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Journal
Future Medicinal Chemistry, 5(12)

ISSN
1756-8919

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Publication Date
2013-08-01

DOI
10.4155/fmc.13.109

Peer reviewed

Published in final edited form as:
Future Med Chem. 2013 August ; 5(12): . doi:10.4155/fmc.13.109.

Insights on how the *Mycobacterium tuberculosis* heme uptake pathway can be used as a drug target

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Abstract

Mycobacterium tuberculosis (Mtb) acquires non-heme iron through salicylate-derived siderophores termed mycobactins whereas heme iron is obtained through a cascade of heme uptake proteins. Three proteins are proposed to mediate Mtb heme iron uptake, a secreted heme transporter (Rv0203), and MmpL3 and MmpL11, which are potential transmembrane heme transfer proteins. Furthermore, MhuD, a cytoplasmic heme-degrading enzyme, has been identified. Rv0203, MmpL3 and MmpL11 are mycobacteria-specific proteins, making them excellent drug targets. Importantly, MmpL3, a necessary protein, has also been implicated in trehalose monomycolate export. Recent drug-discovery efforts revealed that MmpL3 is the target of several compounds with antimycobacterial activity. Inhibition of the Mtb heme uptake pathway has yet to be explored. We propose that inhibitor design could focus on heme analogs, with the goal of blocking specific steps of this pathway. In addition, heme uptake could be hijacked as a method of importing drugs into the mycobacterial cytosol.

Mycobacteria continue to pose significant global health challenges; in particular, ***Mycobacterium tuberculosis*** (Mtb), the etiological agent of tuberculosis (TB), infects a third of the world's population and caused 1.4 million deaths in 2011 [101]. Resistance to frontline anti-TB drugs has risen over the last decade and cases of drug-resistant TB have been documented on all continents except Antarctica. To counteract the spread of drug-resistant TB there exists an urgent need for new anti-TB drugs. We believe that new anti-TB **drug discovery** will hinge on the identification of novel drug targets. In this perspective we present evidence that bacterial heme and non-heme iron pathways may represent viable drug targets, and describe how the mycobacterial heme uptake pathway, in conjunction with the non-heme **iron uptake** pathway, may be inhibited.

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Financial & competing interests disclosure
This work was supported by the grant AI081161 (C.W.G) from the NIH. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.

Bacteria can utilize both non-heme & heme iron

Metal ions are an integral part of life. Within the human body, the most abundant metal ions are Na^+, K^+, Mg^{2+} and Ca^{2+}, which are found in groups 1 and 2 of the periodic table [1]. The most abundant d-block metal ion in humans is $Fe^{2+/3+}$, which is found at approximately 4 mg per kg of body mass. The biological significance of Fe ions is extremely diverse. Iron-containing enzymes are widespread and functionally diverse owing to the metal's physical properties, which makes it a useful cofactor in many biologically important processes [2].

Because of the biological importance of iron, bacteria have devised several strategies to acquire iron from their surroundings. To meet their nutritional iron requirement they have evolved **siderophores**, remarkable small molecules, which are secreted and coordinate iron with extremely high affinity ($K_a > 10^{30}$ M^{-1}) [1,3,4]. The tight Fe binding ability of siderophores is derived from the presence of chemical groups that preferentially bind ferric Fe (Fe^{3+}) ions. Common siderophore functional groups are catecholate, hydroxamate and carboxylate, which are hard Lewis bases [1,3,5]. Furthermore, siderophores often impose a favorable octahedral coordination environment around the Fe center, further increasing their iron-binding affinity [1,3–5]. The Fe sources for siderophores are transferrin, lactoferrin and ferritin, although scavenging from other iron-containing proteins is possible [4]. Once Fe-loaded, siderophores are typically retrieved by bacteria through specific receptors that recognize the Fe-bound form [3]. To prevent bacteria from utilizing host iron, mammals possess a siderophore binding protein, siderocalin, as a component of their innate immune defense system, which sequesters siderophores and disrupts the bacterial iron acquisition pathway [6,7]. In response to siderocalin, some bacteria produce glycosylated siderophores to escape host detection, such as salmochelin from *Salmonella enterica* [8]. In addition, bacteria have also evolved non-siderophore iron uptake mechanisms including transferrin-binding [9] and lactoferrin-binding [10] receptors, and protease-dependent ferritin iron uptake pathways [11]; however, as pathogenic mycobacteria do not appear to possess these pathways they will not be discussed further.

To-date, heme-uptake is the only known non-siderophore mediated iron mechanism know in Mtb [12]. **Heme uptake**, compared with non-heme iron uptake, can draw from a larger iron pool source including host hemoglobin (Hb). Furthermore, it has been postulated that non-heme and heme uptake may have complementary roles, where one of the pathways is activated depending on the infection stage or the host environment in which the bacteria resides [13,14].

Many bacteria can acquire heme and such pathways have been discovered in both Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus* [15], *Mycobacterium tuberculosis* [16], *Bacillus anthracis* [17], *Serratia marcescens* [18], *Pseudomonas aeruginosa* [19] and *Porphyromonas gingivalis* [20]. There are three general strategies for bacterial heme uptake:

- Heme can be scavenged via secreted high affinity heme-binding proteins called **hemophores** [12,21,22];
- Cell-surface receptors bind host hemoproteins and extract the cofactor [23,24];
- Proteases termed hemoglobinases, which degrade host Hb, thereby releasing the heme molecule so it can be imported by the bacterium [20,25].

