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The *Mycobacterium tuberculosis* MEP (2C-methyl-D-erythritol 4-phosphate) pathway as a new drug target

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Abstract

Tuberculosis (TB) is still a major public health problem, compounded by the human immunodeficiency virus (HIV)-TB co-infection and recent emergence of multidrug-resistant (MDR) and extensive drug resistant (XDR)-TB. Novel anti-TB drugs are urgently required. In this context, the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway of *Mycobacterium tuberculosis* has drawn attention; it is one of several pathways vital for *M. tuberculosis* viability and the human host lacks homologous enzymes. Thus, the MEP pathway promises bacterium-specific drug targets and the potential for identification of lead compounds unencumbered by target-based toxicity. Indeed, fosmidomycin is now known to inhibit the second step in the MEP pathway. This review describes the cardinal features of the main enzymes of the MEP pathway in *M. tuberculosis* and how these can be manipulated in high throughput screening campaigns in the search for new anti-infectives against TB.

Keywords

Tuberculosis; 2C-methyl-D-erythritol 4-phosphate pathway; high throughput screening campaigns; anti-infectives

Requirements of novel anti-tuberculosis drugs

The most recent estimates of global tuberculosis (TB) incidence show 8 to 9 million new cases annually and 1.6 million human deaths^{1, 2} and the incidence continues to increase specifically in developing countries, such as in Africa, whereas it is stable or falling in other regions.² The continuing high level of TB risk is in large part attributable to emergence of the human immunodeficiency virus-1 (HIV) pandemic and the development of drug resistant strains. The prevalence of HIV ensures that TB is the second-most prevalent cause of human deaths due to an infectious disease.³ Approximately 10% of infected persons will live with active TB at some point during their lives.⁴ HIV infections of individuals with latent TB accelerate to develop active TB at a rate of 7 to 10% per year, compared to approximately 10% per lifetime for HIV-negative individuals.^{5, 6}

Current conventional directly observed treatment short course (DOTS) therapy is a multiple drug regimen given over a long duration of time, which combines isoniazid (INH), rifampin

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(RIF), pyrazinamide (PZA), and ethambutol (EMB) for 2 months, followed by INH and RIF for 4 months, has been used for decades.^{7, 8} Single drug therapy is now known to result in the rapid emergence of drug resistant *M. tuberculosis* bacilli,⁹ due to the sequential accumulation of spontaneous genetic mutations.^{10–12} Although the DOTS multidrug regimen strategy has contributed to the falling incidence of TB including TB and HIV co-infection,¹³ incomplete implementation of the campaign has been a major cause of the high incidence of drug resistant strains of *M. tuberculosis*.¹⁴ Multidrug-resistant TB (MDR-TB), defined as an isolate that is resistant to at least INH and RIF,^{15, 16} is a phenomenon that is threatening to destabilize TB control. This problem is exacerbated by the recent identification of extensive drug resistant TB (XDR-TB), defined as an isolate showing resistance to INH and RIF and three or more of the six classes of second-line drugs.¹⁷ MDR-TB, or XDR-TB, is much more difficult to treat than sensitive TB, requiring administration of more expensive and less effective second-line antibiotics for up to two years. Consequently, combinatorial treatment with more than one antibiotic is now standard in order to prevent the occurrence of drug resistant strains of *M. tuberculosis*. It is of fundamental importance to enhance TB control infrastructure through development of new generations of anti-TB agents. Recently, many novel inhibitors such as those for ATP synthase,^{18, 19} cell wall assembly,²⁰ isocitrate lyase,²¹ and protein synthesis,²² have emerged as anti-TB targets. In addition, the early steps of isoprenoid biosynthesis in *M. tuberculosis* are considered as ideal targets for new drug development, and are being intensively investigated in this context.

Isoprenoids in *M. tuberculosis*

Isoprenoids are the largest known group of natural products, encompassing over 35,000 known compounds including essential primary metabolites as well as secondary metabolites.²³ Representative examples of isoprenoid structures are listed in Fig. 1. A wide variety of monoterpenes and diterpenes belong to isoprenoid classes, which function as toxins, growth inhibitors, or other secondary metabolites.^{24, 25} However, all known organisms also synthesize isoprenoids that are essential for survival.²⁶

