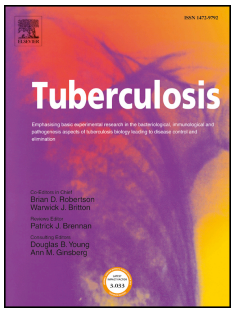
Accepted Manuscript



I3-Ag85 effect on phthiodiolone dimycocerosate synthesis

Céline Rens, Françoise Laval, Ruddy Wattiez, Philippe Lefèvre, François Dufrasne, Mamadou Daffé, Véronique Fontaine

PII: S1472-9792(17)30316-5

DOI: 10.1016/j.tube.2017.10.007

Reference: YTUBE 1636

To appear in: *Tuberculosis*

Received Date: 26 July 2017

Revised Date: 17 October 2017

Accepted Date: 22 October 2017

Please cite this article as: Rens Cé, Laval Franç, Wattiez R, Lefèvre P, Dufrasne Franç, Daffé M, Fontaine Vé, I3-Ag85 effect on phthiodiolone dimycocerosate synthesis, *Tuberculosis* (2017), doi: 10.1016/j.tube.2017.10.007.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

I3-Ag85 effect on phthiodiolone dimycocerosate synthesis

Céline Rens[a], Françoise Laval[b], Ruddy Wattiez[c], Philippe Lefèvre[a], François Dufrasne[d], Mamadou Daffé[b] and Véronique Fontaine[a]

[a]Université Libre de Bruxelles (ULB), Unit of Pharmaceutical Microbiology and Hygiene, CP205/2, Boulevard du Triomphe, 1050 Brussels, Belgium

[b]Institute of Pharmacology and Structural Biology, University of Toulouse, CNRS, University Paul Sabatier (UMR 5089), Department of "Tuberculosis and Infection Biology" 205 route de Narbonne, BP64182, 31077 Toulouse cedex 04, France.

[c]Department of Proteomics and Microbiology, University of Mons, 20, place du Parc, B-7000 Mons, Belgium

[d]Université Libre de Bruxelles (ULB), Therapeutic Chemistry, CP205/05, Boulevard du Triomphe, 1050 Brussels, Belgium

Address correspondence to Véronique Fontaine, vfontain@ulb.ac.be

Keywords: *Mycobacterium tuberculosis*, I3-Ag85, TDM, PDIM

Abbreviations: TDM: trehalose dimycolate, PDIM A: phthiocerol dimycocerosate, PDIM B: phthiodiolone dimycocerosate, MDR: multi-drug-resistant, XDR: extensively-drug-resistant, Mtb: *Mycobacterium tuberculosis*, SL: sulfolipids, DAT: diacyltrehalose, PAT: poly-acyltrehalose, TMM: trehalose monomycolate, MIC: minimal inhibitory concentration, FICI: fractional inhibitory concentration index, *ko*: knock-out, *wt*: wild-type, HP-TLC: High performance thin layer chromatography, MAME: mycolic acid methyl esters, AcPIM: acyl phosphatidylinositol mannoside, TAG: triacylglycerol

ACCEPTED MANUSCRIPT

ABSTRACT

The multiplicity of drug resistant *Mycobacterium tuberculosis* (Mtb) strains is a growing health issue. New therapies are needed, acting on new targets. The I3-Ag85 was already reported to reduce the amount of trehalose dimycolate lipid of the mycobacterial cell wall. This inhibitor of Ag85c increased the mycobacterial wall permeability. We previously showed that *M. tuberculosis* strains, even multi-drug resistant and extensively-drug resistant strains, can be susceptible to vancomycin when concomitantly treated with a drug altering the cell envelope integrity. We investigated the effect of the I3-Ag85 on vancomycin susceptibility of *M. tuberculosis*. Although no synergy was observed, a new target of this drug was discovered: the production of phthiodiolone dimycocerosate (PDIM B).

ACCEPTED MANUSCRIPT

The emergence of multi- and extensively drug resistant (MDR and XDR) *Mycobacterium tuberculosis* (Mtb) strains emphasized the urgent need for new antitubercular drug development [1]. In this perspective, we focused on drugs targeting the external lipid envelope of these bacteria. Mtb, the main causative agent of tuberculosis, has a particular waxy cell wall outward its peptidoglycan layer. The very long chain fatty acids, up to C100 and called mycolic acids, are attached to arabinogalactan, which in turn is covalently bound to peptidoglycan. The giant complex macromolecule interact with extractable waxy lipids, forming a hydrophobic wall [2]. Among these complex lipids, trehalose dimycolate (TDM), sulfolipids (SL), diacyltrehalose (DAT), penta- or poly-acyltrehalose (PAT) and phthiocerol- or phthiodiolone dimycocerosate (PDIM A and PDIM B) are virulence factors important for host interaction. Additionally, both TDM and PDIM play an important structural role. TDM, known as "cord factor", is involved in the host's immune system modulation during granuloma formation, but it is also involved in the mycobacteria wall impermeability, conferring protection against drug entrance [3, 4]. PDIM A and PDIM B have been shown to be involved in mycobacterial wall impermeability against drugs, oxidative stresses and SDS [5-9].