To date, the best-characterized hemophore-mediated heme uptake system is that of the Gram-negative organism *S. marcescens* (Figure 1A). *S. marcescens* acquires heme through the hemophore HasA. HasA binds heme with a high affinity ($K_a = 5.3 \times 10^{10}$ M^{-1}) [26], which is higher than that of human ferric Hb, which has of a K_a of 9×10^9 to 1×10^{12} M^{-1}

depending on its oligomeric state [27–29]. The rate of heme transfer from Hb to HasA was demonstrated to be equal to the rate of heme dissociation from Hb, which suggests that the mechanism of heme acquisition by HasA is through its high heme-binding affinity [30]. However, NMR experiments noted chemical shift perturbations on several HasA residues when mixed with Hb, suggesting protein–protein interactions may also play a role in heme transfer from human Hb to HasA [31].

Cell-surface associated heme uptake systems are found in both Gram-positive and -negative organisms. However, the mechanism of heme uptake differs between these two groups of bacteria. In Gram-negative organisms, unbound heme and host hemoproteins are recognized by membrane-bound receptors [32]. For example, the *P. gingivalis* membrane receptor HmuR binds to both heme and hemoglobin [33,34]. After heme binding, heme is then imported into the periplasm in a TonB-dependent manner [32,33,35]. In Gram-positive organisms, cell-wall anchored proteins extract heme from Hb via protein–protein interactions and subsequently shuttle heme across the cell wall to transmembrane receptors (Figure 1B) [23,24,36].

The final category of bacterial heme uptake pathways, hemoglobinase-mediated heme uptake, has only been thoroughly studied in *P. gingivalis*. In addition to having a hemoglobin receptor, *P. gingivalis* secretes a cysteine protease called gingipain, which proteolytically degrades Hb and results in the release of heme from Hb [25,37]. Heme is then bound by cell-surface heme receptors.

Mtb non-heme iron & heme uptake

Mtb invades the human body via inhalation and attacks the lungs; thus, Mtb's primary infection is in the respiratory organs where it resides in macrophages, and infected macrophages aggregate to form granulomas. During active TB, secondary infections at alternate sites may occur, including the CNS and lymphatic system, and other organs. TB also has an asymptomatic, latent stage, whereby Mtb can lay dormant in granulomas until the host immune system is compromised. Intra-cellular Mtb residing within macrophage phagosomes have been reported to promote iron uptake from transferrin [38], whereas during extracellular infection, Mtb in the bloodstream will encounter iron bound to both transferrin and ferritin proteins [4]. Mtb may also sequester iron from heme or hemoglobin [16], at either intracellular or extra-cellular sites of infection. Intracellular Mtb may encounter heme diffused from the macro phage phagolysosome that degrades Hb containing red blood cells [39]. In the bloodstream, extracellular Mtb may encounter heme or Hb during dissemination from primary to secondary infection sites, as well as blood produced in Mtb-infected lung cavities; in these settings, utilization of heme may be facilitated by Mtb hemolysins [40]. Thus, Mtb has evolved both heme and non-heme uptake pathways (Figure 2) [41–44].

The Mtb siderophore pathway is well studied and consists of secreted siderophores termed **carboxymycobactins** and **mycobactins** [4]. The importance of Mtb iron uptake was highlighted by evidence that the mode of action of one of the earliest anti-TB drugs, *p*-aminosalicylic acid, was inhibition of siderophore biosynthesis [4,45].

Besides non-heme iron uptake, mycobacteria also have a heme iron uptake pathway. Initial evidence for this pathway was based on the observation that a recombinant bacillus Calmette-Guérin (rBCG; an attenuated strain of *Mycobacterium bovis*) harboring a defective siderophore biosynthetic pathway, replicated slowly in SCID mice, suggesting it acquires heme iron [46]. Furthermore, mycobacteria replicate faster in the presence of Hb [47]. However, the heme-analog Ga-protoporphyrin IX (PPIX) is lethal to *Mycobacterium*

smegmatis [48], suggesting uptake of the heme-analog either results in the subsequent release of toxic Ga in the mycobacterial cytosol or alternatively, incorporates into mycobacterial hemoproteins rendering them inactive.

To directly examine whether Mtb has a heme uptake pathway, an Mtb strain (MtbΔ*mbtB*) was constructed, in which the siderophore bio-synthetic pathway was disrupted. Despite being grown in Fe-containing media, MtbΔ*mbtB* replicates poorly. In contrast, addition of heme (Figure 3A) or Hb (Figure 3B) to the media results in restoration of growth to the same level as addition of exogenous mycobactin. In addition; another study confirmed the presence of an Mtb heme uptake system, the attenuated growth of MtbΔ*mbtD* mutant was rescued by supplementation of the media with heme [49]. These results indicate that Mtb has a heme uptake system, prompting the search for proteins involved in this pathway [16].

A pulldown experiment on Mtb culture filtrate using heme–agarose beads followed by mass spectrometry analysis resulted in the identification of a single protein, Rv0203. Rv0203 is predicted to be secreted and in its mature form is 10.6 kDa. To identify additional participants in Mtb heme uptake, the genomic vicinity of Rv0203 was investigated (Figure 4). Two proteins, MmpL3 and MmpL11, were hypothesized to have a role in heme transport across the membrane as they are predicted to be large transmembrane transporters. To directly probe the roles of Rv0203 and MmpL11 in heme uptake the MtbΔ*mbtB*Δ*Rv0203* and MtbΔ*mbtB*Δ*mmpL11* knockout mutants were constructed. In heme supplemented media, both MtbΔ*mbtB*Δ*Rv0203* and MtbΔ*mbtB*Δ*mmpL11* strains displayed a significant growth defect compared with Mtb-Δ*mbtB*, implicating both Rv0203 and MmpL11 in heme uptake (Figure 5) [16].

Of note, direct analysis of the role of MmpL3 in heme uptake was not possible as the mutant MtbΔ*mbtB*Δ*mmpL3* could not be constructed since *mmpL3* is an essential Mtb gene. MmpL3 shares approximately 25% sequence homology with MmpL11 and therefore we propose that MmpL3 is also a component of the Mtb heme uptake system.

