The Mycobacterial Cell Envelope—Lipids

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Mycobacterium tuberculosis (*Mtb*) lipids are indelibly imprinted in just about every key aspect of tuberculosis (TB) basic and translational research. Although the interest in these compounds originally stemmed from their abundance, structural diversity, and antigenicity, continued research in this field has been driven by their important contribution to TB pathogenesis and their interest from the perspective of drug, vaccine, diagnostic, and biomarker development. This article summarizes what is known of the roles of lipids in the physiology and pathogenicity of *Mtb* and the exciting developments that have occurred in recent years in identifying new lead compounds targeting their biogenesis.

The mycobacterial cell envelope is the dominant feature of the biology of *Mycobacterium tuberculosis* (*Mtb*) and other mycobacteria, based on sugars and lipids of exceptional structure. Cell envelope lipids constitute ∼40% of the cell dry mass, although this percentage may vary depending on species/isolate and growth conditions (Goren and Brennan 1979; Minnikin et al. 1982). The particular chemical structures and organization of these lipids between the inner and outer membrane of the mycobacterial cell envelope account for much of its impermeability to biocides and for the unique staining properties of mycobacteria that aid in the diagnosis of infected specimens (Goren and Brennan 1979; Jarlier and Nikaido 1994; Brennan and Nikaido 1995). Being located at the interface between the bacterium and the host, the outer membrane and capsular lipids of *Mtb* also play important roles in directing host–pathogen interactions. Several lipid components of *Mtb* (e.g., isoprenoid lipids, glycerophospholipids, phosphatidylinositol mannosides, lipoarabinomannan, mycolic acids, and trehalose mycolates) are produced by all mycobacterial species, consistent with their requirement for growth. The biosynthesis of some of these lipids is the site of action of anti-TB drugs such as isoniazid (INH) and ethionamide (ETH), as well as multiple other lead compounds under development. Other lipids (e.g., long-chain polymethyl-branched fatty acid–containing acyltrehaloses and long-chain diols) are not essential for growth but are unique to pathogenic mycobacteria and have been involved in pathogenesis.

Studies initiated in 1927 by R.J. Anderson and colleagues mark the beginning of the pio-

neering work that has led to the discovery and early structural definition of some of the most biologically relevant lipids of *Mtb*, including phosphatidylinositol mannosides, mycolic acids, and branched fatty acids and polyols (Goren and Brennan 1979). Studies conducted for decades to follow by generations of outstanding biochemists in France, the United Kingdom, and the United States, in particular, have resulted in a thorough understanding of the chemical structures of these lipids. Their detailed descriptions have been compiled in several recent books and reviews and the reader is referred to these for further reading on this topic (Brennan 1988; Barry et al. 1998; Kaur et al. 2009; Daffé et al. 2014). Developments in the genetic manipulation of mycobacteria in the 1990s and the release of the first complete genome sequence of *Mtb* in 1998 have provided a major impetus to the study of the biosynthesis of these compounds with the result that many of the enzymes and transporters involved in their biogenesis have now been identified. Following the classification of *Mtb* into six major genetic lineages associated with specific geographical regions (Gagneux and Small 2007), recent years have also seen a return of interest in comparative analyses of the lipid content of clinical isolates from different regions of the world in the hope of identifying correlates of pathogenicity. Interestingly, not only are these studies providing new insights into the biochemical diversity of *Mtb* and the putative roles of some lipids in host–pathogen interactions (Daffé et al. 1991; Constant et al. 2002; Reed et al. 2004, 2007; Tsenova et al. 2005; Sinsimer et al. 2008; Huet et al. 2009; Krishnan et al. 2011), they are also shedding light on the genetic basis of observations made some 20 to 40 years ago by early investigators in the field (Goren et al. 1974a; Daffé et al. 1991; Krishnan et al. 2011; Supply et al. 2013). What is known of the biosynthesis of *Mtb* lipids has been reviewed recently (Daffé et al. 2014). This review focuses on our current understanding of the roles of cell wall lipids in the physiology and pathogenicity of *Mtb* and describes ongoing drug discovery efforts aimed at targeting their biosynthesis, export, and regulation.

PHYSICAL ORGANIZATION AND PHYSIOLOGICAL ROLES OF *Mtb* CELL ENVELOPE LIPIDS

Architecture of the *Mtb* Cell Envelope

A schematic representation of the cell envelope's architecture of *Mtb* is presented in Figure 1. The innermost layer is the plasma membrane that seems typical of bacterial membranes. Outside the plasma membrane is a massive cell wall "core" comprised of peptidoglycan (PG) covalently attached to the heteropolysaccharide arabinogalactan (AG), in turn esterified at its nonreducing ends to α-alkyl, β-hydroxy long-chain (C_{60}-C_{90}) mycolic acids. Intercalated within this lipid environment are the "free" (noncovalently linked) lipids and lipoglycans that have intrigued researchers for more than eight decades: the phosphatidylinositol mannosides (PIMs), the phthiocerol dimycocerosates (PDIMs), the phenolic glycolipids (PGLs), a variety of acyltrehaloses, mannose-capped lipoarabinomannan (ManLAM), etc. (Figs. 2 and 3). The covalently bound mycolic acids of the cell wall core and the free lipids form the inner and outer leaflets, respectively, of a highly impermeable asymmetrical bilayer (known as the "mycomembrane" or outer membrane) that confers to mycobacteria their characteristic resistance to many therapeutic agents (Minnikin et al. 1982; Jarlier and Nikaido 1994; Hoffmann et al. 2008; Zuber et al. 2008) (Fig. 1). Finally, a loosely attached capsular-like structure outside the outer membrane of *Mtb* was shown to mainly consist of polysaccharides and proteins with only minor amounts of lipids (2%–3% of the material) (Lemassu and Daffé 1994; Ortalo-Magné et al. 1995; Sani et al. 2010) (Fig. 1). Thus, in spite of being Gram-positive bacteria, mycobacteria possess a cell envelope characterized by the presence of an outer membrane and a periplasmic space.

