid (INH) and ethionamide (ETH) resistance. The *mshA* and *mshC* single deletion mutants were not capable of producing MSH and found to be resistant to INH, whereas *mshB* deletion only decreased MSH levels and was sensitive to INH suggesting that MSH biosynthesis is essential for INH sensitivity in *M. smegmatis* [312]. Further evidence was gained by deleting the gene encoding the MSH S-conjugate amidase, *mca*, from the *mshB* null mutant. The double mutant *mca mshB* of *M. smegmatis* resulted in complete loss of MSH production and was resistant to INH [312]. However, there is considerable difference in redox balance between *M. tuberculosis* and *M. smegmatis*. *M. tuberculosis mshA* and *mshD* strains require exogenous catalase to grow *in vitro* [313], whereas *M. smegmatis* MSH synthetic mutants grow without the addition of catalase indicating the difference in the redox homeostasis between the two species [305, 314]. The *mshA*, *mshC*, and *mshB* single deletion mutants of *M. smegmatis* were also found to be resistant to ETH, indicating that ETH activity is modulated by MSH levels [312, 313]. Interestingly, *M. smegmatis mshD* strain is devoid of MSH but is still sensitive to INH and ETH. It was found that in *M. smegmatis mshD::Tn5* mutant or in *M. tuberculosis mshD* mutant, the reduced form of thiol is novel N-formyl-cys-GlcN-Ins which substitutes for MSH in *M. smegmatis* and likely mediates the drug sensitivity [305, 312]. Taken together, it

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can be inferred from these studies that mycothiol is essential for INH and ETH activity against *M. tuberculosis*.

Mycothiol-dependent Detoxification

Biological degradation of one carbon compounds such as methane, methylated amines and sulfur compounds leads to accumulation of formaldehyde. At lower concentrations, formaldehyde is toxic to cells and suggests the existence of a detoxification mechanism in *M. tuberculosis*. To this end, it has been proposed that MSH or MSH-dependent enzymes are involved in protecting mycobacteria from oxidants/toxins [218, 294] and have led to the study of enzymes that utilize MSH as a cofactor or a substrate for their activity. NAD/MSH-dependent formaldehyde dehydrogenase (*mscR*) was the first enzyme identified as using MSH as a cofactor [315] and is discussed at length in a recent review [284]. Like glutathione, mycothiol spontaneously reacts with formaldehyde to form an *S*-hydroxymethylmycothiol adduct that is converted by the NAD/MSH dependent formaldehyde dehydrogenase (*mscR* encoded by Rv2259) to a mycothiol formate ester. Then an aldehyde dehydrogense becomes likely to convert mycothiol formate ester into CO_2, carbonate ester and mycothiol. Modeling studies reveal that the active sites of GSH- and MSH-dependent formaldehydede hydrogenases have distinct binding sites and are specific to their substrates. Recently, Vogt *et al.* reported that mscR also operates as mycothiol-nitroso reductase, indicating that this enzyme is involved in the protection against oxidative stress posed by reactive nitrogen species. Besides mscR, two other important enzymes involved in MSH metabolism and detoxification are mycothione reductase (*mtr*) and Mycothiol-*S*-conjugate Amidase (*mca*) [see Fig. 6], discussed below.

Mycothione Reductase

To maintain a large cellular pool of reduced MSH, mycothione reductase catalyzes the reduction of oxidized MSH also known as mycothione (MSSM) [see Fig. 6] [284]. *M. tuberculosis* MSH disulfide reductase (*mtr*, encoded by *Rv2855*) was identified by homology to glutathione reductases [316, 317]. *Mtr* is a member of the pyridine nucleotide-disulfide reductase super family. The reductase is a homodimeric flavoprotein disulfide isomerase and requires FAD as a cofactor [284, 316]. NADPH reduces FAD, which then transfers reducing equivalents to the redox-active disulfide in *mtr* to generate a stable two-electron reduced enzyme [316, 317]. Subsequently, *mtr* reduces the disulfide in MSSM via dithiol-disulfide interchange, with concomitant oxidation of NADPH [316, 317].

Phenotypic characterization of an actinomycete *mtr* mutant has not been reported to date and genome-wide transposon mutagenesis has yielded conflicting results. In one study, a transposon mutant in *M. tuberculosis mtr* was reported to be viable [318]. In contrast, another study using high-density Himar-1 transposon mutagenesis reported that *mtr* is essential for *M. tuberculosis* survival [227]. One possible explanation for these conflicting data could be the relative importance of (or requirement for) *mtr* in MSH reduction during different stages of growth. Transcriptional analysis of *M. bovis* BCG reveals that *mtr* mRNA is actively transcribed during exponential bacterial growth [295]. In the same study, *mtr* mRNA expression was absent in the stationery phase suggesting that *mtr* might only be required to maintain the redox balance during intense periods of metabolic activity (*e.g.,*

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during the growth phase) [295]. However, another study found high MSH levels throughout the growth cycle, including the stationery phase [302]. These findings suggest that, in the absence of *mtr*, another thiol reductase might reduce MSSM [284]. Additional experiments will be required to clarify the importance of *mtr* in MSH reduction throughout the mycobacterium lifecycle and to determine whether or not it is essential for bacterial viability.

Mycothiol-S-conjugate Amidase

In mycobacteria, mycothiol-*S*-conjugate Amidase (*mca*) plays a major role in electrophone detoxification [see Fig. 6] [293]. This enzyme was discovered in connection with its ability to detoxify a thiol-specific fluorescent alkylating agent, monobromobimane (mBBr), a compound commonly used for the quantitative determination of thiols. mBBr binds to MSH forming an MSH-mBBr adduct, MSmB, and can be cleaved by *mca* to produce glucosaminyl inositol and acetyl cysteinyl bimane, a mercapturic acid which is rapidly excreted from the cell [293]. *Mca* was first purified from *M. smegmatis* and was found to have an ortholog in the *M. tuberculosis* genome, Rv1082, identified by N-terminal amino acid sequencing [293]. Studies probing the substrate specificity of *mca* indicate that the enzyme specifically recognizes the MSH moiety in the conjugate, but is relatively non-specific for the group attached to the sulfur in the MSH-toxin conjugate [293].

Mca and *mshB* exhibit an overall sequence identity of 32% [300]. Interestingly, *in vitro* studies indicate that *mshB* possesses amidase activity with MSH substrate [293]. Moreover, *mca* can function as a deacetylase [286, 293] and partially restores MSH production when introduced into an *M. Smegmatis mshB* mutant [302]. Based on the sequence identity between *mca* and *mshB* and the crystal structure of *mshB*, a model for the active site of *mca* has been proposed [300, 301]. With the exception of Lys19 in *mca* replaced by Ser20 in *mshB*, other critical catalytic residues, such as the zinc-binding site and an aspartate are perfectly conserved. The Lys to Ser alteration may play an important role in disaccharide binding [300]; a crystal structure of *mca* will be important to define the MSH binding site.