Rv0203 structure & biophysical properties

Initial studies demonstrated that Rv0203 is a heme-binding protein and is dimeric in both its apo- and heme-bound forms in solution. To further understand the function of secreted Rv0203 in heme uptake on a molecular level, its crystal structure was solved (PDB code: 3MAY, Figure 6). It is an entirely α-helical protein that crystallizes as a dimer of dimers, where each monomer consists of five α-helices arranged in a unique structural fold [16]. Interestingly, while the *S. marcescens* hemophore, HasA, has no overall structural or sequence homology to Rv0203, structural comparison reveals that Rv0203 surface residues Tyr59, His63 and His89 are arranged in a similar motif (Figure 6B) as the HasA heme-binding residues His32, Tyr75 and His83 [50]. This observation lead to the hypothesis that Rv0203 residues Tyr59, His63 and His89 may play a role in heme binding. Mutagenesis experiments validated this hypothesis as the Y59A-Rv0203 and H63A/H89A-Rv0203 mutants fail to bind heme efficiently and display different spectroscopic characteristics compared with wild-type Rv0203 [16,51]. The heme-binding affinity of Rv0203 was determined using to stopped flow methods, and the K_a is equal $1.9 \times 10^9\ M^{-1}$ [51]. While Rv0203 has a relatively is K_a lower than high heme-binding affinity, its that of Hb, which implies that if Rv0203 were to scavenge heme from Hb it would not be able to do so using an affinity gradient.

MmpL3 & MmpL11 are multifunctional proteins

MmpL3 and MmpL11 are large transmembrane proteins, which are part of the MmpL protein family. This family of proteins consists of 13 members and is itself a mycobacteria-

specific branch of the diverse Resistance-Nodulation-cell Division (RND) permease superfamily of transmembrane transporters [52]. In Gram-negative bacteria, RND transporters are known to mediate substrate transport across the cell membrane by the action of proton-motive force.

The importance of the MmpL family in mycobacteria has been highlighted in several recent investigations. Studies in mice have demonstrated that deletions of MmpL8 and MmpL11 in Mtb cause mice to survive significantly longer compared with mice infected with wild-type Mtb [52]. In addition, Mtb MmpL4 and MmpL7 knockout mutants are avirulent [52]. Furthermore, MmpL4 and MmpL10 are thought to play a role in Mtb survival in mice lungs [53]. While structural predictions suggest that MmpL proteins are members of the RND family, their respective functions are not well understood. Gram-negative bacteria RND transporters typically are involved in efflux of drugs or toxic compounds [54]; however, only MmpL5 and MmpL7 are thought to be involved in drug efflux [55,56]. Moreover, it was demonstrated that MmpL3, MmpL7 and MmpL8 export trehalose monomycolate (TMM), dimycocerosate and sulfolipid-1 across the bacterial inner membrane, respectively [57–61]. A recent study further identified MmpL4 and MmpL5 as part of the siderophore export machinery [45]. This discovery is highly interesting since heme and siderophore transport proteins often share similar ancestral origins and structural similarity.

Roles of MmpL3 & MmpL11 in heme uptake

Direct characterization of MmpL3 and MmpL11 in heme uptake is complicated by the size of the proteins (944 and 966 amino acids for MmpL3 and MmpL11, respectively), and the fact that both are transmembrane proteins with multiple predicted transmembrane helices. Topology predictions suggest that both MmpL3 and MmpL11 have two extracellular domains and one intracellular domain, termed E1, E2 and C1, respectively (Figure 7). The corresponding MmpL3 and MmpL11 domains were expressed heterologously and purified: MmpL3 and MmpL11 E1 and E2 domains were reported to bind heme whereas the C1 domains did not [16]. Spectroscopic investigation of MmpL3-E1 and MmpL11-E1 revealed that the respective heme coordination environments of the two domains are likely similar; the heme molecules exhibit mixed spin states and are possibly coordinated through a combination of oxygen and nitrogen/oxygen ligands. In contrast, the kinetics of heme binding for MmpL3-E1 and MmpL11-E1 are distinct, with MmpL3-E1 binding heme more tightly than MmpL11-E1 [62]. Based on the evidence that Rv0203, E1 and E2 domains of both MmpL3 and MmpL11 bind heme, heme transfer between Rv0203 and MmpL3 and MmpL11 E1 domains was conducted, which indicated that heme is rapidly transferred to both E1 domains through transient protein–protein interactions [62].

Given the observations that Rv0203, MmpL3 and MmpL11 bind heme, and that Rv0203 and MmpL11 are required for efficient growth when heme is the only available iron source for Mtb, it was proposed that Rv0203 acts as a hemophore or heme transporter, which can donate heme to MmpL3 and MmpL11 through their respective E1 or E2 domains [16,51]. Within the cytosol, an IsdG-like heme degrading protein, MhuD, was identified and reported to release Fe from protoporphyrin IX for cellular uses [63], yielding two isomers of mycobilin with no production of carbon monoxide [64].