A number of isoprenoids have been observed and characterized in *M. tuberculosis*, including polyprenyl phosphate (Pol-P), the prenyl side-chain of menaquinone, and various forms of carotenoids. Polyprenyl phosphate (Fig. 1), which is involved in the biosynthesis of the arabinan portion of arabinogalactan, arabinomannan, and lipoarabinomannan,²⁷ and lipid I and lipid II of peptidoglycan biosynthesis^{28, 29} is a lipid carrier of the activated sugars. Pol-P occurs in the form of undecaprenyl phosphate (C₅₅-P) in many bacteria but the unusual form of Pol-P in *M. tuberculosis* is decaprenyl phosphate (C₅₀-P).^{20, 28} *M. tuberculosis* C₅₀-P is also involved in the biosynthesis of the “linker unit” between two essential cell wall components, arabinogalactan and peptidoglycan.³⁰ It has been reported that the rate of bacterial cell wall biosynthesis in *Staphylococcus aureus* and *Bacillus subtilis* is determined by the level of Pol-P^{29, 31} and this may also be true in *M. tuberculosis*. Several reports have shown that mycobacteria are sensitive to bacitracin.^{32, 33} This antibiotic specifically binds to polyprenyl diphosphate (Pol-PP) by which it blocks formation of Pol-P through dephosphorylation and hence biosynthesis of peptidoglycan.³⁴ Another antibiotic, amphotycin also inhibits mycobacterial growth (unpublished data) presumably inhibiting the synthesis of the Pol-P-glucose, a crucial carbohydrate donor for *M. tuberculosis* cell wall synthesis,^{30, 35} by binding to various forms of Pol-P.³⁶ These observations support the principle that Pol-P, one of the major isoprenoids of *M. tuberculosis* plays a critical role in cell wall biosynthesis.

Another crucial isoprenoid appears in the form of the prenyl side-chain of menaquinone (2-methyl-3-polyprenyl-1,4-naphthoquinone) (Fig. 1), the only lipoquinone found in the *M. tuberculosis* electron transport chain. Menaquinones having nine isoprene units with one

hydrogenated are the major form found in mycobacteria.³⁷ Two components of the electron transport chain, NADH type II dehydrogenase and F₁F₀-ATP synthase have recently been reported as effective drug targets and inhibitors of these enzymes are able to kill MDR-TB under *in vitro* and *in vivo* conditions.^{18, 38} Moreover, the bacterial sterilizing effect of PZA is due to interference in the maintenance of the mycobacterial transmembrane proton gradient,³⁹ resulting in depletion of energy. These results and the observation that mycobacteria require the F₁F₀-ATPase to grow on fermentable and non-fermentable carbon sources,⁴⁰ implying that ATP generation from substrate level phosphorylation is insufficient for survival of the bacteria.^{39, 40} Thus, inhibition of menaquinone synthesis may disrupt electron transport leading to depletion of ATP production in active or persistent *M. tuberculosis* bacilli.

The 2C-methyl-D-erythritol 4-phosphate pathway of *M. tuberculosis*

All isoprenoids are derived from the repetitive condensation of two important precursors, isopentenyl diphosphate (IPP) and dimethyl diphosphate (DMAPP),⁴¹ catalyzed by prenyl diphosphate synthases.⁴² In *M. tuberculosis*, IPP and DMAPP are biosynthesized only through the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway.^{43–45} Subsequently, the skeletons of isoprenoid compounds are completed through rearrangement and/or modification, resulting in enormous diversity.^{46, 47} To date, two distinct pathways have been discovered for the biosynthesis of the two precursors; the mevalonate (MVA) pathway (Fig. 2) and the MEP pathway (Fig. 3). Most bacterial pathogens, including *M. tuberculosis*, exclusively utilize the MEP pathway. However, bioinformatic approaches do identify exceptions; for example, *Coxiella burnetii* and *Staphylococcus aureus* appear to use the MVA pathway and *Listeria monocytogenes* harbors genes encoding orthologs from both pathways for the biosynthesis of isoprenoid precursors.^{48–50} Even bacteria harboring both pathways usually use the MEP pathway to generate essential primary metabolites, with those of the MVA pathway being dedicated to secondary metabolites.^{51, 52}

Multiple lines of evidence indicate that the MEP pathway is a source of viable drug targets. One of the enzymes of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (IspC), is potently inhibited by the phosphonate drug, fosmidomycin.^{44, 53, 54} In addition, only thirty genes with widely distributed orthologs have been identified as essential for the growth of *E. coli* and pathogenic bacteria;⁵⁵ these include all of the MEP pathway genes except for IPP isomerase.^{55–57} The essential nature of the bacterial MEP pathway for *M. tuberculosis* growth was predicted by Himar1-based transposon mutagenesis.⁵⁸ Furthermore, a two-step disruption strategy,⁵⁹ by which nonfunctional mutants were not able to be isolated, confirmed the results of Himar1-based transposon mutagenesis by providing direct evidence that several *M. tuberculosis* MEP pathway enzymes are essential for bacterial survival.^{45, 60, 61} Therefore, the MEP pathway can be considered as a potential source of novel drug targets.

The enzymes of the *M. tuberculosis* MEP pathway

Enzymes known or predicted to be in the *M. tuberculosis* MEP pathway, level of identity with *E. coli* orthologs, and known inhibitors are summarized in Table 1.