Mycolic Acids

The interest in drugs targeting the biosynthesis of mycolic acids is clearly illustrated by the therapeutic efficacy of INH and ETH (Table 1). This essentially owes to their role as key structural elements of the outer membrane. The mycolic
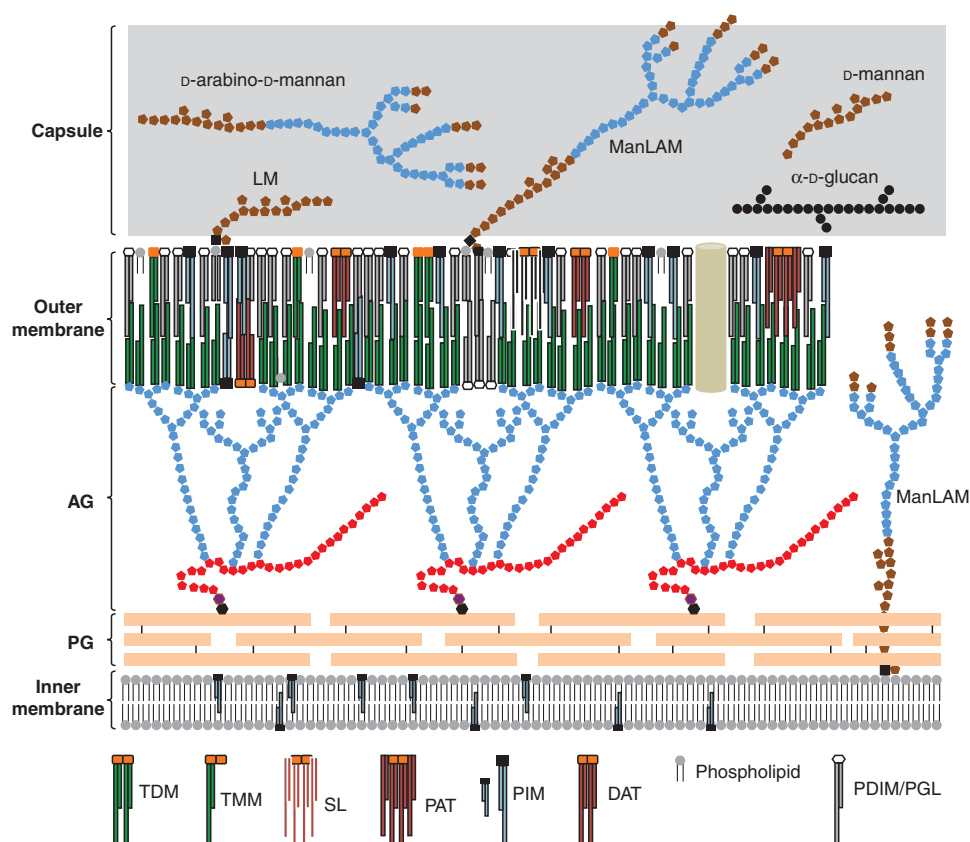
Figure 1. Schematic representation of the *Mtb* cell envelope. Many of the classes of (glyco)lipids discussed in the text are represented schematically and are shown in probable locations in the cell envelope. Light blue symbols represent arabinose residues, red symbols represent galactose residues, brown symbols represent mannose residues, and black circles represent glucose residues. ᴅ-arabino-ᴅ-mannan, ᴅ-glucan, and ᴅ-mannan are capsular polysaccharides. Mycolic acid chains are shown in dark green. LM, lipomannan; ManLAM, mannose-capped lipoarabinomannan; AG, arabinogalactan; PG, peptidoglycan.

acids covalently linked to AG represent about half of the weight of the cell wall core (Draper 1998). Mycolic acids also occur as acyl substituents esterifying a variety of outer membrane (glyco)lipids including trehalose mono- and dimycolates (Fig. 2), glycerol monomycolate, and glucose monomycolate. They play key roles in the permeability of the cell envelope, the ability of *Mtb* to form biofilms (Ojha et al. 2005, 2010; Sambandan et al. 2013), and the pathogenicity of the bacterium during the actively replicating and persistent stages of the infection (Barry et al. 1998; Daffé and Draper 1998; Yuan et al. 1998; Dubnau et al. 2000; Glickman et al. 2000; Sugawara et al. 2002; Vander Beken et al. 2011).

Inner and Outer Membrane Extractable Lipids

Glycerophospholipids, PIM, LM, and ManLAM. Glycerophospholipids (phosphatidylinositol [PI], phosphatidylglycerol, phosphatidylserine [PS], phosphatidylethanolamine [PE], cardiolipin [CL]), and mannosylated forms of PI known collectively as PIMs (Fig. 2) are the major lipid constituents of the plasma membrane of *Mtb*. In addition, the plasma membrane of *Mtb* contains major lipoglycans known as lipomannan (LM) and ManLAM (Fig. 3) (Pitarque et al. 2008), and a number of isoprenoids that play various critical functions in the cell. Among them, decaprenyl phosphate (Dec-P)
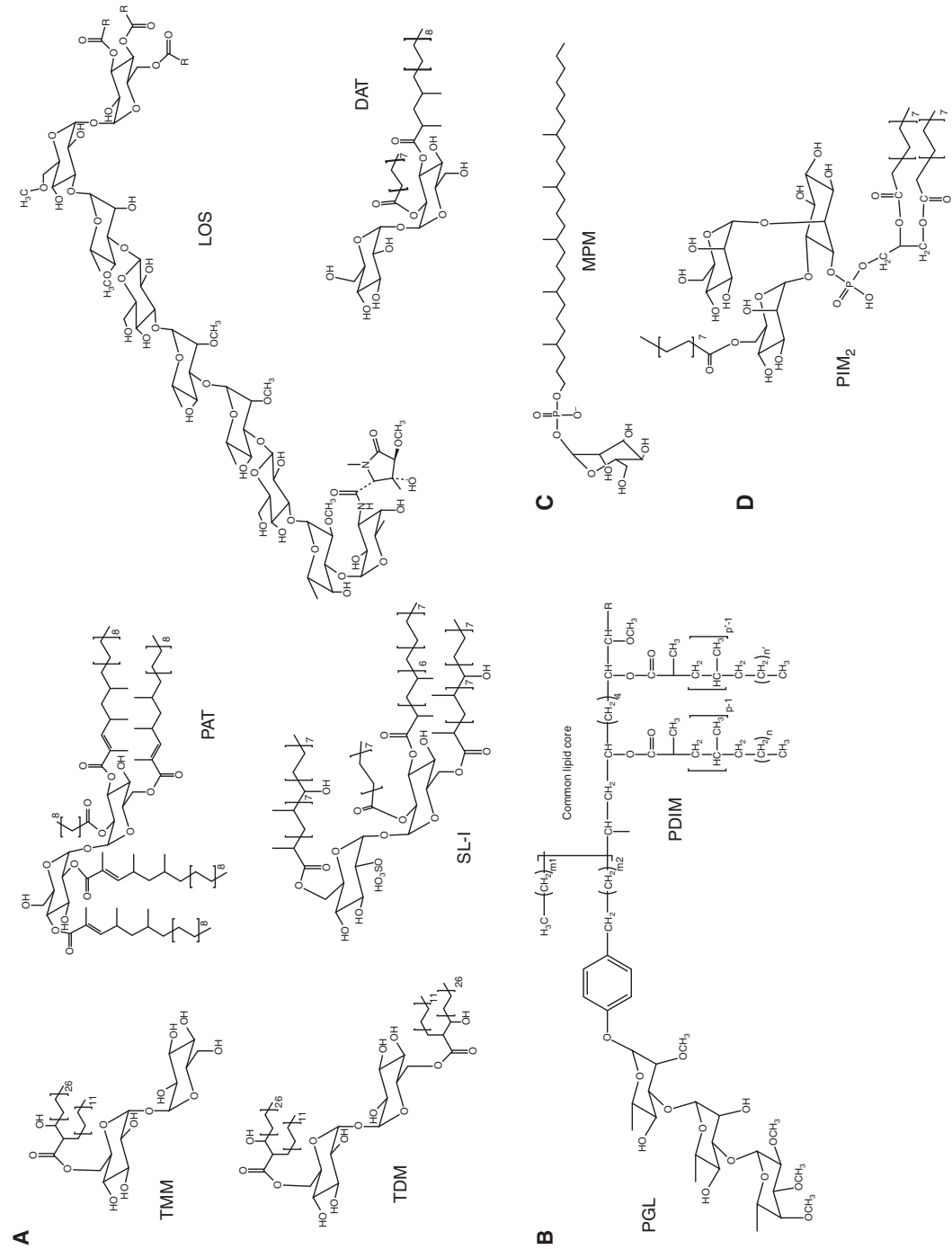
Figure 2. Structures of *Mtb* lipids. (*Legend continues on following page.*)
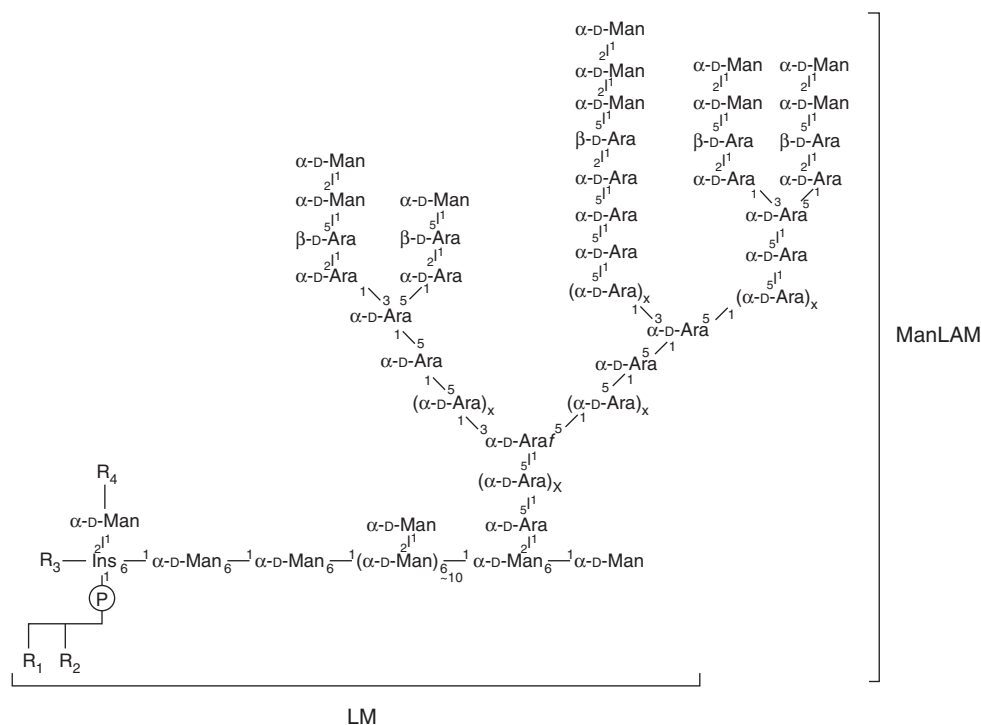
Figure 3. Structures of *Mtb* lipoglycans. Mannose-capped lipoarabinomannan (ManLAM); the mannan moiety of ManLAM consists of ∼20–30 Man*p* residues and the arabinan polymers of ∼60 Ara*f* units; the precise number of arabinan chain(s) attached to mannan is still uncertain. Lipomannan (LM) is devoid of the arabinan chains of ManLAM. R_1, R_2, R_3, R_4 are tuberculostearic acid, oleic acid, or palmitic acid.