Variety in heme-binding sites presents an opportunity for drug discovery

The distance between Tyr59 and His89, the two Rv0203 residues proposed to be part of the heme-binding site, in the apo-Rv0203 structure is 8 Å (Figure 6B & 6C) [16]. This suggests the heme-binding pocket must undergo structural rearrangements to accommodate the heme molecule, which features an α to γ meso-carbon distance of approximately 7 Å. The structural architecture of heme-binding sites within heme uptake proteins range from

solvent-exposed to closed conformations, and reveals surprisingly few shared heme coordination features. The heme-binding sites of HasA and NEAT domains (a common fold found in heme uptake proteins of Gram-positive bacteria) are very different [24,50]. HasA contains a six-coordinate mixed-spin heme ligated by Tyr and His [50], and NEAT domains features high-spin heme coordinated by a single Tyr ligand [32]. Furthermore, even though Rv0203 and HasA share a common heme-binding motif, the overall environment of these sites are dissimilar, where Rv0203 contains a five-coordinate heme ligated by hydroxide, possibly donated by a Tyr [51], compared with the six-coordinate heme–HasA complex. Other proteins involved in heme uptake feature *bis*-Met [65], His-Met [66] and *bis*-His [67] ligation. This diversity may be explained by the necessity to form a temporary interaction with the heme molecule. Unlike catalytic proteins, the structure and chemical properties of the heme-binding site of heme transport proteins is not dictated by the requirement to perform a certain chemical reaction. Therefore, the entire heme uptake protein itself can be seen as the heme ligand, in which the composition and geometry of the first coordination sphere of the heme iron is not as important as the overall structure of the heme-binding site, which mediates protein–protein interactions and governs heme-binding affinity.

The diversity in heme-binding sites and heme coordination may represent an opportunity for synthesizing anti-Mtb compounds that are designed to specifically interact with the Mtb heme uptake proteins while avoiding the human heme transport machinery. As such, this drug-delivery approach is analogous to efforts to inhibit the siderophore-mediated non-heme iron uptake pathway. Siderophore receptors are highly selective proteins, which are susceptible to inhibition by compounds related to their cognate siderophore substrate. For example, the *M. smegmatis* siderophore mycobactin S, which is a stereoisomer to the growth promoting Mtb siderophore mycobactin T, is toxic to Mtb [68]. Furthermore, scandium- and iridium-loaded siderophores were demonstrated to inhibit Mtb growth by competing for the siderophore receptors [69,70].

Heme analogs are porphyrins in which either the central Fe ion has been exchanged for another metal ion or the substituent groups on the tetrapyrrole ring are altered. We previously described the study of bacterial inhibition utilizing GaPPIX by Stojiljkovic *et al.* [48], a pioneer in the field of utilizing modified and non-Fe-porphyrins as antimicrobials [71]. The potential for heme analog-based growth inhibition was also exemplified when SnPPIX, Pd-mesoporphyrin IX (MPIX) and ZnMPIX were found to inhibit growth of *Trypanosoma cruzi* in liquid culture [72]. The effects of SnPPIX and PdMPIX were attributed to inhibition of the ABC transporters used to import heme. A similar disruption of heme uptake was described for *in vitro* heme transfer between NEAT domain-containing *S. aureus* proteins. The cell-membrane associated heme-receptor IsdE is incapable of receiving either CoPPIX or FePPIX-dimethylester (DME) from the heme transport protein, IsdC [73]. Since heme transfer across the membrane hinges upon IsdE, both CoPPIX and FeP-PIX-DME could represent inhibitors for this *S. aureus* heme uptake pathway. The study is especially interesting since it demonstrates that changing either the porphyrin structure or metal content can inhibit heme transfer. Changing the porphyrin structure from PPIX to PPIX-DME most likely prevents the formation of important hydrogen bonds between the heme analog and the restricted heme-binding pocket of IsdE. Transfer inhibition by CoPPIX is most likely explained by the preference of the Co metal center for the hard Tyr ligand from IsdC, compared with the softer His of IsdE [73].

A systematic search for heme analogs with the ability to inhibit mycobacterial heme uptake may provide clues to the utility of this approach for the design of anti-Mtb drugs. To that end, a promising observation that GaPPIX is lethal to *M. smegmatis* [48] suggests that toxicity may be the result of the subsequent release of toxic Ga in the cytosol or inactivation

of hemoproteins with non-Fe heme incorporation, or alternatively, GaPPIX may efficiently disrupt the heme transfer pathway, particularly MmpL3 due to its essentiality.

Besides their potential as antimycobacterial agents, heme analogs may provide insights into the mechanism of heme uptake. If a certain protein of the heme uptake machinery can selectively be disrupted by a heme analog, the function of this component may be investigated. Furthermore, inhibitory heme analogs could be used to assess the importance of heme iron uptake at different stages of mycobacterial growth and infection.

Heme uptake as drug-delivery vehicle

The heme uptake pathway may represent a vehicle for delivering molecules that are toxic to mycobacteria. This antimycobacterial drug-delivery approach resembles efforts to appropriate the siderophore-mediated non-heme iron uptake pathway for the same propose. Siderophores have been linked to antimicrobial agents to facilitate their import into the cytosol, which has led to successfully making potent antimycobacterial agents [74,75].

The heme molecule may easily be modified at its meso positions [76]. However, for heme uptake proteins to bind heme modified with potentially bulky substituents, the heme-binding site needs to be relatively large and flexible. IsdX1 from *B. anthracis* [77] and HasA [50], feature solvent accessible heme-binding sites, which could potentially accommodate modified heme molecules. Similarly, based on the apo-structure of Rv0203, the heme-binding site is likely to be surface-exposed, suggesting it may be able to transport modified heme molecules (Figure 6C).

To utilize heme analogs as basis for an antimicrobial drug, the molecules' insolubility and harmful oxidative effects on the human host need to first be overcome. Drug encapsulation has been studied as an avenue for antimycobacterial drug delivery [78], with encouraging results. For example, micelles have been demonstrated to successfully deliver the potent anti-mycobacterial drug SQ641 in a murine model [79]. Furthermore, the feasibility of encapsulation for heme analogs has been demonstrated for ZnPPIX delivery to a variety of cancer cells in cell culture [80–82]. Taken together, these results highlight the potential for delivery of heme analogs exists *in vivo*.