A. 1-Deoxy-D-xylulose 5-phosphate synthase

A.1. General characteristics of 1-deoxy-D-xylulose 5-phosphate synthase—The MEP pathway begins with the condensation of glyceraldehyde 3-phosphate (GAP) and pyruvate forming 1-deoxy-D-xylulose-5-phosphate (DXP) which is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS).⁶² This reaction is similar to that observed in the transketolases (TK).⁶³ In parallel with the catalytic mechanism of TK,⁶⁴ DXS requires both thiamine diphosphate (TPP) and divalent cations such as Mg²⁺ or Mn²⁺ for activity,⁶⁵

probably employing a ping-pong mechanism in which pyruvate binds to TPP, CO₂ is released, then GAP binds next and, finally, DXP and TPP are released from the DXS as seen in *E. coli*.⁶⁶ The *dxs* gene was first isolated from *E. coli*^{65, 67} and has subsequently been cloned from a variety of plants and microorganisms.^{43, 68–70} All cognate enzymes have a conserved thiamine-binding domain (DRAG sequence).^{65, 71} The *dxs* gene of *E. coli* was reported to be essential for bacilli survival.⁵⁶ The product of DXS is used as not only a biosynthetic intermediate of IPP but also the precursors of thiamin (vitamin B₁) and of pyridoxol (vitamin B₆) in *E. coli*,^{65, 67, 72} indicating that DXS is not a committed step in IPP synthesis.

A.2. *M. tuberculosis* 1-deoxy-D-xylulose 5-phosphate synthase—Sequence alignment with *E. coli* DXS demonstrated that Rv2682c has approximately 38% identity with a conserved DRAG motif and a key catalytic amino acid (His49 of the *E. coli* enzyme).⁷¹ Recombinant Rv2682c is capable of producing DXS by condensation of pyruvate and GAP in the presence of TPP,⁴³ indicating that Rv2682c encodes *M. tuberculosis* DXS. Optimal activity of *M. tuberculosis* DXS was observed between pH 7.0 and pH 8.0, in the presence of divalent cations, Mg²⁺, having 40 μM for K_m^{Pyruvate} and 6.1 μM for K_m^{GAP} . Like *E. coli* DXS,⁷³ *M. tuberculosis* DXS is capable of using other substrates such as D-glyceraldehyde, L-glyceraldehyde and D-erythrose 4-phosphate, all of which have much greater K_m values than that of GAP (6.1 μM).⁴³ Interestingly, *M. tuberculosis* contains another homolog of *E. coli* DXS, Rv3379c. Thus far, two other organisms have been reported to have two homologs of DXS, the photosynthetic *Rhodococcus capsulatus* and the soil bacterium *Streptomyces coelicolor*.^{43, 74} In each case, both proteins are functional. However, an alignment with *E. coli* DXS indicated, despite relatively high level of identity (38%), that the 65 amino acid N-terminus residues of Rv3379c are truncated due to the positioning of the insertion element (IS6110) and, more importantly, His49 of the *E. coli* enzyme amino acid is missing in Rv3379c.⁴³ Recombinant Rv3379c showed no DXS activity,⁴³ suggesting that Rv2682c is the only functional *M. tuberculosis* DXS.

A.3. HTS campaign for 1-deoxy-D-xylulose 5-phosphate synthase—Most reported DXS assays utilize either TLC or HPLC to monitor DXS activity through measurement of the reaction product.^{65, 67, 68, 74–76} Both of these methods are time and labor intensive and thus are of limited use in the large-scale of screening inhibitors. However, coupled spectrophotometric⁷⁷ and colorimetric assays⁷⁸ have been reported for rapid screening of DXS activity and these methods are likely to be more amenable to HTS. A radiometric, anion exchange assay was also developed and adapted to medium-throughput screening for inhibitors.⁴³ This assay was used to identify compounds inhibiting *M. tuberculosis* DXS and build a preliminary structure-activity relationship (SAR) of several potential inhibitors against *M. tuberculosis* DXS identified by this screen. 2-methyl-3-(4-fluorophenyl)-5-(4-methoxyphenyl)-4H-pyrazolo[1,5-a]pyrimidin-7-one was the most potent inhibitor of the enzyme and bacterial growth, but did show some toxicity in mammalian cell toxicity.⁷⁹

B. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase

B.1. General characteristics of 1-deoxy-D-xylulose 5-phosphate reductoisomerase—1-Deoxy-D-xylulose 5-phosphate reductoisomerase (IspC), the second enzyme in the cascade of the MEP pathway, catalyzes the rearrangement and reduction of DXP dependent upon the presence of nicotinamide adenine dinucleotide phosphate (NADPH), forming 2C-methyl-D-erythritol 4-phosphate (MEP).⁸⁰ The finding that 2C-methyl-D-erythritol (ME) is incorporated into the prenyl side chain of ubiquinone in *E. coli*⁸¹ suggested that the branched chain of the linear carbon skeleton of DXP requires an intramolecular rearrangement to yield the intermediate product, 2C-methyl-D-erythrose 4-phosphate and subsequent reduction forming MEP.⁸² Studies of *E. coli* mutants in which MEP or ME was required for survival indicated that defects within the *yaeM* gene were responsible

for the phenotype, and incubation of YaeM with DXP and NADPH resulted in formation of MEP.^{80, 83} YaeM was renamed later IspC.⁷³ The importance of IspC is emphasized by the fact that fosmidomycin, a phosphonate antibiotic naturally isolated from *Streptomyces lavendulae* inhibits this enzyme.⁸⁴ Fosmidomycin acts through competitive slow-binding with regard to DXP and uncompetitive inhibition with regard to NADPH.⁸⁵ It shows inhibitory effects against *E. coli* IspC at nanomolar levels.^{86, 87} Genetic disruption of the IspC in *E. coli* strain showed a lethal phenotype.⁸⁸ As mentioned earlier, DXP is a precursor not only of IPP and DMAPP but also for thiamine and pyridoxol. Therefore, IspC catalyzes the first committed step for biosynthesis of IPP and DMAPP.^{82, 89}

B.2. *M. tuberculosis* 1-deoxy-D-xylulose 5-phosphate reductoisomerase—Blast searches with *E. coli* IspC indicated that the primary structure of Rv2870c of *M. tuberculosis* is 25% identical to that of the *E. coli* IspC with conserved amino acid residues.^{44, 90, 91} The crucial amino acids participating in forming the catalytic pocket (His153, His209, Glu231, and His257) and substrate and cofactor binding areas (Trp212 and Met214 for DXP; Asp150, Glu152, Glu231, and Glu234 for divalent cations; and Thr10 and Tyr37 for NADPH) in the enzyme from *E. coli* are all well conserved in Rv2870c.^{44, 68, 92} Recombinant Rv2870c efficiently catalyzes the conversion of DXP to MEP in the presence of NADPH and the reverse reaction in the presence of NADP⁺. *M. tuberculosis* IspC harboring mutations in two crucial amino acids (corresponding to Asp150 and Glu231 of the *E. coli* enzyme) lost catalytic activity,^{90, 92} suggesting that the active site of *M. tuberculosis* IspC is similar to that of *E. coli* IspC and that divalent cations are absolutely required for the activity. The K_m^{DXP} value for *M. tuberculosis* IspC in the presence of Co²⁺, Mg²⁺ or Mn²⁺ is reported to be $42 \pm 7 \mu\text{M}$, $100 \pm 4 \mu\text{M}$, or $4.0 \pm 0.3 \mu\text{M}$, respectively,⁹² and similar values were obtained in our laboratory.⁴⁴ The K_m values are somewhat lower than those reported for *E. coli* IspC in the presence of Mn²⁺ ($73 - 250 \mu\text{M}$).⁹³⁻⁹⁵ *M. tuberculosis* IspC has a sharp pH optimum between pH 7.5 and 7.9.⁴⁴ Interestingly, it was reported that fosmidomycin shows no inhibitory effect on Gram-positive bacteria or anaerobic species including *M. tuberculosis* bacilli.^{44, 96} However, we and others have shown that the *in vitro* *M. tuberculosis* IspC catalytic reaction is inhibited by fosmidomycin, with IC₅₀ values of 80 or 300 nM reported.^{44, 90} Moreover, *Salmonella enterica* with an inactivated chromosomal IspC copy when complemented with Rv2870c showed sensitivity to fosmidomycin treatment, indicating that Rv2870c is not intrinsically resistant to fosmidomycin.⁴⁴ Genetic evidence of the essentiality of *M. tuberculosis* IspC has been provided recently.⁶¹ The data indicate that fosmidomycin does not accumulate in the cytosol of *M. tuberculosis*, suggesting that the main reason for *M. tuberculosis* resistance is lack of translocation of this antibiotic through the *M. tuberculosis* membrane.^{44, 61}

B.3. HTS campaign for 1-deoxy-D-xylulose 5-phosphate reductoisomerase—A continuous assay format was developed to monitor IspC activity by following the oxidation of NADPH or reduction NADP⁺ in either forward or reverse directions, respectively.^{44, 92} IspC reduces DXP to produce MEP, resulting in oxidation of NADPH and a decrease of the optical density signal at A_{340 nm}. This assay is well established and has been used to characterize IspC from many different sources.^{74, 77, 85, 97-100} For HTS, the reverse IspC assay has been suggested.⁴⁴ In this assay, IspC reaction is coupled to NADPH oxidase and horseradish peroxidase in order to increase the sensitivity by producing resorufin, a stable fluorescent dye with excitation/emission wavelengths of 570/590 nm. The assay can be miniaturized and automated for use in multi-well plate formats.