Apart from the mBBr model substrate, the substrates for *mca* include the MSH conjugate of cerulenin, an antibiotic that inhibits fatty acid synthetase and other antibiotic adducts. *Mca* homologs have been found in several antibiotic biosynthesis operons such as those for avermectin (*Streptomyces avermitilis*) and eythromycin (*Saccharopolyspora erythrae*) [219, 319]. In addition, it has been demonstrated that MSH forms a conjugate with Rifamycin SV and this complex is a substrate for *M. tuberculosis mca* [310]. Treatment of *mca* mutant and wild-type *M. smegmatis* strains with Rifamycin SV showed that the MSH-Rifamycin SV adduct is converted to mercapturic acid only in the wild-type [297]. Taken together, these findings demonstrate that MSH and *mca* in mycobacteria work together to detoxify antibiotics [219].

Drug Targets in Mycothiol Metabolism

Mca plays a critical role in mycobacterial detoxification of antibiotics. Therefore, inhibitors of *Mca* could enhance the sensitivity of MSH-producing bacteria to antibiotics, establishing *Mca* as a promising new drug target. Toward this end, 1,500 natural product extracts and

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synthetic libraries were screened to identify lead compounds [320–322]. Two classes of bromotyrosine-derived natural products were competitive inhibitors of *Mca*. Non-competitive inhibitors were also identified in this screen [see Fig. 8 *mca inhibitors* (non-competitive)]. These results motivated the total synthesis of a competitive inhibitor [see Fig. 8 *mca inhibitors*] that inhibits *mca* with an IC_{50} value of 30 μM [323].

Recently, a series of compounds based on the structure of the natural product bromotyrosine inhibitor was synthesized and screened against mycobacteria and other gram-positive bacteria [324]. One of the lead compounds identified from this study termed, EXEG1706 [see Fig. 8 *mca inhibitors*], exhibited low minimum inhibitory concentrations (1.5 – 15.5μg ml^{-1}) for *M. smegmatis, M. bovis* and against methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*, and *S. aureus*. However, this class of compounds was also active against mycobacterial *mca* mutant strains and against gram-positive bacteria that do not produce MSH. Thus, in addition to *mca*, it appears that these compounds inhibit other protein targets *in vivo*. Another approach used to identify *mca* inhibitors has been the synthesis of MSH analogs. Synthesis of simplified thioglycosidic analogs of MSH [see Fig. 8] [325] and a variety of amide-functionalized MSH analogs synthesized from quinic acid led to the identification of inhibitors with modest inhibitory activities (IC_{50} values around 50 μM) [326] [see Fig. 8].

Naphtoquinone derivatives like plumbagin, juglone and alkyl plumbagin derivatives were shown to inhibit *M. tuberculosis* growth. Since napthoquinone derivatives were known to interact with electron transport or related biochemical reactions [327],Gammon et al. utilized these moieties to create plumbagin conjugated glucopyranoside derivatives as inhibitors of *mca* and *mshB* [328]. The maximum potent inhibition of *mca* and *mshB* was observed when the plumbagin moiety was separated by 5-carbon spacer from glucopyranoside moiety [328]. However, testing of the molecule in disease models of *M. tuberculosis* is yet to be reported.

In addition to *mca,* other possible drug targets that could block MSH biosynthesis are the enzymes encoded by *mshA* and *mshC* (both essential genes in *M. tuberculosis* [77, 304]). The identification of inhibitors for *mshC* has been initiated [286] and recently Newton *et al.* reported a novel inhibitor, NTF1836, inhibiting MshC in micromolar range (IC_{50} 85μM) [329]. They also reported that inhibition of *M. tuberculosis* growth by NTF1836 was accompanied by a decrease in mycothiol and an increase in GlcN-Ins cellular levels, which is consistent with the targeting mshC. Moreover, NTF1836 was also found to inhibit non-replicating *M. tuberculosis* in the carbon starvation model of latency [329]. Subsequent structure activity relationship studies revealed that five structurally related compounds to NTF1836 have similar activity towards clinical strains of *M. tuberculosis* and paved the way for designing second generation MshC inhibitors [329]. In a similar effort by Gutierrez-Lugo *et al.,* dequalinium chloride was identified through high-throughput screening, inhibiting *M. tuberculosis* mshC with an IC_{50} of 24μM. Further studies showed dequalinium chloride as an ATP-competitive inhibitor of mshC which inhibits the growth of *M. tuberculosis* under aerobic and anaerobic conditions [330]. In another study, Gutierrez-Lugo *et al.* screened two groups of known aminoacyl tRNA synthetase inhibitors for inhibition of *M. tuberculosis* mshC including aminoacyl adenosine analogs and natural products. Using

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enzyme assays, isothermal titration calorimetry and NMR of this group showed that *M. Smegmatis* mshC can be selectively inhibited by cysteinyl sulfamoyl adenosine (CysSA) with an IC50 of 10nM [331]. Derivatives of naphthoquinone, juglone and plumbagin were shown to be subversive substrates of mycothiol disulfide reductase having anti-tubercular activity. 7-methyljuglone derivatives are the most potent with MIC of 0.5 μg/ml against *M. tuberculosis* [332]. However, the lack of correlation between anti-tubercular activity and mycothiol disulfide reductase activity suggests that these compounds are not selective for mycothiol disulfide reductase and instead target multiple enzymes *in vivo*. Identification of inhibitors for another UDP-GlcNAc-dependent glycosyltransferase, *murG* [333] suggests that *mshA* is also likely to be a druggable target. Blanchard *et al*. recently reported the three-dimensional structure and basic kinetic characterization of the retaining glycosyltransferase *mshA* from *Corynebacterium glutamicum* (Cg*mshA*) [334]. The same group recently reported a slow-binding competitive inhibitor, UDP-(5F)-GlcNAc, for Cg*mshA* with K_i 1.6μM [335]. Although high-density transposon mutagenesis studies have identified *mshD* as nonessential for the growth of *M. tuberculosis* in minimal culture medium [227], the survival of *M. tuberculosis mshD* mutants is severely compromised in activated and non-activated macrophages [225]. Thus, *mshD* could be a promising drug target and further analysis of this mutant in animal models of TB infection may be warranted.

METHIONINE BIOSYNTHESIS AND REVERSE TRANSSULFURATION

In *M. tuberculosis*, *Rv1079* (annotated as *metB*) encodes a bi-functional cysteine γ-lyase (CGL)-cystathionine γ-synthase (CGS) enzyme, converts cysteine to cystathionine [249]. Cystathionine is then transformed into methionine by two subsequent reactions: *MetC* (Rv3340) converts cystathionine into homocysteine [249, 336] and *metE/metH* (Rv1133c/Rv2124c) catalyzes methylation of homocysteine to produce methionine [249, 337].

Interestingly, mutation of *metB* in *M. tuberculosis* results in a prototrophic methionine mutant [338]. In other words, *metB* is not absolutely required for methionine production. This finding can be explained by the action of *metZ* (Rv0391), an *O*-succinylhomoserine sulfurylase, which bypasses the requirement for *metB* and *metC* by condensing S^{2-} with *O*-succinylhomoserine to produce homocysteine directly [see Fig. 3]. In mice, a *metB* strain was slightly attenuated [338]. However, no differences in bacterial load in the lungs, liver or spleen, were observed between the *metB* mutant and wild-type *M. tuberculosis* in immunocompetent mice up to 80 days post-infection [338]. This growth phenotype contrasts that of *cysH* mutants in *M. tuberculosis* where viability decreases significantly, specifically during the persistence phase of infection [220].