A further potential target for heme analog-based compounds is MhuD, a non-conventional heme-degrading enzyme [63,64], which produces a heme degradation product, mycobilin, unique to mycobacteria [64]. MhuD binds a single heme molecule per monomer in its active form, but can accommodate a second heme molecule in its heme-binding pocket in an inactive state [63]. To exploit MhuD as a potential drug target, a Trojan horse strategy could be employed to deliver GaPPIX whereby MhuD catalysis would release the toxic metal to Mtb. The fate of intracellular heme in Mtb has not yet been investigated and further research should clarify whether heme is directly incorporated in Mtb heme proteins or degraded, thereby making toxic metal release possible; however, related research on *P. aeruginosa* heme uptake has demonstrated that exogenous heme is degraded [83]. Alternatively, a covalently linked dimeric heme-type molecule could be used to inactivate MhuD by trapping it in its inactive conformation, thereby limiting the iron availability of Mtb.

MmpL3 is the target of several antimycobacterial compounds

To date, Mtb heme uptake has not been investigated as a drug target. However, during small-molecule high-throughtput mycobacterial growth inhibition screening, several structurally dissimilar compounds have been found to target MmpL3 and disrupt Mtb growth. The 1,5-diarylpyrrole derivative BM212 has been demonstrated to have antimycobacterial activity against multidrug resistant strains of Mtb [84] with an MIC of 1.5

μg/ml [85]. A single mutation within MmpL3 at position 215 (L215S) caused resistance against BM212 (Figure 7). The intracellular levels of BM212 within this mutant strain were similar to that of wild type Mtb, which suggests that MmpL3 is the target of BM212 and furthermore, that drug resistance is not due to BM212 efflux.

Adamentyl urea compounds, derived from 1-adamantyl-3-phenyl urea cores were found to inhibit Mtb growth in liquid culture [86,87]. The most active compound (1-(2-adamantyl)-3-(2,3,4-trifluorophenyl)urea (AU1235) has an MIC of 0.2 μg/ml. However, investigation of Mtb growth inhibition in mice in a related adamentyl urea compound yielded no protection [87]. A search for resistant mutants against AU1235 demonstrated that resistance arises from the MmpL3 mutation G253E and G758A in the avirulent H37Ra, the MmpL3 mutation (Figure 7) [60]. As with BM212, resistance did not arise from drug efflux, suggesting MmpL3 is the target of AU1235. In a separate study, a benzimidazole derivative (C215) was found to inhibit Mtb by targeting MmpL3 with a MIC of 24 μg/ml. Several amino acid mutations in MmpL3 were found to cause resistance against C215-L320P, T667A, V684A and V51A (Figure 7) [88].

A 1,2-diamine related to ethambutol, SQ109 was found to inhibit cell wall assembly by targeting MmpL3 with a MIC of 0.5 μg/ml [59,89]. Two MmpL3 mutations, Q40R and A700T (Figure 7), provided resistance against SQ109 *in vitro*. Furthermore, SQ109 appears to exhibit synergistic effects with the antimycobacterial compounds TMC207 [90] and PNU-100480 [91]. SQ109 has low cytotoxicity and has been reported to be effective against Mtb infected mice [89]. It also has good pharmacological properties and was found in the lungs of Mtb infected rats [92], leading to its evaluation in early trials in patients with pulmonary TB.

The identification of MmpL3 as the target of several antimycobacterial compounds led to additional insights on its function. Besides being part of heme uptake, MmpL3 has been demonstrated to be part of the TMM export machinery [16,51,60,61,85]. TMM is an important mycobacterial cell wall component. Treatment of Mtb with SQ109 resulted in TMM accumulation in the cytoplasm and lower levels of trehalose 6,6′-dimycolate in the membrane [59]. Furthermore, the morphology of Mtb treated with SQ109 differs from wild type Mtb as it displays ruffling along the surface. This phenotype has been attributed to the lack of trehalose 6,6′-dimycolate in the membrane [59]. Taken together, these results suggest MmpL3 is a bifunctional transporter with roles in both heme import and TMM export.

The mechanism of inhibition of the above compounds and their effects on heme uptake is not yet known. The MmpL3 mutations that confer resistance to the various antimycobacterial compounds are located on different domains. For example, residue 215 (BM212 resistance) is located on the third predicted transmembrane helix, whereas residues 40 and 700 (SQ109 resistance) are located on two predicted soluble domains. Structural information is urgently required to provide clues on how the MmpL3 inhibitors bind and whether the seemingly distant resistance mutation sites are located in proximity to each other.

Evolution of heme uptake pathways

Several lines of evidence suggest that heme uptake in Mtb has distinct evolutionary origins. Rv0203 contains a novel fold and other than the location of the three heme-binding residues, which are similar to the hemophore HasA heme-binding residues, the heme-binding site has no structural similarity with any known hemo protein. Thus, the heme-binding ability of Rv0203 is likely to have evolved separately from other heme uptake proteins and also did not evolve from a known hemoprotein origin. While MmpL3 and MmpL11 do not have

shared origins with non-mycobacterial heme uptake proteins, it is related to siderophore export proteins. MmpL4 and MmpL5 are suggested to export siderophores and are virulence factors [45]. Proteins involved in heme and siderophore transport are known to share common ancestral origins [93]. Therefore, while the heme uptake function of MmpL3 and MmpL11 appear to have evolved from a unique origin, these proteins do share ancestral roots with siderophore transport proteins.