serves as a carrier of activated sugars in the synthesis of essential cell envelope polymers including PG, AG, LM, and ManLAM (Kaur et al. 2009), and menaquinones are essential components of the respiratory chain (Weinstein et al. 2005; Dhiman et al. 2009). Thus, as in other bacteria, plasma membrane lipids play a vital role in *Mtb*, providing a selective permeability barrier around the cell and participating

in a number of critical physiological processes, including electron transport, ATP formation, DNA replication, and the biosynthesis of a variety of cell envelope components.

Glycerophospholipids (particularly PE), PIM, LM, and ManLAM are also found in the outermost layers of the cell envelope of *Mtb* and other *Mycobacterium* species where they may interact with the host (Ortalo-Magné et al.

Figure 2. (*Continued*) (*A*) Structures of acyltrehaloses: trehalose monomycolate (TMM); trehalose dimycolate (TDM); sulfolipid (SL-I); diacyltrehalose (DAT); polyacyltrehalose (PAT); and the lipooligosaccharide (LOS) of *Mtb* Canettii (R=Ac). The major sulfolipid SL-I (2,3,6,6′-tetraacyl α-α′-trehalose-2′-sulfate) is represented. In SL-I, trehalose is sulfated at the 2′ position and esterified with palmitic acid and the multimethyl-branched phthioceranic and hydroxyphthioceranic acids. In DAT (2,3-di-*O*-acyltrehalose), trehalose is esterified with palmitic acid and the multimethyl-branched mycosanoic acid. In PAT, trehalose is esterified with palmitic acid and the multimethyl-branched mycolipenic acids. (*B*) Structures of the phthiocerol dimycocerosate (PDIM) and phenolic glycolipid (PGL) of *Mtb*. p, p′ = 3–5; n, n′ = 16–18; m1 = 20–22; m2 = 15–17; R = CH$_2$-CH$_3$ or CH$_3$. (*C*) Structure of the predominant mannosyl-β-1-phosphomycoketide (MPM) from *Mtb* H37Rv. (*D*) Structure of a triacylated phosphatidylinositol dimannoside (PIM$_2$), one of the major forms of PIMs produced by *Mtb*.

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Table 1. TB drug discovery pipeline showing the inhibitors targeting lipid biosynthesis

Pathway targeted	(Proposed) target protein	Inhibitor		
		Discovery	Preclinical development	Clinical development
Mycolic acids				
	InhA	CD117 (Vilchèze et al. 2011) Pyridomycin (Hartkoorn et al. 2012) Triclosan derivatives (Pan and Tonge 2012; North et al. 2013) Novel structural classes (GlaxoSmithKline)		
	HadABC	ISO and TAC analogs (Phetsuksiri et al. 1999; Bhowruth et al. 2006; Alahari et al. 2007; Shahab et al. 2010; Coxon et al. 2013) NAS-21, NAS-91 and analogs (Bhowruth et al. 2008; Gratraud et al. 2008)		
	KasA/KasB	Thiolactomycin and analogs (Pan and Tonge 2012; North et al. 2013)		
	Pks13	Thiophene compounds; Compound 3′ (Ioerger et al. 2013; Wilson et al. 2013)		
	FadD32	4,6-diaryl-5,7-dimethyl coumarins (Stanley et al. 2013)		
	Antigen 85 A, B, and C	Ebselen (Favrot et al. 2013) I3-AG85 (Warrier et al. 2012)		
	MmpL3	Adamantyl ureas (Grzegorzewicz et al. 2012a) BM212 and analogs (La Rosa et al. 2012; Poce et al. 2013) C215 (Stanley et al. 2012) SPIRO2 and THPP1 (Remuinan et al. 2013) Compound 2 (Ioerger et al. 2013)		SQ109 (Tahlan et al. 2012) (Sequella)
	Unknown (Inhibition of oxygenated mycolic acid biosynthesis)			PA-824 (Stover et al. 2000) OPC-67683 (Matsumoto et al. 2006)