The interesting observation that defects in sulfate transport or its reduction could be rescued by methionine supplementation suggested that a functional reverse transsulfuration pathway [see Fig. 3], used to produce cysteine from methionine, was present in mycobacteria [221, 249, 250]. Indeed, the existence of this pathway has recently been confirmed [249]. Although a methionine transporter has not yet been identified in mycobacteria, an apparent K_m of 80 μM for the transporter has been estimated in *M. bovis* BCG [250] and the estimated concentration of methionine in humans is 20–27 μM [339].

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Once methionine is transported into the bacteria, three enzymatic steps are required for conversion into homocysteine [see Fig. 7] [246]. In the first step, *S*-denosyl methionine transferase or *S*-adenosyl methionine synthase which is denoted by *metK*, catalyzes the conversion of methionine to *S*-adenosine-L-methionine [340]. *S*-adenosyl-L-methionine dependent methyl transferase (Rv2118c) catalyzes the conversion of *S*-adenosyl-L-methionine to *S*-adenosine homocysteine [341]. *S*-adenosyl-L-homocysteine hydrolase (*sahH*) catalyzes the reversible hydrolysis of *S*-adenosyl-L-homocysteine into free adenosine and homocysteine and maintains the intracellular balance between *S*-adenosylhomocysteine and *S*-adenosylmethionine [342]. Adenosyl homocysteine hydrolase has been validated as a "druggable" target [343], is essential for growth *in vitro* and is up-regulated in mouse models of TB infection [344]. Subsequently, *cysM2* (Rv1077) converts homocysteine to cystathionine and the bifunctional enzyme, *Rv1079* (annotated as *metB*) encodes a bifunctional cysteine γ-lyase (CGL)-cystathionine γ-synthase (CGS) enzyme catalyzes the cystathionine to cysteine [249]. Thus, *M. tuberculosis* can shuttle the sulfur source between the important sulfur containing metabolites to meet cellular demands. This pathway also provides support for *M. tuberculosis* survival against oxidative stress by scavenging the methionine from host cell. Also, sulfate assimilation in *M. tuberculosis* appears to be dynamic and can quickly adapt environmental changes.

OTHER SOURCES OF REDUCED SULFUR

Consistent with the requirement for sulfur in mycobacterial survival, the ability of mycobacteria to scavenge reduced sulfur from its host has been confirmed in *M. bovis* BCG and in *M. tuberculosis* [220, 249, 250]. Several potential sources of reduced sulfur in the human host are discussed below.

Cysteine and Cystine

Mutation of *cysH* in *M. smegmatis* and *M. tuberculosis* produces a cysteine auxotroph and this defect can be rescued by the addition of cysteine to the growth medium [220, 221]. The finding that growth of *cysH* mutant can be restored by the addition of cysteine suggests that cysteine or cystine (the oxidized form of cysteine) can be transported into *M. smegmatis* and *M. tuberculosis*. In addition, when [^{35}S] cysteine is added to a growing culture of *M tuberculosis*, more than 70% of the radioactive sulfur taken up by the bacteria is found in methionine, also consistent with import of cysteine [249]. While genes that encode the cysteine/cystine transporter in mycobacteria have not yet been identified, cysteine and cystine uptake systems have been characterized in other prokaryotes [345, 346]. In humans, cystine is the preferred form of cysteine for the synthesis of glutathione in macrophages and is present in plasma at ~25 – 35 μM [347].

Genetic screens for amino acid auxotrophs in *M. bovis* BCG (an attenuated version of bovine bacillus) have not isolated cysteine auxotrophs [87, 250]. In the first report, only three auxotrophs were identified, one for methionine and two for leucine [87]. A subsequent study isolated two auxotrophs, both for methionine [250]. Since isolation of mycobacterial auxotrophs depends on the growth medium composition [348], it is possible that the use of casamino acids to rescue the growth of transposon mutants in these studies selects only a small subset of amino-acid auxotrophs. Consistent with this hypothesis, the approximate

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concentration of sulfur-containing amino acids in 1% (w/v) casamino acids is expected to be ~900 μM methionine and ~60 μM cystine.

The methionine auxotrophs identified by transposon mutagenesis in *M. bovis* BCG are mapped to genes in the sulfate assimilation pathway, in particular to sulfate transport genes, *subI* [87] and *cysA* [250]. Since sulfate serves as the precursor for cysteine synthesis, defects in the sulfate assimilation pathway should result in cysteine auxotrophy. Surprisingly, however, growth of *M. bovis* BCG *subI* or *cysA* mutants could not be rescued by supplementation with cysteine (in contrast to the *cysH* knockout in *M. smegmatis* and *M. tuberculosis*) and instead, required methionine supplementation. In addition to the inability of cysteine to rescue defects in sulfate transport, the same study also reported that wild-type *M. bovis* BCG grew slowly on growth media supplemented with 0.3 mM cysteine and not at all in the presence of 0.5 mM cysteine. In contrast, toxicity has not been observed in wild-type strains of *M. smegmatis* [221] or *M. tuberculosis* [220] grown in the presence of 1 – 2 mM cysteine. Hence, it is possible that mutation of sulfate transport genes, *subI* or *cysA*, impacts cysteine/cystine import directly or that *M. bovis* BCG does not transport cysteine/cystine efficiently. Further investigations into the differences between mycobacterial strains, growth media and other critical factors, such as inoculum densities, for the requirements of sulfur-containing compounds are warranted.

Glutathione

In mycobacteria, a large amount of the reduced sulfur in cells is used to make mycothiol, the dominant low molecular weight thiol used to maintain redox equilibrium and scavenge reactive oxygen species in the cell [269]. Similarly, GSH – a tripeptide, γ-glutamylcysteinylglycine found in many prokaryotes and eukaryotes – is also present at high intracellular levels [284] and may provide a source of reduced sulfur for mycobacteria in the host. Estimates of GSH concentration in human cells and macrophages range from 1 – 7 mM [349, 350]. An analog of GSH, nitrosoglutathione (GSNO), is bactericidal in *M. bovis* [351] and *M. tuberculosis* [352]. Use of GSNO has facilitated identification and characterization of the ABC transporter dipeptide permease (Dpp, Rv3663 – Rv3666) responsible for GSH catabolism and utilization [351, 352]. Interestingly, GSH is not transported into mycobacterial cells as the tripeptide, but rather as the dipeptide, Cys-Gly [352]. Hence, import of GSH involves proteolysis by a γ-glutamyl transpeptidase (*ggtA*, Rv0773c) and subsequent transport via *dpp*. Consistent with the proposed route of GSH catabolism and import, mutants in the transpeptidase or the permease are resistant to the toxic effects of GSNO [352]. In culture, it has been reported that GSH exhibits bacteriostatic activity at a concentration of 5 mM [353]. This effect appears to be mediated intracellularly since mutations in the *dpp* or *ggtA* relieve this phenomenon [353].