The dual roles of MmpL3 in heme and TMM transport suggest it is bifunctional. Bifunctionality has been observed among other heme uptake transport proteins. For example, the *S. aureus* transmembrane protein, HtsA, displays dual roles as both a heme and siderophore receptor [94,95]. The lack of evolutionary relationship with non-mycobacterial heme uptake systems has lead to a poor understanding of the regulation of heme uptake. It is not known how Mtb heme uptake is regulated. Typically, non-heme and heme iron uptake is governed by a regulatory element termed the Fur box, which is recognized by iron-dependent transcription factors [12,24]. However, to-date no Fur box has been identified in the vicinity of the Mtb heme uptake region, which suggests a different form of regulation may exist. Furthermore, the presence of two iron uptake pathways raises the question of how Mtb senses which iron source is available.

Future perspective

While heme uptake pathways have been characterized in many pathogens, their role in virulence is often unclear. The importance of heme uptake in virulence has only been demonstrated in few cases. In *S. aureus,* heme uptake is critical for early infection *in vivo* [14] and evidence exists that pathogenic *Escherichia coli* require heme uptake during early colonization events [96]. However, the interplay between heme and non-heme iron uptake in Mtb is not yet established. Upon infection, the human immune system launches a nutritional immunity attack against Mtb by restricting transferrin levels [97], suggesting heme uptake may be required during early infection. Neither heme uptake nor non-heme iron uptake pathways alone are necessary for Mtb survival as long as both sources of iron are available, suggesting the pathways serve redundant roles. However, while siderophores are necessary for virulence, the role of heme uptake upon virulence is not yet understood. Drug design would benefit from a better understanding of the underlying importance of the respective pathways during the Mtb infection cycle. Furthermore, additional structural characterization of the mycobacterial heme uptake system, and in particular MmpL3, would provide avenues for informed structure-based inhibitor design.

Key Terms

Mycobacterium tuberculosi	A pathogenic bacteria that is the causative agent of the infectious disease tuberculosis
Drug discovery	The process by which knowledge, in this instance the *Mycobacterium tuberculosis* heme uptake pathway, is exploited for novel inhibitor design with the intent of therapeutic treatment
Iron uptake	The ability for organisms to import external sources of iron
Siderophores	Secreted small molecules by organisms with high affinities for iron
Heme uptake	The ability for organisms to import external sources of heme to utilize iron from heme

Hemophores	Secreted proteins able to scavenge heme from host hemoproteins
Carboxymycobactins	Secreted mycobacterial specific siderophores
Mycobactins	Membrane-associated mycobacterial specific siderophores

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Executive summary

- *Mycobacterium tuberculosis* has a mycobacterial-specific heme uptake pathway.
- Rv0203 is a secreted heme transport protein and MmpL3 and MmpL11 are proposed transmembrane heme importers.
- MmpL3 is also a trehalose monomycolate exporter.
- MmpL3 is the target of several compounds with antimycobacterial activity.
- Heme uptake inhibitors could be based on heme analogs.
- Heme could be functionalized to facilitate transport of antimycobacterial drugs.
- Characterizing the differential roles of heme iron and non-heme iron uptake is critical for targeted drug development.

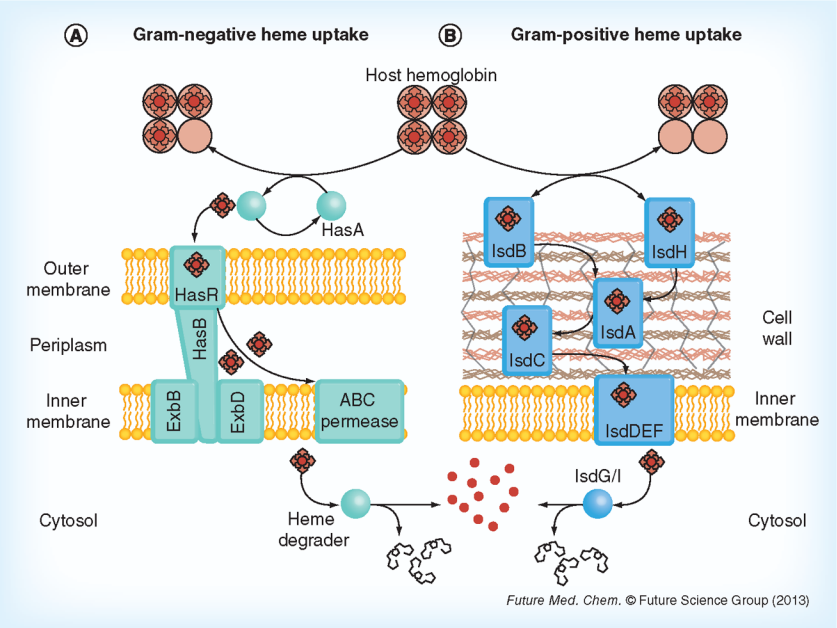

Figure 1. Heme uptake in *Serratia marcescens* (Gram-negative) and *Staphylococcus aureus* (Gram-positive)

(A) *Serratia marcescens* sequesters heme via the HasA hemophore. The HasB–ExbB–ExbD multiprotein complex is required for proton-motive force transport of diverse molecules through the outer membrane, where HasB is the *S. marcescens* TonB paralogue. Heme is eventually transported into the cytosol via a membrane-associated ABC protein and degraded by a heme degrader to liberate iron. **(B)** In *Staphylococcus aureus*, IsdB and IsdH are host hemoglobin receptors, IsdA and IsdC are heme transport proteins and the IsdDEF complex is responsible for ATP-dependent transmembrane heme transport. Imported cytosolic heme is degraded by IsdG or IsdI.

(A) Adapted from [21].

(B) Adapted from [23].