Continued

Table 1. *Continued*

Pathway targeted	(Proposed) target protein	Inhibitor		
		Discovery	Preclinical development	Clinical development
	EthR	ETH "boosters": BDM 31343 and analogs (Willand et al. 2009; Villemagne et al. 2012)		
Decaprenyl phosphate				
	IspC	Fosfomidomycin analogs (Obiol-Pardo et al. 2011; Uh et al. 2011)		
Isoprenoid-based biosynthetic precursors				
PG biosynthesis	MraY	Capuramycin analogs (Sequella)	SQ641 (lead capuramycin analog) (Sequella)	
PG biosynthesis	WecA	Caprazamycin derivatives (CPZEN-45) (Ishizaki et al. 2013)		
LAM (and AG) biosynthesis	DprE1	DNB1 (Christophe et al. 2009) VI-9376 (Magnet et al. 2010) 377790 (Stanley et al. 2012) TCA-1 (Wang et al. 2013) Several synthetic compounds (TB Alliance, Scripps)	Benzothiazinones (BTZ043) (Makarov et al. 2009)	
Menaquinones				
	MenA	Aurachin RE, Ro 48-8071 and analogs (Dhiman et al. 2009; Debnath et al. 2012)		
	MenB	1,4-benzoxazines; 4-oxo-4-phenylbut-2-enoates (Li et al. 2010; 2011)		
	MenE	Sulfonyladenosine analogs (Lu et al. 2008; 2012)		
PGL				
	FadD22	5'-O-[*N*-(4-hydroxybenzoyl)sulfamoyl]-adenosine (Ferreras et al. 2008)		
Ser/Thr kinases				
	PknA PknB PknG	Several chemical scaffolds (Vertex Pharmaceuticals Inc.) (Magnet et al. 2010; Danilenko et al. 2011; Lougheed et al. 2011; Chapman et al. 2012)		

See text and the Working Group for New Drugs, Stop TB Partnership (http://www.newtbdrugs.org) for details.

1996; Pitarque et al. 2008; Dhiman et al. 2011). Whether *Mtb* requires phosphatidylglycerol, CL, PS, and PE for growth is at present not known. PI, PIM, LM, and ManLAM, in contrast, are all required for the viability of *Mtb* in vitro (Kaur et al. 2009; Guerin et al. 2010). Emerging data indicate that PIM play important roles in the permeability of the cell envelope, inner membrane integrity, and regulation of cell septation and division (Parish et al. 1997; Kordu-láková et al. 2002; Patterson et al. 2003; Morita et al. 2005, 2006). Likewise, structural defects in LM and ManLAM were shown to affect the β-lactam susceptibility and acid-fast staining properties of mycobacterial cells, indicating an equally important role of these molecules in cell envelope integrity (Fukuda et al. 2013).

Trehalose esters, PDIM, and PGL. Other outer membrane/surface-exposed lipids include trehalose monomycolates (TMM) and trehalose dimycolates (TDM), PDIM, sulfolipids (SL), diacyltrehaloses (DAT), polyacyltrehaloses (PAT), phenolic glycolipids (PGL), and lipooligosaccharides (LOS) (Fig. 2). In addition to serving as a mycolic acid donor in the formation of TDM and the cell wall core, TMM was recently identified as the likely form under which mycolic acids, which are synthesized in the cytoplasm, translocate to the periplasm or outer membrane (Grzegorzewicz et al. 2012a). Consistently, compounds that prevent TMM translocation are potent inhibitors of *Mtb* (Grzegorzewicz et al. 2012a; Tahlan et al. 2012; Stanley et al. 2012; La Rosa et al. 2012; Remuinan et al. 2013). In contrast to TDM and TMM, which are considered essential lipid components of the cell envelope of all mycobacteria, SL, DAT, PAT, and PDIM are more restricted in their distribution and are not essential for the growth of *Mtb* in vitro, although they have been involved in pathogenicity (see the section Roles of *Mtb* Lipids in Pathogenicity). PDIM and closely related phthiocerol diphthioceranates are essentially confined to pathogenic *Mycobacterium* species (Daffé and Lanéelle 1988). Consistent with their important roles in the permeability of the cell envelope (Camacho et al. 2001) and pathogenicity, PDIMs are apparently produced by all virulent clinical isolates of *Mtb*. DAT and

PAT are confined to virulent isolates of the *Mycobacterium tuberculosis* complex (Goren and Brennan 1979; Jackson et al. 2007), and SL are exclusively found in the human pathogen *Mtb* (Goren 1990). Structural similarities between SL, DAT, and PAT and, to some extent PDIM, particularly the fact that they all are esterified with long-chain polymethyl-branched fatty acids, is suggestive of at least partially redundant functions in the cells. One of them is to alleviate the propionate-mediated stress undergone by *Mtb* when the bacterium switches to host cholesterol and fatty acids as major carbon sources (Singh et al. 2009; Lee et al. 2013). The propionyl-CoA generated upon β-oxidation of cholesterol is converted to methylmalonyl-CoA by the propionyl-CoA carboxylase and then used by dedicated polyketide synthases in the elongation of the polymethyl-branched fatty acids esterifying PDIM, SL, DAT, and PAT. Independent from this metabolic function and suggestive of their collective role in the permeability of the cell envelope, *Mtb* mutants deficient in the synthesis of more than one polymethyl-branched fatty acid–containing lipid (either PDIM and SL or DAT, PAT, and SL) but not mutants lacking only one lipid species, lose the ability to stain with neutral red (Cardona et al. 2006). Finally, DAT and PAT have been involved in the retention of the capsular material at the surface of *Mtb* (Rousseau et al. 2003a).

PGL and LOS are other lipids acylated by polymethyl-branched fatty acids produced by *Mtb* and related tubercle bacilli, albeit not all lineages (Fig. 2). LOS have thus far only been found in *Mycobacterium canettii* (Daffé et al. 1991), a representative of smooth tubercle bacilli that seem to have originated from the same pool of ancestors as *Mtb* but rarely causes human disease (Supply et al. 2013). Based on studies performed in *Mycobacterium marinum* (Ren et al. 2007), the presence of LOS in the cell envelope of *M. canettii* may account for the particular smooth colonial morphotype of this strain. The ability to synthesize PGL, on the other hand, has only been retained by *M. canettii* and some *Mtb* isolates of the East Asian/Beijing lineage (Daffé et al. 1987; Constant et al. 2002; Reed et al. 2004; Huet et al. 2009). PGL have

been involved in pathogenicity (see the section Roles of *Mtb* Lipids in Pathogenicity). In an attempt to correlate the lipid content with the virulence of *Mtb* isolates, Goren et al. characterized a methoxylated phenolphthiocerol (a nonglycosylated variant of PGL), the so-called "attenuation indicator lipid" (Goren et al. 1974a). This lipid and its unmethylated form were detected in East Asian/Beijing isolates and found to accumulate in all Indo-Oceanic *Mtb* strains examined (Krishnan et al. 2011). Similarly, Beijing strains were reported to accumulate variants of PDIM and eventually PGL, known as phthiotriol and glycosylated phenolphthiotriol dimycocerosates (Huet et al. 2009). The correlation between the occurrence of these lipids and variations in virulence remains, however, unclear (Huet et al. 2009; Krishnan et al. 2011). PGL and LOS are clearly not essential for growth but they are potent B-cell antigens making them potentially useful, albeit lineage-specific, serodiagnostics (Brennan 1988; He et al. 1991; Vera-Cabrera et al. 1994; Simonney et al. 1995, 1996; Constant et al. 2002; Julian et al. 2002).