The large molecular size of the glycopeptides prevents them from penetrating the waxy Mtb cell wall. However, in previous articles, we showed that drugs inhibiting PDIM synthesis could increase the inhibitory action of vancomycin on Mtb [9,10]. The report of Warrier et al., on a TDM inhibitor specifically targeting the Ag85C on MDR and XDR Mtb clinical strains and able to improve Mtb permeability to glycerol, raised our attention [11]. The Ag85C is part of an enzymatic complex including Ag85A and Ag85B, and the most active enzyme involved in the transfer of mycolic acid residues, carried by trehalose monomycolate (TMM), on arabinogalactan [12].

Based on Warrier *et al.* results, we tested the susceptibility of Mtb to vancomycin in the presence of this inhibitor, I3-Ag85, in order to investigate a potential synergistic effect of this combination [11]. The I3-Ag85 was synthetized as previously described [13]. We performed drug susceptibility assay following the agar proportion method on the Mtb H37Rv strain [14]. Vancomycin and I3-Ag85 were serially diluted alone or in combination in 24-well plates and inoculated with 10 µl 10^{-1} to 10^{-4} dilutions of McFarland No. 1 turbidity culture. The obtained minimal inhibitory concentration (MIC) were used to calculate the fractional inhibitory concentration index (FICI) following the Checkerboard method: FICI= MIC_{ab}/MIC_a + MIC_{ba}/MIC_b [15]. MIC_a were 50 µg/ml for vancomycin and MIC_b was 44 µg/ml for I3-Ag85.

Vancomycin serially diluted with 4.4 µg/ml I3-Ag85 fix concentration still gave an MIC_{ab} of 50 µg/ml vancomycin. Similarly, I3-Ag85 serially diluted with 10 µg/ml vancomycin fixed concentration gave a MIC_{ba} of 44 µg/ml I3-Ag85. A FICI of 2 was obtained, showing no synergistic effect of the two drugs.

Since these results suggested that the I3-Ag85 targets, including Ag85C [11], are not involved in vancomycin resistance, we verified the vancomycin susceptibility by the agar proportion method of a strain lacking this enzyme (KO), MT0137, obtained by transposon insertion, compared to the CDC1551 wild type Mtb strain (WT) [16]. The absence of the expression of the Ag85C in the MT0137 strain was confirmed by proteomic analysis (Fig. S1). In contrast to the WT strain, no specific peptide corresponding to the Ag85c was identified and sequenced from the KO sample. The WT and KO strains showed similar MIC for vancomycin (50-200 µg/ml for the WT and 100-200 µg/ml for the KO). Considering that the vancomycin susceptibility was unchanged in the KO strain compared to WT strain, we considered that Ag85C is not an interesting target to potentiate glycopeptide effect. It is worth noting that we observed the same susceptibility to the I3-Ag85 (22-44 µg/ml) in both strains, as previously reported by Warrier et al. [11].

I3-Ag85 inhibitory effect should therefore rely on the inhibition of additional targets, including potentially orthologous Ag85A or B proteins, given that the KO strain is devoid of Ag85C but shows the same MIC to the inhibitor as the WT strain. We therefore analyzed their lipid composition by high-pressure thin-layer chromatography (HPTLC) as previously described [10], comparing midlog-phase growing Mtb CDC1551 WT and KO cultures (with inoculum size 100 fold higher compared to drug susceptibility assays) , treated or untreated 24 h with 44 µg/ml I3-Ag85 [17]. As described by Warrier *et al*., we observed a slight decrease of TDM and an increase of DAT+TMM in the treated WT strain (Fig. 1A and B) [11]. Additionally, we observed a decrease of acylated phosphatidylinositol hexamannoside (Ac_2PIM_6) and an increase of phosphatidyl ethanolamine (PE) (Fig. 1A and B). Although both the I3-Ag85 treated KO and the WT strains showed an increase of DAT+TMM, the mutant additionally exhibited a decrease of the triacylglycerol (TAG) and PDIM B (Fig. 1A and C). This decrease of PDIM B by the I3-Ag85 treatment in the KO strain is highlighted by the stronger PDIM B HPTLC signal intensity compared to the untreated WT strain (Fig. 1A).

As reported by Warrier et al, mycolic acid methyl esters (MAME) were not notably changed by the I3-Ag85 treatment, suggesting that other enzymes, including the orthologous Ag85A or

B proteins could rescue mycolic acid transfer on arabinogalactan (data not shown). Considering that Warrier et al. also reported a free mycolic acid change, these authors suggested that a specific effect on the TDM synthesis by this inhibitor [11].