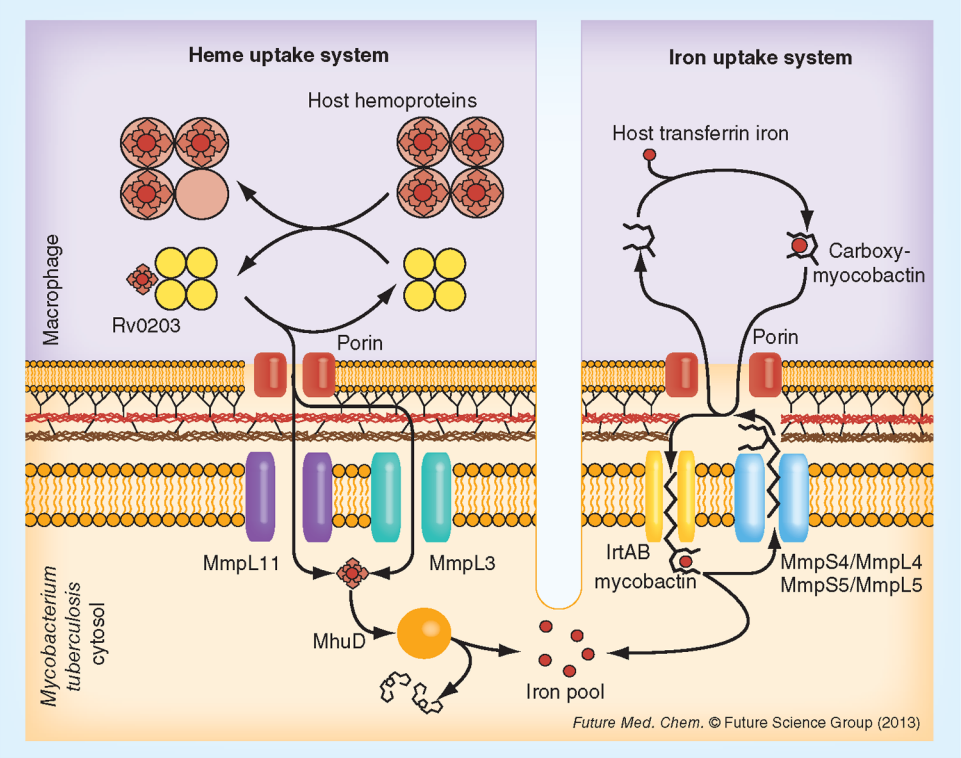

Figure 2. *Mycobacterium tuberculosis* heme and non-heme iron uptake pathways

Within the iron uptake pathway, IrtAB [41,42] and Esx-3 [43] are thought to be involved in iron-mycobactin retrieval, while MmpS4/L4 and MmpS5/L5 are thought to be involved in mycobactin export [44]. Within the heme uptake pathway, Rv0203 may function as a hemophore, MmpL3 and MmpL11 are the proposed heme transport proteins and MhuD is the heme-degrading protein.

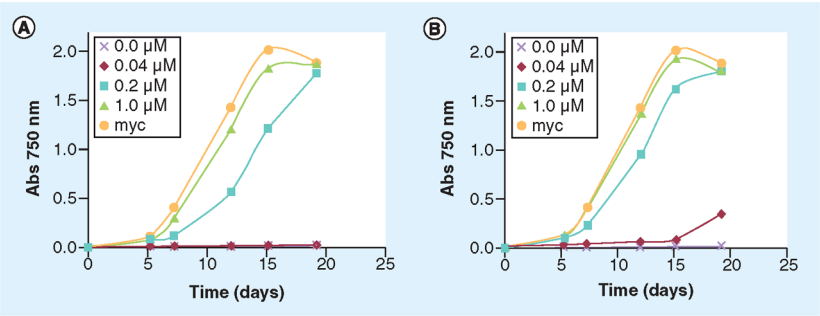

Figure 3. Growth of MtbΔ*mbtB*

In presence and absence of **(A)** heme and **(B)** hemoglobin, compared with growth with exogenously added myc.

myc: Mycobactin J.

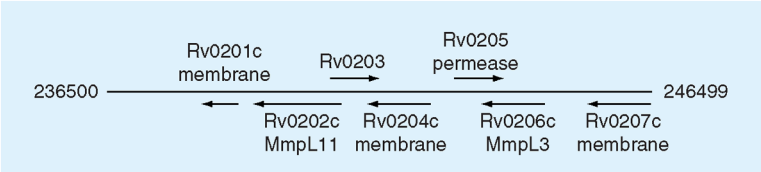

Figure 4.
Mycobacterium tuberculosis genomic region within the vicinity of Rv0203.

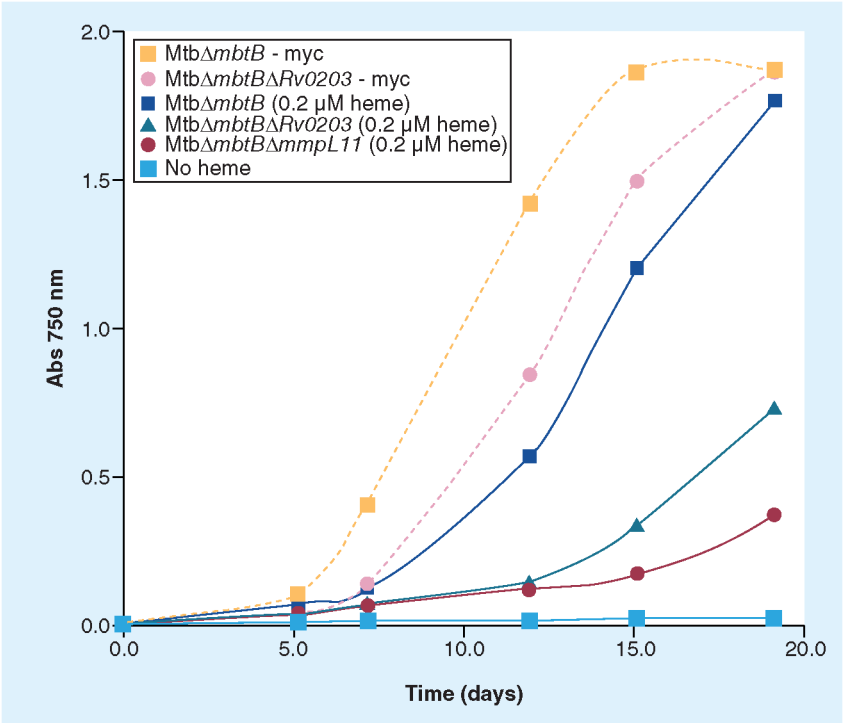

Figure 5.