Other Lipids

Another family of lipids known as the mannosyl-β-1-phosphomycoketides (MPM) are produced by slow growing mycobacteria including *Mtb*, *Mycobacterium avium*, and *M. marinum* (Fig. 2). They are found inside the cells and released in the culture medium. They are not essential for the growth of *Mtb* but are thought to be involved in pathogenicity (Matsunaga and Sugita 2012).

Triglycerides (TAG) are considered intracellular lipids of *Mtb*. They represent the main apolar lipid when glycerol serves as the major carbon source in the medium (Brennan 1988). TAGs are thought to serve as an energy reserve for the long-term survival of *Mtb* during the persistence phase of infection (Brennan 1988; Daniel et al. 2004), as well as a means by which free fatty acids are detoxified. *Mtb* Beijing family isolates accumulate large quantities of TAG, possibly as a result of the up-regulation of the TAG synthase, Rv3130c (Reed et al. 2007).

Regulation of Lipid Synthesis

The physiological status of mycobacterial bacilli (i.e., age of the culture, actively replicating vs. persistent) and a number of environmental factors including temperature, oxygen tension, and nitrogen and carbon sources are known to impact their lipid composition, particularly phospholipids, TAG, PIM, LM, ManLAM, mycolic acid–based glycolipids, and polymethyl-branched fatty acid–containing lipids (Brennan 1988; Barry et al. 1998; Daffé and Draper 1998; Singh et al. 2009; Dhiman et al. 2011; Lee et al. 2013; Yang et al. 2013). Less is known of the regulation of lipid synthesis during host infection. PDIM and mycolic acids are apparently produced in abundant quantities by in vivo–grown tubercle bacilli (Kanai et al. 1970; Bhamidi et al. 2012). Yet, variations in the acid-fast characteristics of *Mtb* during mouse and guinea pig infection suggest that the bacterium undergoes drastic adaptive changes of its lipid composition in vivo (Ryan et al. 2010). Increased production of SL and DAT during host infection is supported by the up-regulation of biosynthetic genes on phagocytosis of *Mtb* by human primary macrophages or during progressive pulmonary TB infection in mice (Graham and Clark-Curtiss 1999; Rodriguez et al. 2013). The virulence-associated two-component regulator PhoP-PhoR may be involved in this up-regulation as it has been found to coordinately and positively regulate the synthesis of SL, DAT, and PAT in *Mtb* (Gonzalo-Asencio et al. 2006; Walters et al. 2006). The metabolic switching of *Mtb* to host lipids as carbon sources during infection and the associated increase in the production of PDIM, SL, DAT, and PAT were shown to be facilitated by the regulatory protein WhiB3 (Singh et al. 2009). In recent years, PknA, PknB, and other members of the Ser/Thr kinase family were found to regulate the activity of multiple enzymes and transporters involved in the biosynthesis of mycolic acids, ManLAMs, and PDIMs (Molle and Kremer 2010). It is thus apparent that lipid synthesis in *Mtb* is tightly controlled and that regulation occurs both at the transcriptional and post-translational levels.

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ROLES OF *Mtb* LIPIDS IN PATHOGENICITY

Several excellent reviews have been published about the biological activities of *Mtb* lipids, including very recently (Daffé and Draper 1998; Russell et al. 2002; Karakousis et al. 2004; Bertozzi and Schelle 2008; Britton and Triccas 2008; Gilleron et al. 2008; Glickman 2008; Guilhot et al. 2008; Guenin-Macé et al. 2009; Neyrolles and Guilhot 2011; Russell 2011; Daffé et al. 2014). Thus, only a brief overview of what is known and remains to be investigated about the involvement of lipids in the pathogenesis of TB will be presented here.

The existence of a possible correlation between the lipid content of *Mtb* clinical isolates and their pathogenicity was noted early on by Middlebrook and Goren among others. These studies led to the identification of SL, TDM, and PDIM and derivatives as lipids of potential relevance to TB immunopathogenesis (Bloch 1950; Goren et al. 1974a,b; Daffé and Draper 1998). Further work on the biological activities of purified lipids in vitro and in vivo followed, in the postgenomic era, by an examination of the virulence phenotype and immune responses induced by *Mtb* mutants deficient in various aspects of their biosynthesis lent further support to the involvement of lipids in host–pathogen interactions. Their contribution to the infection process accompanies every step of the life cycle of the bacterium. The entry of *Mtb* inside host macrophages and dendritic cells is essentially mediated by specialized phagocytic receptors including complement receptors, scavenger receptors, and C-type lectins, such as the mannose receptor, DC-SIGN, and Mincle. PIM, TDM, and ManLAM are among the predominant glycolipids mediating *Mtb*'s interactions with these receptors (Villeneuve et al. 2005; Schlesinger et al. 2008; Ishikawa et al. 2009; Neyrolles and Guilhot 2011). Once inside phagocytic cells, *Mtb* resides in a phagosome that fails to fuse with lysozymes. The isoprenoid-derived compounds, isotuberculosinol and tuberculosinol, ManLAM, TDM, PDIM, SL, and DAT all have been implicated in the impairment of phagosome maturation, although the mechanisms involved and amount of support-

ing data varies for each compound (Daffé and Draper 1998; Brodin et al. 2010; Neyrolles and Guilhot 2011; Russell 2011; Mann and Peters 2012). Inside the cells, *Mtb* releases significant amounts of proteins and lipids, among them TMM, TDM, PIM, PGL, PE, phosphatidylglycerol, CL, and ManLAM, that traffic within the cell and may be released through exocytosis (Russell et al. 2002; Russell 2011). These molecules are not only taken up by bystander antigen-presenting cells, they also act as modulators of the function of the host cell and surrounding tissue, impacting the secretion of pro- and anti-inflammatory cytokines (e.g., PGL, TDM, mycolic acids, SL, PIM, LM, ManLAM, and lipoproteins), apoptosis (lipoproteins, PIM, ManLAM, and TDM), T-cell functions (ManLAM, DAT, and PAT), the induction of foamy macrophages (oxygenated mycolates and TDM), and granuloma formation (TMM, TDM, and ManLAM) (Daffé and Draper 1998; Jackson et al. 2007; Gilleron et al. 2008; Peyron et al. 2008; Guenin-Macé et al. 2009; Neyrolles and Guilhot 2011; Rajni et al. 2011; Russell 2011; Vander Beken et al. 2011; Li et al. 2012; Sakamoto et al. 2013; Vir et al. 2013). Importantly, several *Mtb* lipids, including diacylated forms of SL, PIM, ManLAM, MPM, and mycolyl lipids, represent themselves as antigens for immune recognition and are presented to T lymphocytes by MHC-I-like molecules of the CD1 family (Matsunaga and Sugita 2012; Arora et al. 2013; Ly et al. 2013; Van Rhijn et al. 2013). Some of these lipids may show potential as subunit vaccines (Guiard et al. 2009; Arora et al. 2013). Finally, ManLAM, SL, TDM, PGL, LOS, DAT, and lipoproteins are also potent inducers of humoral immune responses.