Recently Guerra *et al*. characterized the mechanisms by which glutathione (GSH)-enhanced natural killer cells inhibit the growth of *M. tuberculosis* inside human monocytes. In healthy individuals, treatment of natural killer cells with N-acetyl cysteine (NAC), a GSH pro-drug in conjunction with cytokines such as interleukin (IL)-2 + IL-12, resulted in enhanced expression of natural killer cell cytotoxic ligands (FasL and CD40L) with subsequent inhibition in the intracellular growth of *M. tuberculosis*. Neutralization of FasL and CD40L

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in IL-2, IL-12 and NAC-treated natural killer cells resulted in abrogation in the growth inhibition of *M. tuberculosis* inside monocytes. Interestingly, the levels of GSH are decreased significantly in natural killer cells derived from individuals with HIV infection compared to healthy subjects and this decrease is correlated with several fold increase in growth of *M. tuberculosis* inside monocytes [354].

OUTLOOK

The emergence of antibiotic resistance and the problem of mycobacterial persistence in *M. tuberculosis* urgently stress the need for new target identification. Toward this end, mycobacterial sulfur metabolic pathways represent a promising new area for anti-TB therapy. In the last several years, excellent progress has been made, leading to the identification and validation of several potential drug targets in sulfate assimilation and MSH metabolism. At the same time, many aspects of mycobacterial sulfur metabolism remain poorly understood and represent exciting areas of new or continued investigation. Significant work remains to validate additional targets, improve inhibitor potency for existing targets and to further define the roles that sulfated and many reduced sulfur-containing metabolites play in mycobacterial virulence and persistence. Finally, a wide variety of microbes including *Pseudomonas aeruginosa*, *Bacillus anthracis*, and *Yersinia pestis* also relies on unique sulfur metabolic pathways for their own survival. Hence, in the fight against multidrug resistant microbes, investigation of microbial sulfur metabolism in mycobacteria and other pathogens should be fertile scientific ground in the years to come.

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ABBREVIATIONS

TB	Tuberculosis
MDR	Multidrug resistant
CoA	Coenzyme A
$-SO_3^-$	Sulfuryl moiety
SO_4^{2-}	Sulfate
APS	Adenosine-5’-phosphosulfate
PAPS	3’-Phosphoadenosine-5’-phosphosulfate
SO_3^{2-}	Sulfite
S^{2-}	Sulfide
BCG	Bacillus Calmette-Guérin
ATP	Adenosine 5’-triphosphate
GTP	Guanosine 5’-triphosphate

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SAC	Sulfate-activating complex
ST	Sulfotransferase
SL-1	Sulfolipid-1
T2S	Trehalose-2-sulfate
PAP	3'-Phosphoadenosine-5'-phosphate
EST	Estrogen ST
TPST	Tyrosyl protein ST
GST	Golgi-resident ST
β-AST	β–arylsulfotransferases
cAMP	Cyclic adenosine-5'-phosphate
IC_{50}	Half maximal inhibitory concentration
$O_2^{\cdot -}$	Superoxide
NO·	Nitric oxide
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NOS2	Inducible nitric oxide synthase
ROI	Reactive oxygen intermediate
RNI	Reactive nitrogen intermediate
SOD	Superoxide dismutase
H_2O_2	Hydrogen peroxide
OH·	Hydroxyl radical
NOHLA	*N*-ω-hydroxy-L-arginine
$ONOO^-$	Peroxynitrite
TLR	Toll-like receptor
Phox	Phagocyte NADPH oxidase
AMP	Adenosine-5'-phosphate
Trx	Thioredoxin
Cys-Sγ–SO_3^-	*S*-sulfocysteine
[4Fe-4S]	Four iron-four sulfur cluster
MSH	Mycothiol
AcCys	*N*-acetylcysteine
GlcNAc-Ins	1D-myo-inositol 2-acetamido-2-deoxy-α-D-glucopyranoside
GSH	Glutathione

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1L-Ins-1-P	1L-*myo*-inositol 1-phosphate
UDP-GlcNAc	UDP-*N*-acetylglucosamine
GlcNAc-Ins-P	1-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol 3-phosphate
GlcNAc-Ins	1-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol
GlcN-Ins	1D -*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside
Cys-GlcN-Ins	1-*O*-[[(2R)-2-amino-3-mercapto-1-oxopropyl] amino]-2-deoxy-α-D- (glucopyranosyl)-D-*myo*-inositol
fCys-GlcN- Ins	*N*-formyl-Cys-GlcN-Ins
succ-Cys-GlcN- Ins	*N*-succinyl-Cys-GlcN-Ins
MSSM	Mycothione
mBBr	Monobromobimane
CGL	Cysteine γ-lyase
CGS	Cystathionine γ-synthase
GSNO	Nitrosoglutathione

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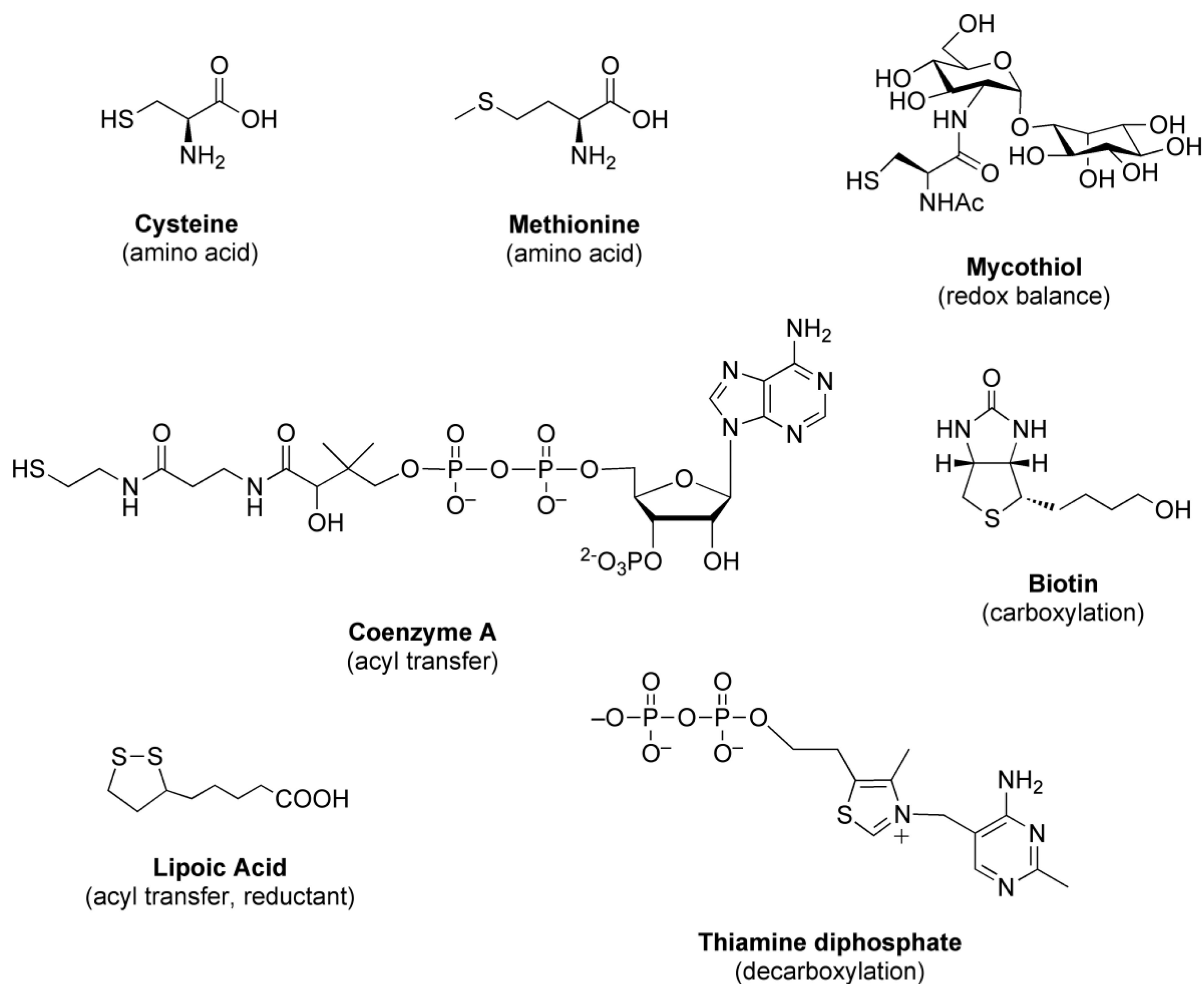