Growth curves of MtbΔ*mbtB*Δ*Rv0203* and MtbΔ*mbtB*Δ*mmpL11* in presence of 0.2 μM heme, demonstrating attenuated growth compared with that of MtbΔ*mbtB* grown in media containing either 0.2 μM heme or exogenous mycobactin J

myc: Mycobactin J.

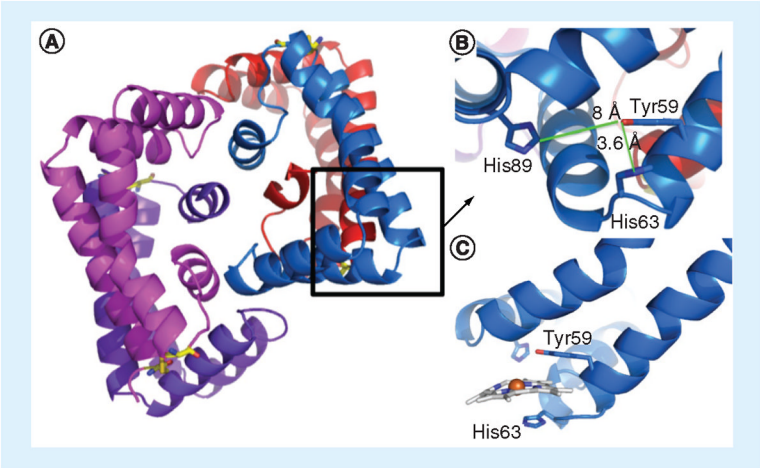

Figure 6. Crystal structure of Rv0203 (PDB code: 3MAY)

(A) Rv0203 is a dimer of dimers. **(B)** A close-up view of the proposed heme-binding site. **(C)** Iron-tetrapyrrole is modeled into the predicted heme-binding site. Residues are illustrated as sticks, where oxygen is colored red, sulfur yellow and nitrogen blue. Heme carbons are colored white and heme iron is depicted as an orange sphere. This figure can be viewed in full color at: www.future-science.com/doi/full/10.4155/FMC.13.109

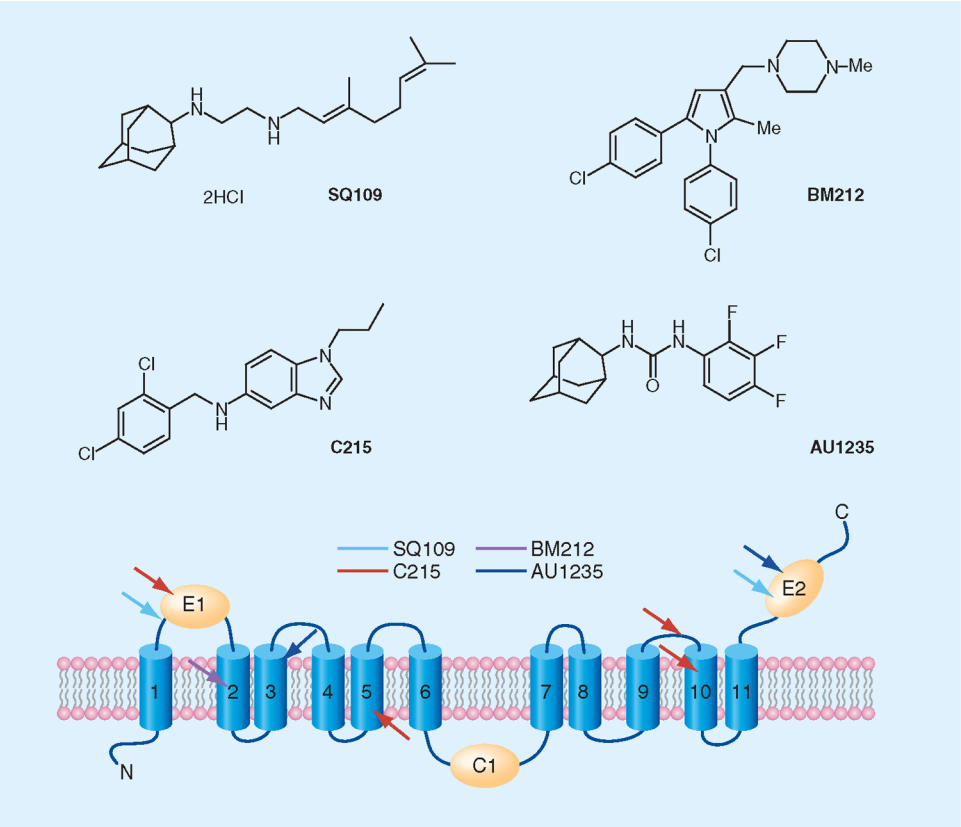

Figure 7. Molecular structures of *Mycobacterium tuberculosis* inhibitors BM212, SQ109, C215 and SQ109 – all target MmpL3

Mycobacteirum tuberculosis strains resistant to each inhibitor have single point mutations in MmpL3. The predicted secondary structure depiction of MmpL3 is shown, whereby arrows indicate where each resistance point mutation occurs.