C. 4-Diphosphocytidyl-2C-methyl-D-erythritol synthase

C.1. General characteristics of 4-diphosphocytidyl -2C-methyl-D-erythritol synthase—Incubation of MEP with crude cell free extracts of *E. coli* in the presence of

cytidine 5'-triphosphate (CTP) produces 4-diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME).¹⁰¹ Acs1 of *Haemophilus influenza*, functions as a phosphocytidyl transferase and harbors an N-terminal domain for the transfer of a cytidine 5'-monophosphate (CMP) moiety to ribitol 5-phosphate producing 5-pyrophosphocytidyl ribitol.¹⁰¹ Therefore, when Acs1 sequence similarity searches were conducted, they uncovered a relatively large number of orthologs from various organisms, including an *E. coli* *ygbP* gene,¹⁰¹ which was later renamed IspD.⁷³ Recombinant *E. coli* IspD catalyzes the conversion of MEP into CDP-ME with corresponding release of inorganic pyrophosphate (PPi) in the presence of CTP.¹⁰¹ Divalent cations are absolutely required for IspD activity; for example, optimal activity of *E. coli* IspD requires Mg²⁺, Mn²⁺ or Co²⁺, however, Cu²⁺, Ni²⁺, Ca²⁺, Fe²⁺, or Zn²⁺ could not serve as cofactors.^{85, 101} The crystal structure has shown that Mg²⁺ forms coordinated bonds to the α , β , and γ phosphates of CTP in homodimer formation of *E. coli* IspD in the native condition,¹⁰² and one report showed that L-erythritol-4-phosphate weakly inhibits *E. coli* IspD with an IC₅₀ of 1.4 mM.¹⁰³

C.2. *M. tuberculosis* 4-diphosphocytidyl-2C-methyl-D-erythritol synthase—Blast searches showed that the *Rv3582c* gene product has approximately 31% identity with *E. coli* IspD and conserved amino acids which are involved in formation of the substrate binding site and the catalytic active site. However, the amino acid corresponding Ser88 of the *E. coli* enzyme active site has been changed to a Thr in *Rv3582c*. Recombinant *Rv3582c* protein is clearly a functional IspD in *M. tuberculosis*.⁴⁵ Himar1-based transposon mutagenesis has shown that *M. tuberculosis* IspD is essential for bacilli survival⁵⁸ and the inability to isolate a non-functional mutant using the two step disruption confirmed the essentiality.⁴⁵ *M. tuberculosis* IspD is active over a relatively broad pH range (6.0 – 9.0) as was previously reported for the *E. coli* IspD.¹⁰⁴ A divalent cation, such as Mg²⁺ or Mn²⁺, is absolutely required for this cytidyltransferase activity. Zn²⁺ supports the *M. tuberculosis* IspD activity at concentrations lower than 5 mM⁴⁵; however *E. coli* IspD activity was not supported by Zn²⁺.¹⁰¹ *M. tuberculosis* IspD shows a high degree of specificity for CTP as a substrate, unlike other nucleotide 5-phosphates (GTP, UTP, TTP, or ATP). A discussion of the structural basis of this specificity has been reported.^{102, 104}

C.3. HTS campaign for 4-diphosphocytidyl-2C-methyl-D-erythritol synthase—Characterization of IspD orthologs from various organisms has been performed using ¹H- or ¹³C nuclear magnetic resonance spectroscopy^{101, 105, 106} or TLC based assays,^{102, 104} which require either expensive equipment or are protracted, and are incompatible with HTS. However, a spectrophotometric assay, in which released PPi is coupled to inorganic pyrophosphatase and purine nucleoside phosphorylase could be amenable to HTS. This assay has already proven robust enough to be utilized as a HTS assay (unpublished data), yielding an excellent Z'-factor value.¹⁰⁷

D. 4-Diphosphocytidyl -2C-methyl-D-erythritol kinase

D.1. General characteristics of 4-diphosphocytidyl -2C-methyl-D-erythritol kinase—The fourth step in the MEP pathway involves the conversion of CDP-ME to 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (CDP-ME2P) in the presence of ATP catalyzed by IspE, which was initially identified in *E. coli* and tomatoes.^{108, 109} Recombinant *E. coli* IspE was capable of catalyzing phosphorylation at tertiary hydroxyl group of CDP-ME.¹⁰⁸ A sequence alignment revealed that *E. coli* IspE has a high level of similarity to mammalian ATP-dependent kinases such as galactose kinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase (members of the GHMP kinase superfamily). An *E. coli* IspE crystal structure in complex with substrate and the hydrolysis resistant ATP analogue (adenosine 5'-(β , γ -imino)triphosphate) also showed a close structural relationship between *E. coli* IspE and members of the GHMP kinase superfamily^{110–112} in terms of forming

homodimers and following an α/β fold-subunit arrangement.¹¹³ This structural characteristic of IspE was corroborated by the crystal structure of *Thermus thermophilus* ortholog.¹¹⁴ Despite this high structural similarity with the GHMP kinase superfamily, prominent differences can be observed in the catalytic sites and ATP binding sites.^{113, 115} Interestingly, the conserved residues thought to coordinate the divalent cation (Mg^{2+}) and the triphosphate moiety of ATP are missing in *E. coli* IspE¹¹³; however, divalent cations are required for optimal *E. coli* IspE activity.⁹⁸ These conformational differences between bacterial and mammalian members of the GHMP kinase superfamily support the hypothesis that IspE is an attractive and potential drug target.