Although the critical involvement of PDIM and TDM in TB pathogenesis is now supported by a wealth of in vitro and in vivo data (Guilhot et al. 2008; Neyrolles and Guilhot 2011; Rajni et al. 2011; Li et al. 2012), the role(s) played by other prominent (glyco)lipids including (iso)-tuberculosinol, SL, DAT, PAT, PGL, some PDIM variants, and ManLAM during host infection are far from being as clear. Their roles in pathogenicity typically come into question when their demonstrated biological activities in vitro

or in vivo (e.g., inhibition of phagosome maturation and immunomodulatory activities), usually as purified molecules, fail to translate into a clear "virulence" phenotype in *Mtb* strains genetically engineered to produce or, on the contrary, lose the ability to produce them. Classical examples of this include, for instance, SL, whose plethora of biological activities (Goren and Brennan 1979; Goren 1990; Daffé and Draper 1998; Bertozzi and Schelle 2008) fail to translate into a clear replication or persistence advantage in infected mice or guinea pigs (Rousseau et al. 2003b); DAT and PAT, whose absence from *Mtb* H37Rv does not hamper the ability of this strain to grow in mice (Rousseau et al. 2003a); or Man-LAM, the suppression of the bioactive mannose caps of which fails to translate into any detectable phenotype in macrophages and animal models of infection (Afonso-Barroso et al. 2012). A combination of factors may account for this. First, no pathogenic trait of *Mtb* is attributable to a single virulence factor and so compensatory mechanisms within certain lipid families (e.g., polymethyl-branched fatty acid–containing lipids) or cell ligands (e.g., Man-LAMs and mannosylated glycoproteins) are known to exist (Pitarque et al. 2005; Passemar et al. 2014). Second, an inherent flaw associated with studies that use purified molecules is that they may not accurately mimic qualitatively and/or quantitatively the way these molecules are presented to the host when carried by whole bacilli. Furthermore, the genetic background of the *Mtb* strain in which they are produced is likely to affect the way they interact with the host and their subsequent contribution to pathogenicity (e.g., PGL) (Sinsimer et al. 2008). Third, lipids such as SL and (iso)tuberculosinol are restricted to the human pathogen, *Mtb*, and may only show a clear phenotype in this specific host. The facts that the ability of *Mtb* to produce some PDIM variants and that PGL is relatively restricted to East-Asian isolates (Constant et al. 2002; Reed et al. 2004; Huet et al. 2009) suggest that the different *Mtb* lineages may even have evolved regionally to tailor their lipid composition to the genetic background of their human host (Neyrolles and Guilhot 2011). From a mechanistic standpoint, concluding to the di-

rect effect of a lipid on a specific host function can be challenging. This owes to the roles that lipids often play in the structure and permeability of the cell envelope in addition to potentially interacting with the host. Thus, a deficiency in their production may not only cause *Mtb* to become more susceptible (i.e., more permeable) to host defense mechanisms (e.g., PDIMs and their impact on the resistance of *Mtb* to oxidative stress) (Rousseau et al. 2004), it may also profoundly alter the way other immunomodulatory antigens are presented at the cell surface and interact with host functions.

Mtb LIPIDS IN THE CONTEXT OF DRUG DISCOVERY

General Considerations

The previous sections have highlighted the diversity, unique structures, and essential roles played by cell envelope lipids in the physiology and pathogenicity of *Mtb*. These features account for the specificity and therapeutic efficacy of first- and second-line TB drugs such as INH and ETH, which inhibit the biosynthesis of mycolic acids (Banerjee et al. 1994; Vilchèze and Jacobs 2007; Grzegorzewicz et al. 2012b) (Fig. 4), and those of ethambutol (EMB), which inhibits the formation of the arabinan domains of ManLAM (Fig. 3) and AG (Mikušová et al. 1995). In addition, pyrazinamide was reported to be an inhibitor of the fatty acid synthase I (FAS-I) (Zimhony et al. 2000). With multidrug resistance on the increase, recent years have seen a marked intensification of TB drug discovery efforts with the result that many new lead compounds are now at various stages of the drug discovery and preclinical development pipeline. Interestingly, these efforts keep pointing at cell envelope lipids as one of the Achille's heels of *Mtb* (Table 1) (Jackson et al. 2013). As importantly, they are leading the TB field to revisit earlier impressions that drug targeting the biogenesis of the cell envelope lipids may not be synergistic with other drugs, useful against MRD-*Mtb* isolates, or active against persistent bacilli. This section summarizes recent developments that have occurred in the discovery of drugs targeting lipid synthesis in *Mtb*.
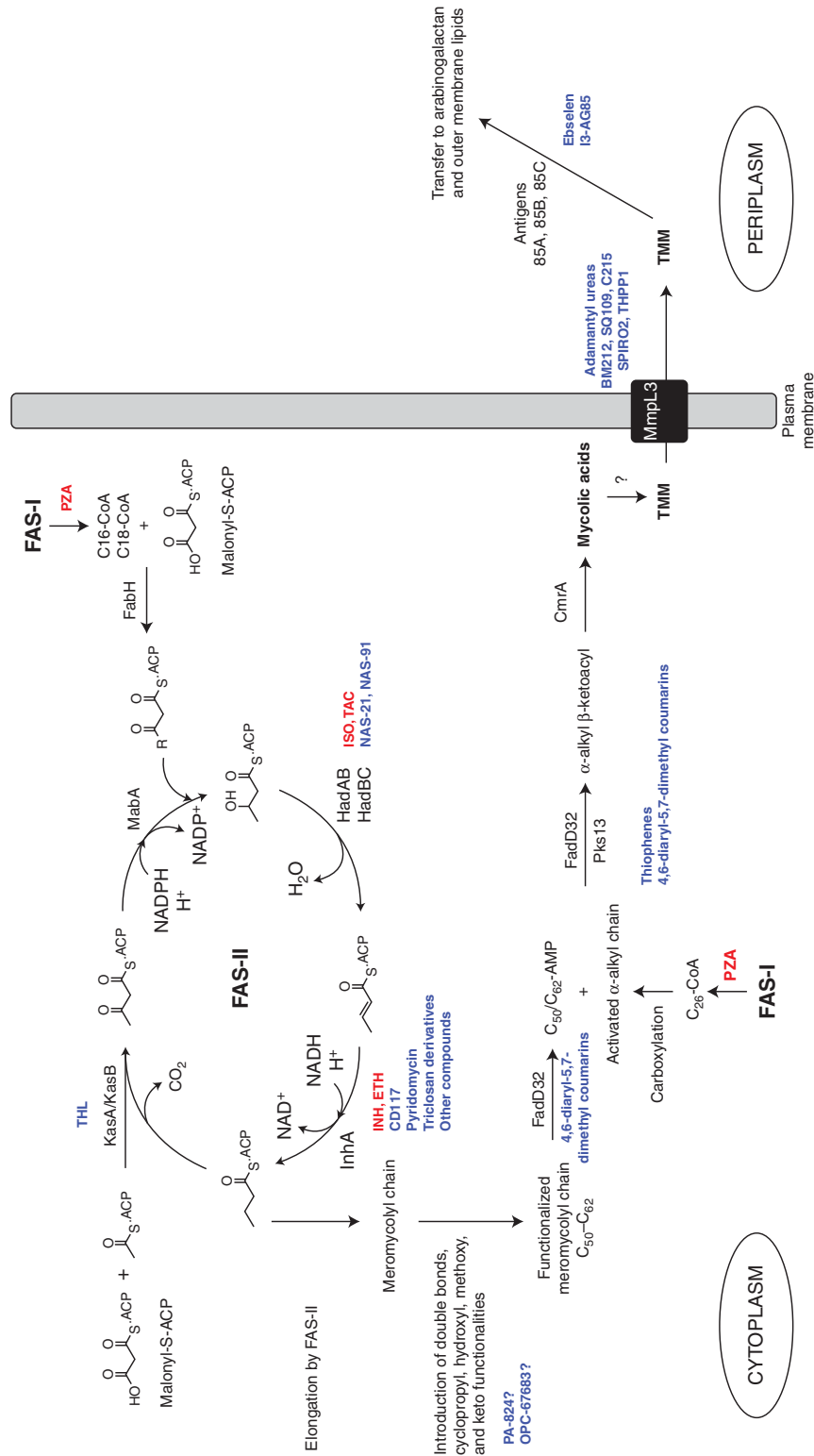
Figure 4. Biosynthetic pathway of mycolic acids in *Mtb* and site of action of anti-TB drugs. The saturated α-alkyl chain (C_{24} or C_{26} in *Mtb*) and the meromycoloyl chain (C_{50}–C_{62}) are synthesized by fatty-acid synthase-I (FAS-I) and fatty-acid synthase-II (FAS-II), respectively. The initial substrates of FAS-II are medium length keto-acyl-ACP resulting from the condensation by the *Mtb* FabH protein of the acyl-CoA products of FAS-I with malonyl-ACP. (*Legend continues on following page.*)