Figure 1.
Reduced sulfur–containing metabolities in mycobacteria.

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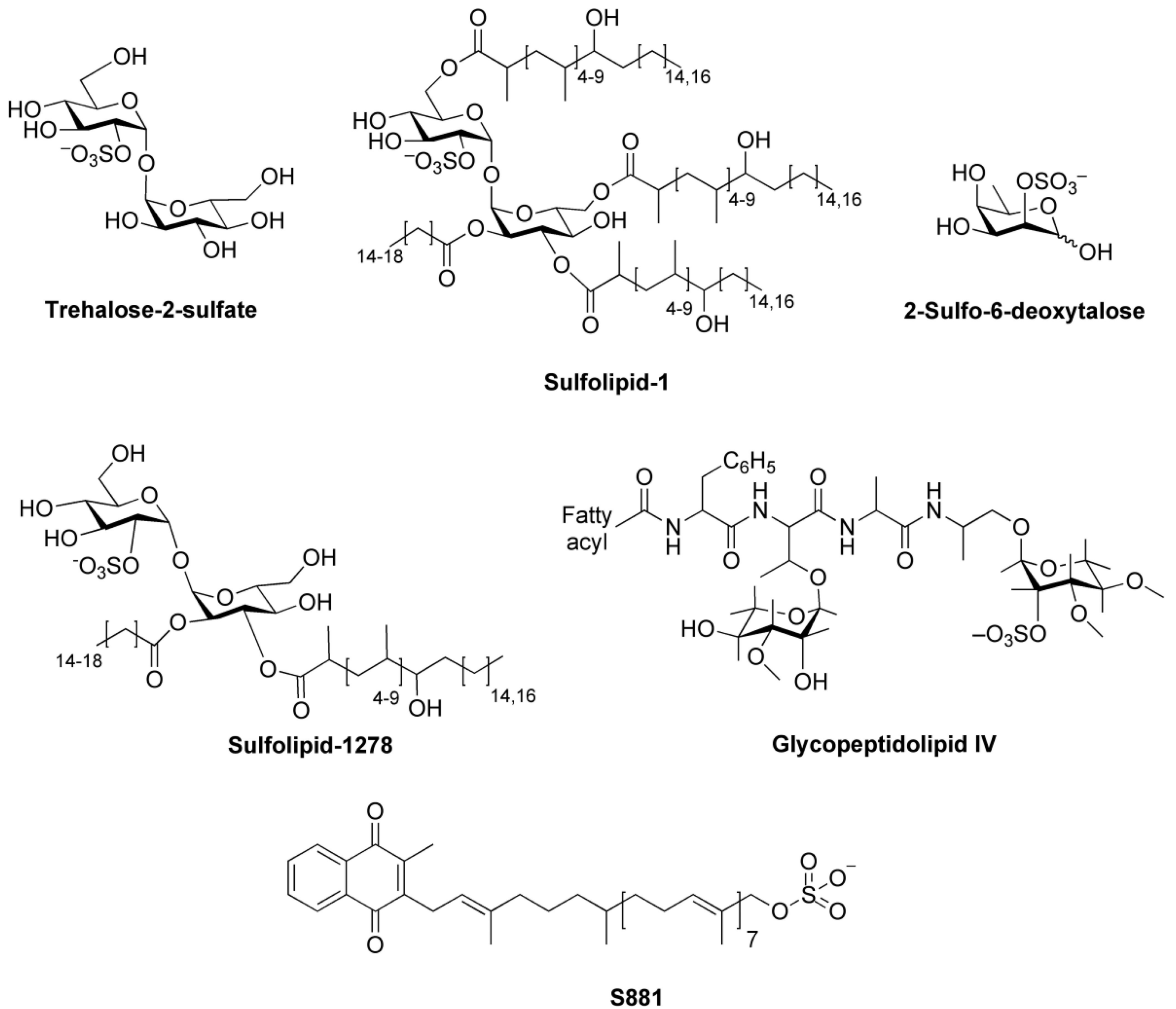

Figure 2.
Sulfated metabolites in mycobacteria.