D.2. *M. tuberculosis* 4-diphosphocytidyl -2C-methyl-D-erythritol kinase—

Alignment of *E. coli* IspE with genes of the *M. tuberculosis* genome indicates that *Rv1011* encodes a protein which harbors around 22% identity with conserved amino acids involved in forming the CDP-ME and ATP binding and crucial active sites.¹¹³ Recombinant Rv1011 is able to catalyze CDP-ME phosphorylation in an ATP-dependent manner (unpublished observations). Rv1011 is predicted to be cytosolic and soluble, but the intact Rv1011 failed to be expressed in *E. coli* at reasonable levels, presumably because of low solubility. Soluble and active *M. tuberculosis* IspE could be obtained by truncating six amino acid residues (Ser-Ala-Pro-Thr-Glu-Val) at the C-terminal end. Alignment analysis and *in vitro* enzyme assay reveal that the six amino acids at the C-terminal of Rv1011 add little functional or structural contribution to the catalytic activity as compared to *E. coli* IspE, where the C-terminal end was reported to be involved in forming the ATP-binding motif.¹¹³

D.3. HTS campaign for 4-diphosphocytidyl -2C-methyl-D-erythritol kinase—

E. coli IspE was previously characterized by TLC analysis using $[2-^{14}C]$ CDP-ME,¹⁰⁸ which is presumably not amenable to HTS. For screening purposes, ADP released from the *M. tuberculosis* IspE step can be coupled by pyruvate kinase, pyruvate oxidase, and horseradish peroxidase. This assay has been validated with an excellent Z'-factor value (unpublished observations), suggesting that this coupled assay is sufficiently robust to screen for inhibitors.

E. 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase

E.1. General characteristics of 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase—

The fifth step of the MEP pathway involves the unusual enzymatic formation of a metabolite containing a cyclodiphosphate moiety. The product of IspE, CDP-ME2P, is converted into 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) with a corresponding release of cytidine 5'-monophosphate (CMP) by the *ispF* gene product.¹¹⁶ Orthologous IspF proteins from *E. coli* and *Plasmodium falciparum* were shown to generate not only MECDP from CDP-ME2P but also the 2C-methyl-D-erythritol 3,4-cyclophosphate using CDP-ME, albeit at a reduced rate.^{116, 117} The crystal structures of the IspF from *E. coli*, *H. influenzae*, and *T. thermophilus* are available and have shown that IspF proteins form homotrimers containing three active sites, each of which is formed in a cleft between pairs of subunits.^{118–120} The subunit pairs recognize and bind Zn^{2+} with tetrahedral coordination.^{118–122} The Zn^{2+} supports the correct binding of the substrate at the active site and facilitates the nucleophilic attack of the 2-phosphate group.¹¹⁸ However, *E. coli* IspF also requires Mg^{2+} or Mn^{2+} for optimal activity.¹¹⁹

E.2. *M. tuberculosis* 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase—

Rv3581c likely encodes *M. tuberculosis* IspF as *Rv3581c* appears to be transcriptionally coupled with *M. tuberculosis* *ispD* and blast analysis shows that the primary sequence of *Rv3581c* has 40% identity with *E. coli* IspF with conserved amino acids reported to be involved in binding divalent cations and catalysis.^{118, 121, 123} It has recently been reported that *M. tuberculosis* IspF is essential for bacillary survival.⁶⁰ The crystal structure of *M. smegmatis*

IspF, harboring around 73% amino acid sequence identity with *M. tuberculosis* IspF, is available.⁶⁰ The overall architecture of the active site formation and the divalent cation binding patterns resembles that of *E. coli* IspF.^{119, 121}

E.3. HTS campaign for 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase—

The IspF proteins from *E. coli* and *P. falciparum* has been characterized by TLC and HPLC methods, respectively.^{116, 117} The reaction catalyzed by IspF generates CMP, which, theoretically, can be coupled to commercially available enzymes to generate a highly sensitive assay that is likely to be amenable to multi-well plate formats.