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Drugs Targeting Mycolic Acid Biosynthesis and Export

The biosynthesis of mycolic acids is shown schematically in Figure 4. In the past 10 years, both target-based and whole cell–based approaches have been used toward the identification and development of inhibitors acting on virtually all portions of this biosynthetic pathway (Fig. 4 and Table 1). A comprehensive review of these efforts was recently published by North et al. (2013), and we will here only briefly describe some of the most exciting developments that have occurred in the last few years. Target-based screening uses biochemical and structural approaches to design, screen, and further evaluate a compound's activity against a specific molecular target. This type of approach has been used extensively to rationally optimize inhibitors of the FAS-II elongation cycle—in particular, analogs of thiolactomycin as inhibitors of KasA/KasB and triclosan derivatives as inhibitors of InhA (Pan and Tonge 2012). The screening of inhibitors showing activity against the *Plasmodium falciparum* enoyl reductase and β-hydroxyacyl-ACP dehydratase, FabZ, identified compounds that appear to target the corresponding FAS-II enzymes in *Mtb* and display an MIC against whole mycobacterial cells (Bhowruth et al. 2008; Gratraud et al. 2008; Vilchèze et al. 2011). Pyridomycin, a cyclic depsipeptide isolated from *Streptomyces* with an MIC value of 0.4 μg/mL against *Mtb*, is now known to also target InhA (Haatkoorn et al. 2012), as are novel structural classes of inhibitors in the lead-optimization phase reported by the Working Group on New TB Drugs (Table

1). Incidentally, the recent elucidation of the mode of action of isoxyl (ISO) and thiacetazone (TAC), two prodrugs formerly used in the clinical treatment of *Mtb*, revealed that they both inhibit the dehydration step of the FAS-II cycle catalyzed by HadAB and HadBC (Grzegorzewicz et al. 2012b). The finding that relatively simple structural modifications of the parent ISO and TAC compounds can increase the potency of analogs more than 20-fold is encouraging in that it suggests that it may be possible to identify compounds that could be administered at a lower dose, thereby reducing side effects (Phetsuksiri et al. 1999; Bhowruth et al. 2006; Alahari et al. 2007; Shahab et al. 2010; Coxon et al. 2013).

Because of their pivotal roles in the final assembly of mycolic acids and their transfer to cell envelope acceptors, Pks13, FadD32, and the mycoloyltransferases of the antigen 85 complex are also receiving a lot of attention from the perspective of drug development (Fig. 4). High-throughput screening assays were reported for FadD32 (Galandrin et al. 2013) and the antigens 85 (Boucau et al. 2009; Elamin et al. 2009; Sanki et al. 2009; Favrot et al. 2013). The latter assays led to the identification of a number of inhibitors of antigens 85, one of them, known as ebselen, also shows activity against *Mtb* in culture (MIC of 20 μg/mL). The molecular mechanism of inhibition of antigens 85 by ebselen was elucidated and found to be particularly interesting in the sense that it is unlikely to lead to the selection of drug-resistant isolates (Favrot et al. 2013). Small molecule binders of antigen 85C identified by magnetic resonance spectroscopy also showed activity against *Mtb* in

Figure 4. (*Continued*) After reduction by the β-keto-acyl-ACP reductase MabA, dehydration by the (*3R*)-hydroxyl-dehydratases HadAB and HadBC and reduction by the enoyl-CoA reductase InhA, either the β-ketoacyl-ACP synthase KasA or KasB catalyzes the condensation of the resulting product with malonyl-ACP units, thereby initiating the next round of elongation. Methyltransferases and other as yet unknown enzymes modify the meromycolyl chain introducing double bonds, cyclopropyl, hydroxy, methoxy, and keto functionalities. These two chains are coupled together via Claisen condensation by the acyl-AMP ligase FadD32 and the polyketide synthase Pks13. Upon release from Pks13, reduction by CmrA yields mycolic acids, which are then translocated to the periplasm under the form of TMM by partially elucidated translocation machinery involving the essential integral membrane transporter, MmpL3, and attached to AG or another molecule of TMM to form TDM, by the antigen 85 complex. The sites of action of *Mtb* inhibitors are indicated. Drugs presently or formerly used in the clinical treatment of TB are in red; inhibitors under development are in blue (see Table 1). THL, thiolactomycin; PZA, pyrazinamide.

vitro and inside macrophages in the 100-μM range (Warrier et al. 2012). The return of interest in whole cell–based screening that the TB field has witnessed in recent years has led to the identification of thiophenes and other compounds active against Pks13 (Ioerger et al. 2013; Wilson et al. 2013), diaryl coumarin–based compounds active against FadD32 (Stanley et al. 2013), and diverse chemotypes active against the TMM transporter, MmpL3 (Grzegorzewicz et al. 2012a; Stanley et al. 2012; Ioerger et al. 2013; Remuinan et al. 2013). BM212 and derivatives (La Rosa et al. 2012; Poce et al. 2013) and SQ109, a drug candidate originally designed to be an EMB analog and currently undergoing phase II clinical trials (Sacksteder et al. 2012; Tahlan et al. 2012), were also shown to kill *Mtb* through the inhibition of MmpL3. The reason why so many different chemical scaffolds apparently inhibit the MmpL3-mediated translocation of TMM is unclear. Establishing a validated assay for the direct measurement of MmpL3 inhibition is a high priority, because it would greatly facilitate the further development of these inhibitors and could potentially reveal new scaffolds for development.