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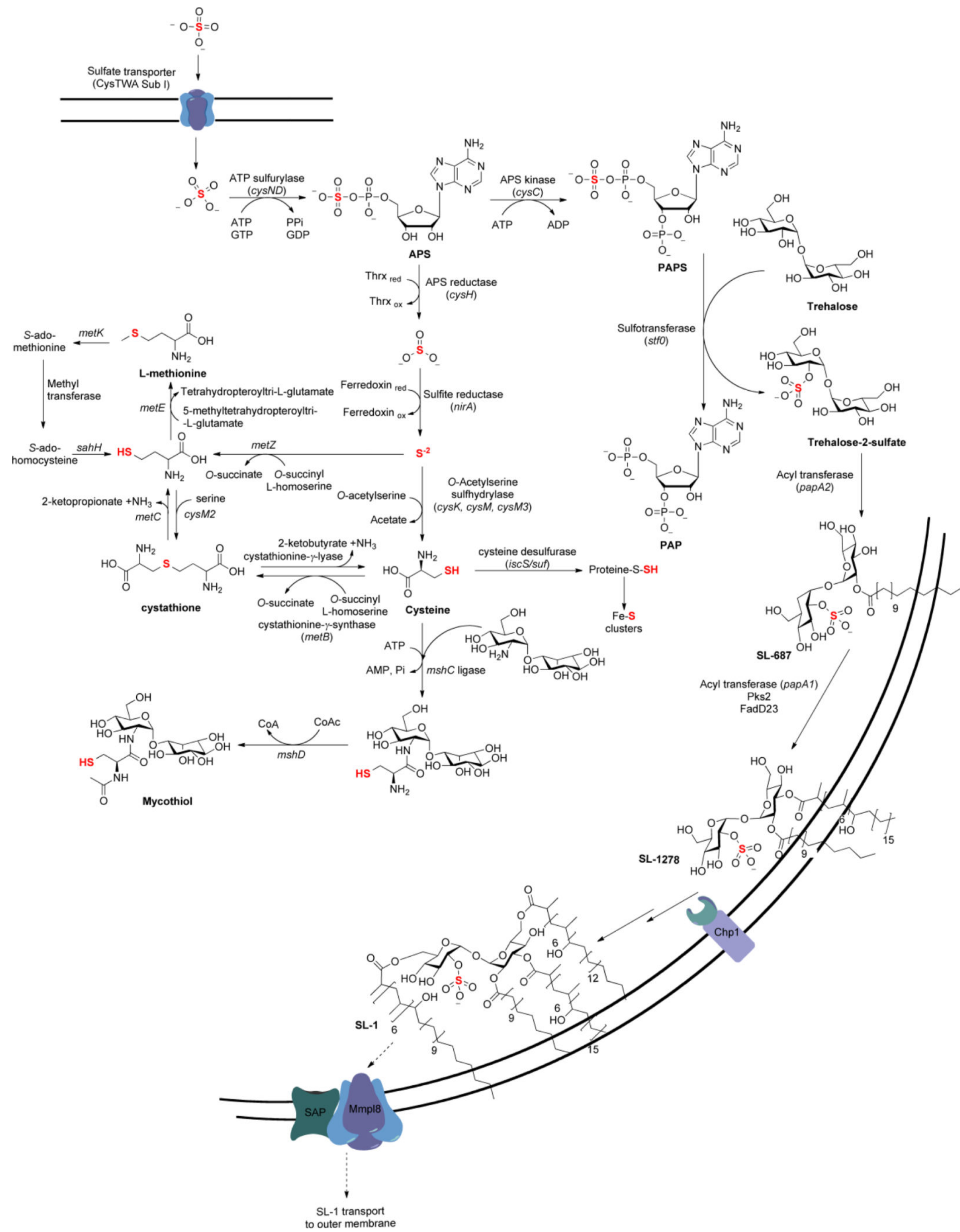

Figure 3.

The sulfate assimilation pathway in mycobacteria.

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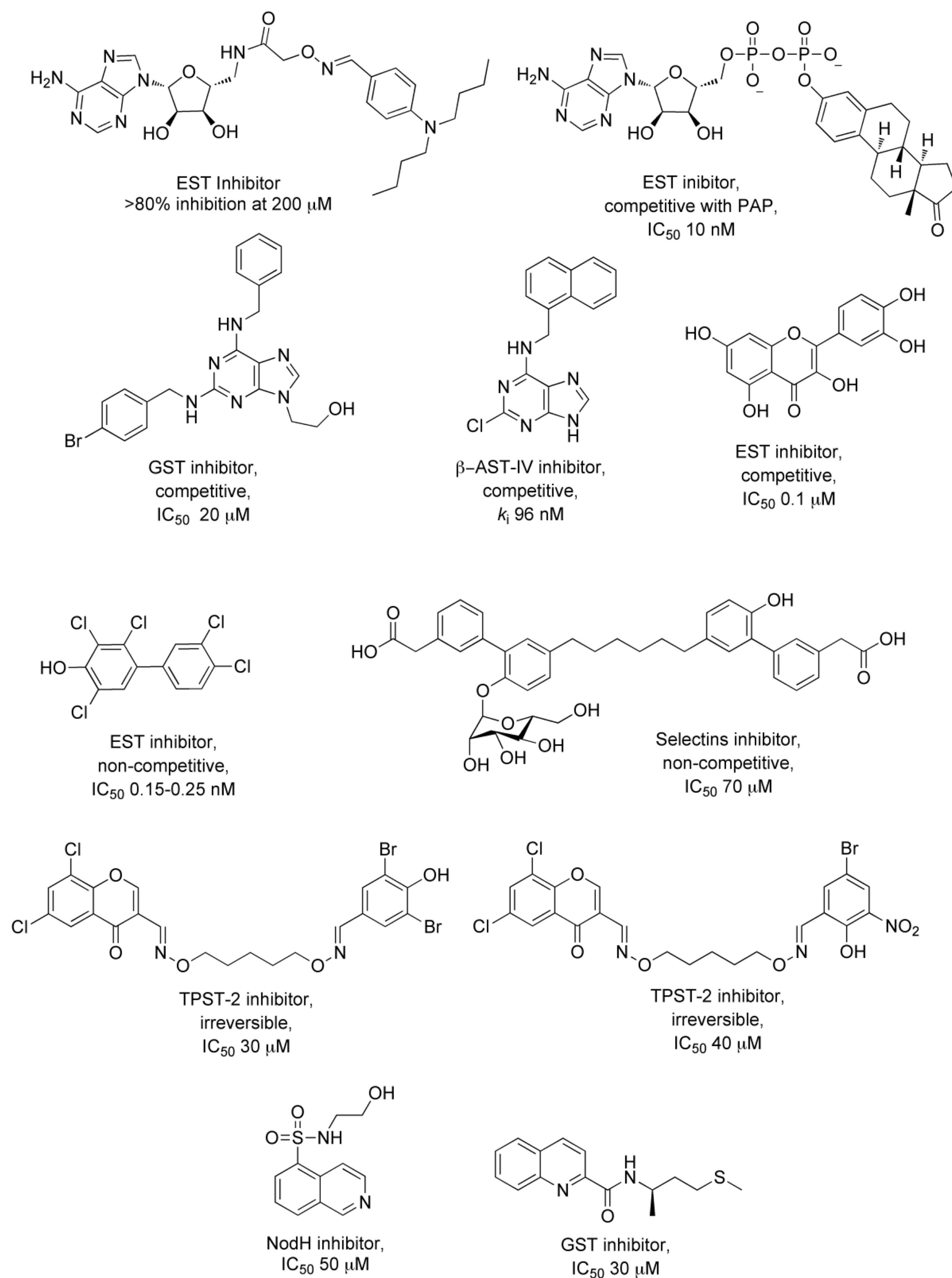