F. 1-Hydroxy-2-methyl-2(E)-butenyl-4-diphosphate synthase and reductase

F.1. General characteristics of 1-hydroxy-2-methyl-2(E)-butenyl-4-diphosphate synthase and reductase—

Recombinant *E. coli* overexpressed *ispC*, *ispD*, *ispE*, *ispF*, and *ispG* catalyzed the conversion of exogenous 1-deoxy-D-xylulose (DX) into 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate (HMBPP)¹²⁴ and the *ispH* gene product is responsible for the conversion of HMBPP into IPP and DMAPP. Therefore, HMBPP is the product of IspG and the substrate of IspH.¹²⁵ Recombinant IspG catalyzes the reduction of MECDP resulting in opening of the cyclodiphosphate ring structure using a photoreduced deazaflavin derivative as an artificial electron donor.^{126, 127} *In vivo* experiments using an *E. coli* strain overexpressing the *ispH* gene yield IPP and DMAPP from HMBPP at a molar ratio of 5:1.¹²⁸ The functions of these two enzymes were previously determined to be essential in the survival of *E. coli* as well as *Synechocystis* sp.^{129, 130} IspG and IspH have prosthetic [4Fe-4S]²⁺ clusters, suggesting that the reduction reaction involves a free radical.^{127, 131} Reduction of MECDP could also occur via the shuttle of redox equivalents from NADPH through flavodoxin reductase.^{126, 127, 132} Notably, the IspG protein from *T. thermophilus* has been shown to catalyze the reaction more efficiently with an artificial electron donor system comprised of dithionite as compared to NADPH, flavodoxin and flavodoxin reductase.¹³² Recombinant *E. coli* IspH showed no activity without stimulation by *E. coli* crude extract, suggesting optimal IspH activity requires unknown cofactors, likely composed of NADH, flavin-adenine dinucleotide (FAD), and at least one more unidentified electron shuttles.¹³³ The IspH enzyme can also be activated under anaerobic conditions, restoring reduction capacity in the presence of flavodoxin, flavodoxin reductase and NADPH.^{127, 131} Blast searches of *E. coli* IspG or IspH with the *M. tuberculosis* genome indicates that *Rv2868c* is the likely *M. tuberculosis* IspG and *Rv1110* or *Rv3382c* are likely candidates for the gene encoding *M. tuberculosis* IspH (unpublished observations).

F.2. HTS campaign for 1-hydroxy-2-methyl-2(E)-butenyl-4-diphosphate

synthase and reductase—IspG and IspH from *E. coli* have been assayed using HPLC,¹²⁷ which is not amenable to HTS. As mentioned earlier, both enzymes require a flavodoxin/flavodoxin reductase system for their activities. Such a system was cloned from *E. coli* and shown to stimulate the activity of both IspG and IspH.¹²⁷ However, the flavodoxin/flavodoxin reductase system may be unnecessary as IspG and IspH could be chemically activated by using a photoactivatable chemical reductant, 10-methyl-5-deaza-isoalloxazine.¹²⁷ In many cases, an enzyme which requires a flavodoxin/flavodoxin reductase system and can be activated by 10-methyl-5-deaza-isoalloxazine, can also be converted to the fully active form by sodium dithionite,^{134, 135} which can be more easily obtained.

H. Isopentenyl diphosphate isomerase

Upon biosynthesis of IPP and DMAPP by IspH in *Arabidopsis thaliana*, IPP isomerase (Idi) catalyzes the conversion of the two isoforms, however, the equilibrium favors the forward reaction, from IPP to DMAPP.¹³⁶ After the first description of the responsible enzyme from yeast,¹³⁷ orthologs of the enzyme have been characterized from bacteria including *E. coli*.

^{57, 138} In organisms capable of synthesizing isoprenoid units by the MVA pathway, Idi was reported to be an essential enzyme ¹³⁹ because pyrophosphomevalonate decarboxylase in the MVA pathway produces only IPP (Fig. 2). Idi orthologs are also found in many organisms including *E. coli* that utilize the MEP pathway, and *E. coli* Idi is non-essential for the bacillary survival, ⁵⁷ as IspH of the MEP pathway produces both IPP and DMAPP. In this case, Idi may serve as accessory enzyme in order to balance the pools of IPP and DMAPP to the specific needs of the downstream enzymes, as the molar ratio of IPP to DMAPP varies from 1:1 in monoterpenes to 2:1 in sesquiterpenes and sterols, 3:1 in diterpenes and carotenoids, and much higher for long-chain polyprenols, polyprenyl phosphate and polyterpenes. ¹³⁶