In summary, recent drug discovery efforts have yielded a number of chemotypes inhibiting various aspects of the synthesis of mycolic acid, some of which are promising candidates for further development. Inhibiting the FAS-II system and MmpL3 currently appear to be the most attractive/druggable targets for TB drug discovery in this pathway.

Isoprenoids and Related Lipids

Inhibitors of the MEP pathway. As a lipid carrier of activated saccharide subunits, polyisoprenyl phosphate (Dec-P) is involved in the biosynthesis of the arabinan portion of AG, the mannan and arabinan domains of arabinomannan, LM, and ManLAM, the lipid I and lipid II precursors of PG, and the biosynthesis of the "linker unit" between AG and PG (Kaur et al. 2009; Daffé et al. 2014) (Fig. 1). The discovery that Dec-P synthesis in *Mtb* proceeds through the methylerythritol phosphate pathway, which has no homolog in humans, has provided stimulus for the charac-

terization and identification of inhibitors of the relevant enzymes. Fosmidomycin, which is currently in clinical studies for the treatment of malaria, is a competitive inhibitor of the second enzyme of the pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (IspC). Several derivatives of this antibiotic have been synthesized, some of which have shown promising results against *Mtb* (Jackson et al. 2013) (Table 1).

Inhibitors of lipid-linked biosynthetic precursors. The phospho-*N*-acetylmuramyl pentapeptide translocase (MraY) catalyzes the exchange of UDP-N-acetylmuramic acid pentapeptide from UDP to Dec-P thereby generating lipid I in PG biosynthesis. The synthesis of capuramycin analogs as inhibitors of this enzyme has led to the identification of several compounds with 2- to 4-μg/mL MICs against drug-susceptible and MDR *Mtb* isolates (Jackson et al. 2013). Although its mode of action has yet to be confirmed in whole *Mtb* cells, one of them, SQ641, is at the stage of preclinical development (Table 1).

Very recently, the caprazamycin derivative CPZEN-45 was shown to be an inhibitor of *Mtb*'s decaprenyl-phosphate-GlcNAc-1-phosphate transferase, WecA, in the biosynthesis of AG (Ishizaki et al. 2013).

Decaprenylphosphoryl arabinose (DPA) is the only known arabinose donor in the building of the arabinan domains of the two essential cell envelope (lipo)polysaccharides, AG, and ManLAM. In the last 4 years, whole cell–based screening of compounds against *Mtb* has produced several inhibitors of the essential epimerase DprE1 required for the formation of DPA (Table 1). The molecular mechanism of action of some of these compounds has been thoroughly investigated and several DPA inhibitors are now reported to be in the hit-to-lead or preclinical development phases (Table 1) (Jackson et al. 2013).

Menaquinones. The lipoquinones involved in the respiratory chains of mycobacteria consist of menaquinones (2-methyl-3-polyprenyl-1,4-naphthoquinones) (Daffé et al. 2014). Evidence based on transcriptomics profiling and the inhibitory activity of menaquinone inhibitors against *Mtb* in culture has highlighted the requirement of this bacterium for electron trans-

port and, therefore, menaquinone synthesis both during active replication and microaerophilic persistence (Dhiman et al. 2009). Accordingly, three key enzymes of the menaquinone biosynthetic pathway of *Mtb*, MenB (1,4-dihydroxy-2-napthoic acid synthase), MenE (O-succinylbenzoyl-CoA synthase), and MenA (1,4-dihydroxy-2-napthoic acid octaprenyl transferase) have been studied as potential drug targets and a number of promising inhibitors synthesized (Table 1).

Inhibition of PIM, LM, and ManLAM Biosynthesis

The essential character of PIM, LM, and ManLAM, their restricted distribution to mycobacteria and closely related Actinomycetes, and their demonstrated impact on the structure and permeability of the cell envelope of *Mtb* make the biosynthetic enzymes of these molecules attractive candidates for the development of specific *Mtb* inhibitors with the potential to synergize with or potentiate the activity of other drugs used in combination. Accordingly, target-to-drug approaches are pursuing various essential enzymes acting both at early (e.g., PimA, PimB') and late (e.g., Emb proteins and other lipid-linked sugar-utilizing glycosyltransferases) stages of the pathway. Innovative high-throughput screening assays have been developed for some of these enzymes (Zhang et al. 2010, 2011). The most advanced ManLAM inhibitors to date are those targeting DPA synthesis, as described in the previous section.

Targeting Lipids Involved in the Replication and Persistence of *Mtb* In Vivo

Targeting TAG synthesis through inhibition of the primary TAG synthase of *Mtb*, Rv3130c (Daniel et al. 2004; Sirakova et al. 2006), may represent a viable approach to the eradication of persistent bacilli for the key role TAG is likely to play as an energy reserve for the long-term survival of *Mtb* during the persistence phase of infection.

As detailed in the previous section, PDIMs are critical components of the permeability bar-

rier surrounding *Mtb* and contribute to a significant extent to the survival and ability of the bacterium to replicate in vivo. To the best of our knowledge, no specific PDIM inhibitors have yet been reported but promising compounds targeting the Ser/Thr kinases of *Mtb* that regulate their biosynthesis (among other physiological processes) are under development (Jackson et al. 2013) (Table 1). Another regulatory system of interest in the context of drug development is the two-component transcriptional regulator PhoP-PhoR, which regulates multiple virulence-associated processes in *Mtb*, including the biosynthesis of SL, DAT, and PAT (Gonzalo-Asencio et al. 2006). Finally, a compound inhibiting the production of PGL in whole *Mtb* cells has been reported (Ferreras et al. 2008).

CONCLUDING REMARKS

Mtb lipids have been for the last eight decades and still remain today the object of much research activity. One of the highlights of this research in the last five years is undoubtedly the discovery of a variety of novel chemical scaffolds inhibiting various aspects of lipid synthesis, particularly mycolic acids, ManLAM, and AG. Beyond their therapeutic applications, lipids involved in the immunopathogenesis of TB may also represent useful targets for future preventive interventions, as illustrated by current studies aimed at investigating the vaccine potential of CD1-restricted lipid antigens (G Puzo, pers. comm.) and that of an attenuated *phoP*-based *Mtb* knockout mutant (Nambiar et al. 2012). Although the interest in developing TB serodiagnostics based on *Mtb* lipids seems to have somewhat waned in the last decade, important efforts are currently focusing on their potential as biomarkers to monitor TB reactivation, as well as the efficacy of treatment and vaccination (Shui et al. 2012; Wallis et al. 2013; Chan et al. 2013).

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