Figure 4.
Sulfotransferase (ST) inhibitors

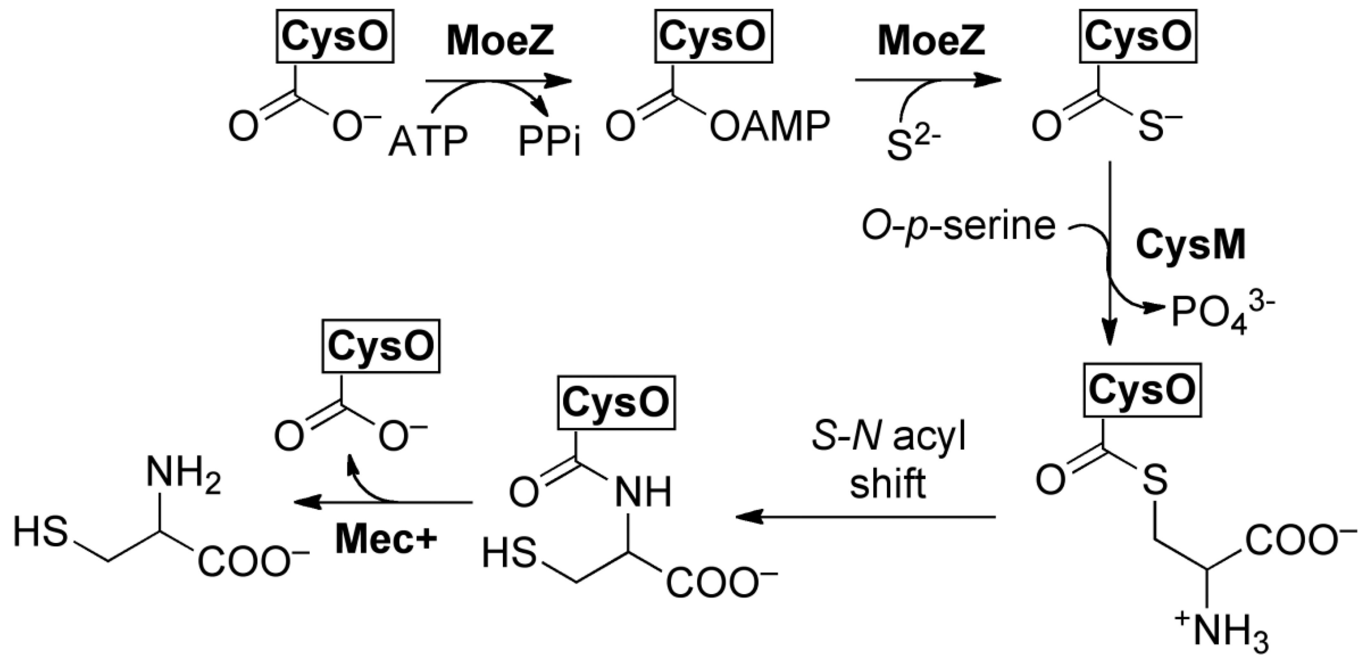

Figure 5.
Alternate cysteine biosynthetic pathway.

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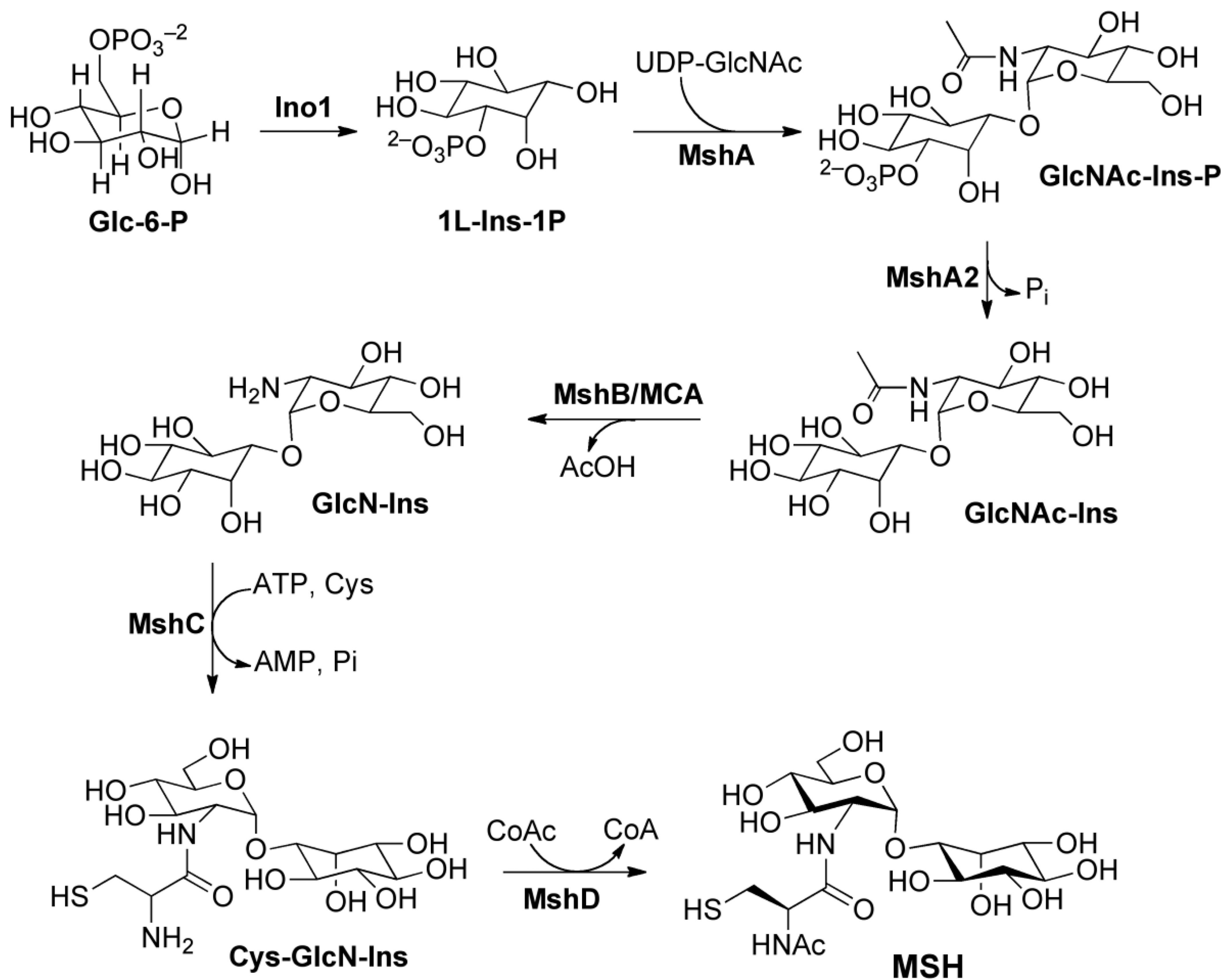

Figure 6.
Mycothiol biosynthetic pathway.