Two forms of Idi have been reported, to date. The Type I enzyme including the Idi from *E. coli* has an absolute requirement for divalent cations such as Mg^{2+} or Mn^{2+} and Zn^{2+} . Mg^{2+} or Mn^{2+} facilitates substrate binding and Zn^{2+} is required for its catalytic activity. ^{137, 140} A crystal structure of *E. coli* Idi shows that three His residues and two Glu residues are crucial in forming an octahedral metal binding site. ¹⁴¹ The catalytic reaction involves protonation at the double bond and deprotonation at the second carbon of IPP to form DMAPP. ¹⁴¹ As mentioned earlier, the demonstration that a mutant *E. coli* strain disrupted in the *idi* gene can survive on M9 minimal medium confirmed the non-essential nature of *E. coli* Idi. ⁵⁷ An other form of Idi (Type II) was identified in *Streptomyces* sp. strain CL190 ¹⁴² and homology searches using the sequence of the *Streptomyces* Idi as a query sequence revealed that archaeobacteria and some Gram-positive bacilli harbor Type II Idi enzymes. ¹³⁹ The crystal structure of Type II Idi enzymes ^{143, 144} and alignment data showed that they are unrelated to Type I Idi and require a divalent cation, reduced flavin mononucleotide and NADPH for the activity. ^{143, 144} *M. tuberculosis* Idi has 38% identity with the enzyme from *E. coli* and belongs to Type I.

Conclusions

There has been intensive interest in several chemical entities as potential TB drug candidates including fluoroquinolones ¹⁴⁵, PA-824 ^{146–148} and the diarylquinolone R207910. ^{149–151} New TB drugs would benefit standard DOTS programs if they could shorten the treatment period from the current 6 months or be used to treat patients infected with MDR-TB or XDR-TB. More are likely to become available in the near future adding to the armament capable of managing the resistance problem as we currently know it. However, resistance continually evolves and, therefore, all possible drug targets should be identified and evaluated. In this context, the *M. tuberculosis* MEP pathway is potentially attractive. The final products of the MEP pathway are obligatory precursors of all isoprenoids, which are involved in many of the essential biochemical transformations required for *M. tuberculosis* survival including biosynthesis of the cell wall and production of ATP.

We propose a series of HTS amenable assays for each step in the *M. tuberculosis* MEP pathway, to be used to screen for and identify specific inhibitors. Bioinformatic approaches have shown that all enzymes in the MEP pathway are devoid of transmembrane motifs and are, therefore, probably water-soluble cytosolic proteins, and thus readily purified using current recombinant techniques for further *in vitro* enzyme assay development. Lead compounds can be sought through HTS campaigns using the recombinant purified enzymes. Each of the resulting inhibitors then represents a starting point by which structures can be further modified to improve binding and other important characteristics. ¹⁵² To date, the *M. tuberculosis* MEP pathway has not been targeted by any promising new anti-TB drug. Research opportunities are ripe for the identification of inhibitors against the *M. tuberculosis* MEP pathway that can be potential weapons contributing to increase the quality of the DOTS program.

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Fig 1. Various isoprenoids

Examples of isoprenoid compounds. Isopentenyl diphosphate and dimethylallyl diphosphate contribute the carbon architectures. (a) Phytol, (b) β -carotene or xanthophyll, (c) menaquinone, (d) ubiquinone, and (e) prenyl phosphate.

Fig 2. The mevalonate pathway for IPP biosynthesis

This pathway is an important cellular metabolic pathway present in all higher eukaryotes and some eubacteria. DMAPP and IPP serve the repeating units for the biosynthesis of isoprenoids¹⁵⁹. Abbreviation: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, HMG-CoA reductase.

Fig 3. The 2C-methyl-D-erythritol 4-phosphate pathway

Most eubacteria utilize this pathway for the biosynthesis of IPP and DMAPP. Abbreviations: The names of structures are in the text. DXS, 1-deoxy-D-xylulose 5-phosphate synthase; IspC, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; IspD, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; IspE, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; IspF, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG, 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase; IspH, 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase; Idi, Isopentenyl diphosphate isomerase ¹⁵⁹.

Table 1The enzymes of *M. tuberculosis* MEP pathway

enzyme	ORF	Identity with <i>E. coli</i> ortholog	Known inhibitor
DXS	Rv2682c	38 %	Clomazone ¹⁵³ 2-Methyl-3-(4-fluorophenyl)-5-(4-methoxy-phenyl)-4H-pyrazolo[1,5-a]pyrimidin-7-one ⁷⁹
IspC	Rv2870c	39 %	Fosmidomycin ⁴⁴
IspD	Rv3582c	31 %	L-Erythritol 4-phosphate ¹⁰³
IspE	Rv1011	22 %	Cytidine derivatives ¹⁵⁴ Tetrahydrothiophenyl derivatives ¹⁵⁵
IspF	Rv3581c	40 %	Fluorescent ligand ¹⁵⁶
IspG	Rv2868c *	41 %	N.A.
IspH	Rv1110 * or Rv3382c *	50 % each	N.A.
Idi	Rv1745c	35 %	Diene analogues ¹⁵⁷ 3,4-epoxy-3-methylbutyl diphosphate ¹⁵⁸

* Putative candidates based on the alignment data with *E. coli* orthologs.

N.A., not applicable.