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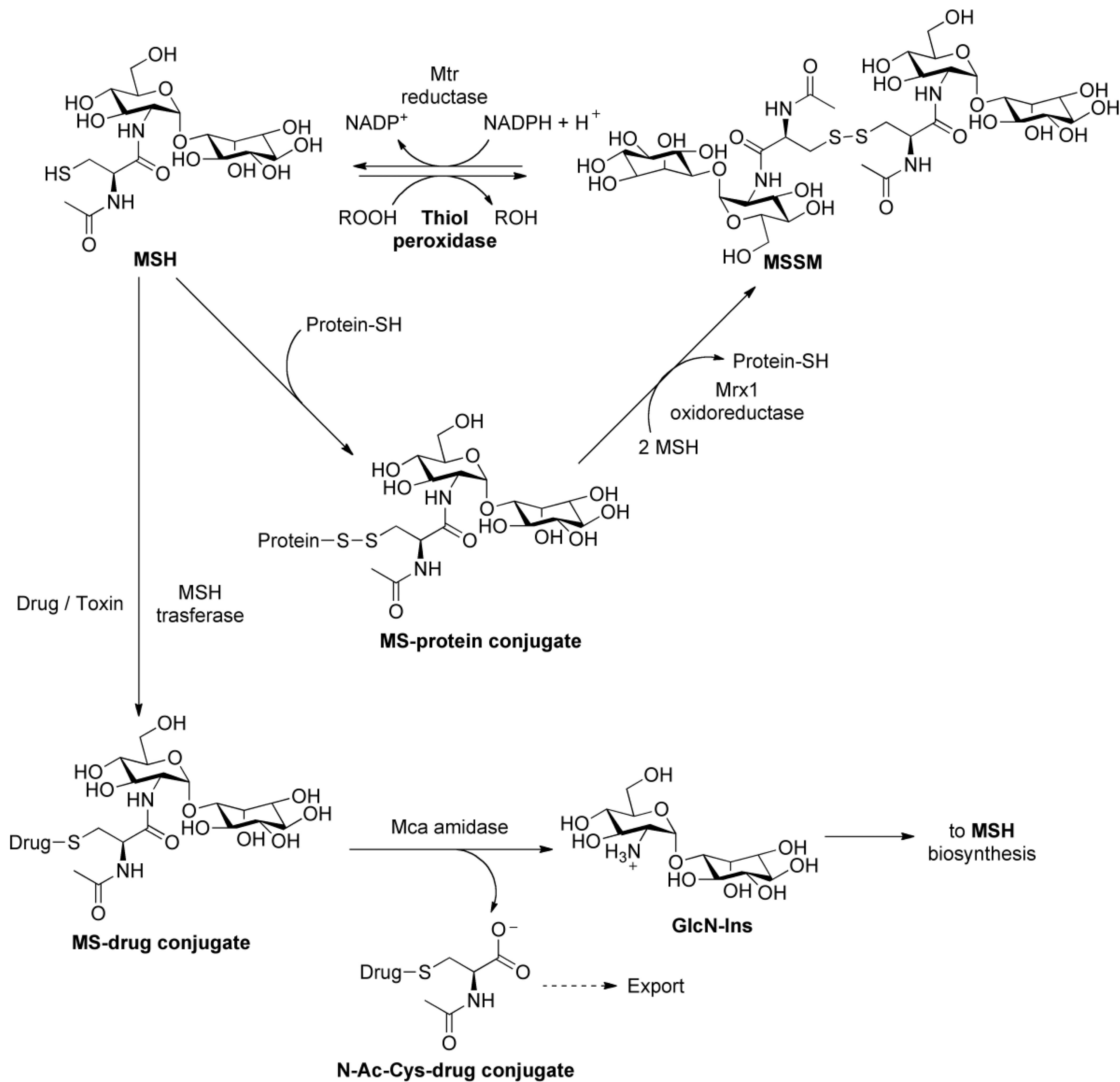

Figure 7.
Mycothiol mediated detoxification pathway.

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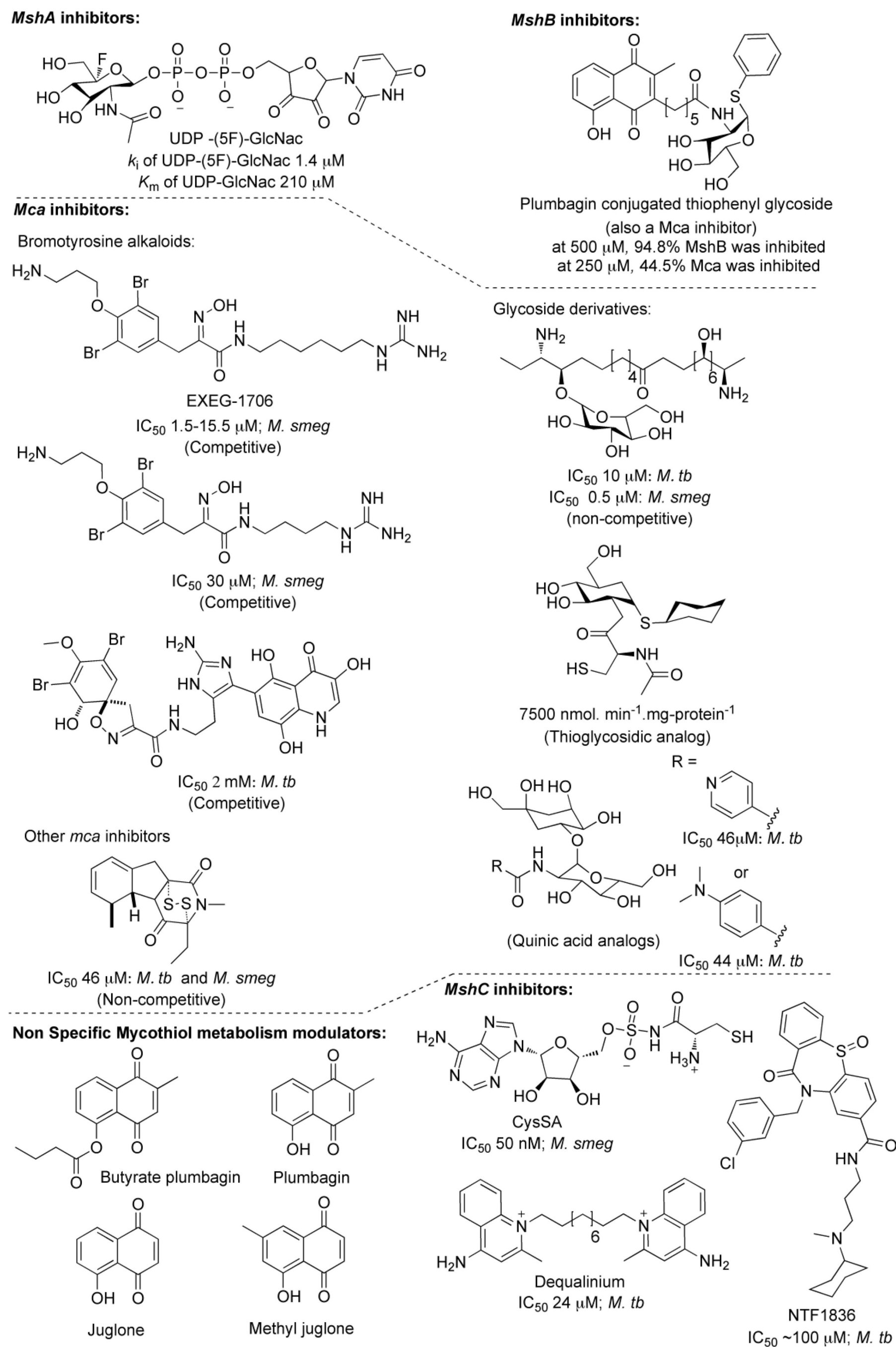

Figure 8.

Mycothiol metabolism inhibitors.