Our lipid analyses, especially on the KO strain lacking Ag85C, suggest that the I3-Ag85 has an additional effect by reducing the PDIM B production, either directly or indirectly . Indeed, a change in the balance between an acetyl-CoA derived lipid (e.g. TMM) and a propionyl-CoA derived lipids (e.g. PDIM B), as observed by the I3-Ag85 treatment, has been already reported in a Δ*mce*1 KO mutant strain [18]. Propionyl-CoA derived lipid synthesis could protect bacteria against propionate induced toxicity [2]. The inhibition of PDIM synthesis could therefore be harmful for Mtb and explain the I3-Ag85 susceptibility of both WT and MT0137 Δ*fbp*C strains.

ACKNOWLEDGEMENT

Céline Rens was supported by “Les amis des Instituts Pasteurs à Bruxelles” asbl. We thank Alain Baulard for providing us biosecurity level 3 facilities.

ACCEPTED MANUSCRIPT

120 REFERENCES

121 [1] World Health Organization. WHO report 2016. Global tuberculosis control.

122 [2] Daffé, M., Crick, D. and Jackson, M. Genetics of capsular polysaccharides and cell
123 envelope (glyco)lipids. Microbiol Spectrum 2014; 2:MGM2-0021-2013.

124 [3] Welsh KJ, Abbott AN, Hwang SA, Indrigo J. A role for tumour necrosis factor-alpha,
125 complement C5 and interleukin-6 in the initiation and development of the mycobacterial cord
126 factor trehalose 6,6'-dimycolate induced granulomatous response. Microbiology 2008;
127 154:1813-24.

128 [4] Katti MK, Dai G, Armitige LY, Rivera Marrero C. The Delta fbpA mutant derived from
129 *Mycobacterium tuberculosis* H37Rv has an enhanced susceptibility to intracellular
130 antimicrobial oxidative mechanisms, undergoes limited phagosome maturation and activates
131 macrophages and dendritic cells. Cell Microbiol 2008; 10:1286-303.

132 [5] Chavadi SS, Edupuganti UR, Vergnolle O, Fatima I. Inactivation of tesA Reduces Cell
133 Wall Lipid Production and Increases Drug Susceptibility in Mycobacteria. J Biol Chem 2011;
134 286: 24616–25

135 [6] Wang XM, Lu C, Soetaert K, S'Heeren C. Biochemical and immunological
136 characterization of a cpn60.1 knockout mutant of *Mycobacterium bovis* BCG. Microbiolog
137 2011; 157:1205-19.

138 [7] Camacho LR, Constant P, Raynaud C, Laneelle MA. Analysis of the phthiocerol
139 dimycocerosate locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in
140 the cell wall permeability barrier. J Biol Chem 2001; 276:19845-54.

141 [8] Siméone R, Constant P, Malaga W, Guilhot C. Molecular dissection of the biosynthetic
142 relationship between phthiocerol and phthiodiolone dimycocerosates and their critical role in
143 the virulence and permeability of *Mycobacterium tuberculosis*. FEBS J 2007; 274:1957-69.

144 [9] Soetaert K, Rens C, Wang XM, De Bruyn J. Increased Vancomycin Susceptibility in
145 Mycobacteria: a New Approach To Identify Synergistic Activity against Multidrug-Resistant
146 Mycobacteria. Antimicrob Agents Chemother 2015; 59:5057-60.

147 [10] Rens C, Laval F, Daffé M, Denis O. Effects of Lipid-Lowering Drugs on Vancomycin
148 Susceptibility of Mycobacteria. Antimicrob Agents Chemother 2016; 60:6193-9.

[11] Warrier T, Tropis M, Werngren J, Diehl A. Antigen 85C Inhibition Restricts *Mycobacterium tuberculosis* Growth through Disruption of Cord Factor Biosynthesis. Antimicrob Agents Chemother 2012; 56: 1735–43.

[12] Jackson M, Raynaud C, Lanéelle MA, Guilhot C. Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. Mol Microbiol 1999; 31:1573-87.

[13] Scheich, C, Puetter, V, Schade, M, Novel Small Molecule Inhibitors of MDR *Mycobacterium tuberculosis* by NMR Fragment Screening of Antigen 85C. J Med Chem 2010; 53: 8362-7

[14] National Committee for Clinical Laboratory Standards, Wayne, PA. Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes. Approved standard M24-A 2003.

[15] Hsieh MH, Yu CM, Yu VL, Chow JW. Synergy assessed by checkerboard. A critical analysis. Diagn Microbiol Infect Dis 1993; 16:343-9.

[16] Lamichhane G, Zignol M, Blades NJ, Geiman DE, Dougherty A, Grosset J, Broman KW, Bishai WR. A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A 2003; 100:7213-8.

[17] Simeone R, Huet G, Constant P, Malaga W. Functional Characterization of Three O-methyltransferases Involved in the Biosynthesis of Phenolglycolipids in *Mycobacterium tuberculosis*. PLoS One 2013; 8:e58954.

[18] Queiroz A, Medina-Cleghorn D, Marjanovic O, Nomura DK, and Riley LW. Comparative metabolic profiling of mce1 operon mutant vs wild-type *Mycobacterium tuberculosis* strains. Pathog Dis 2015; 73: ftv066.

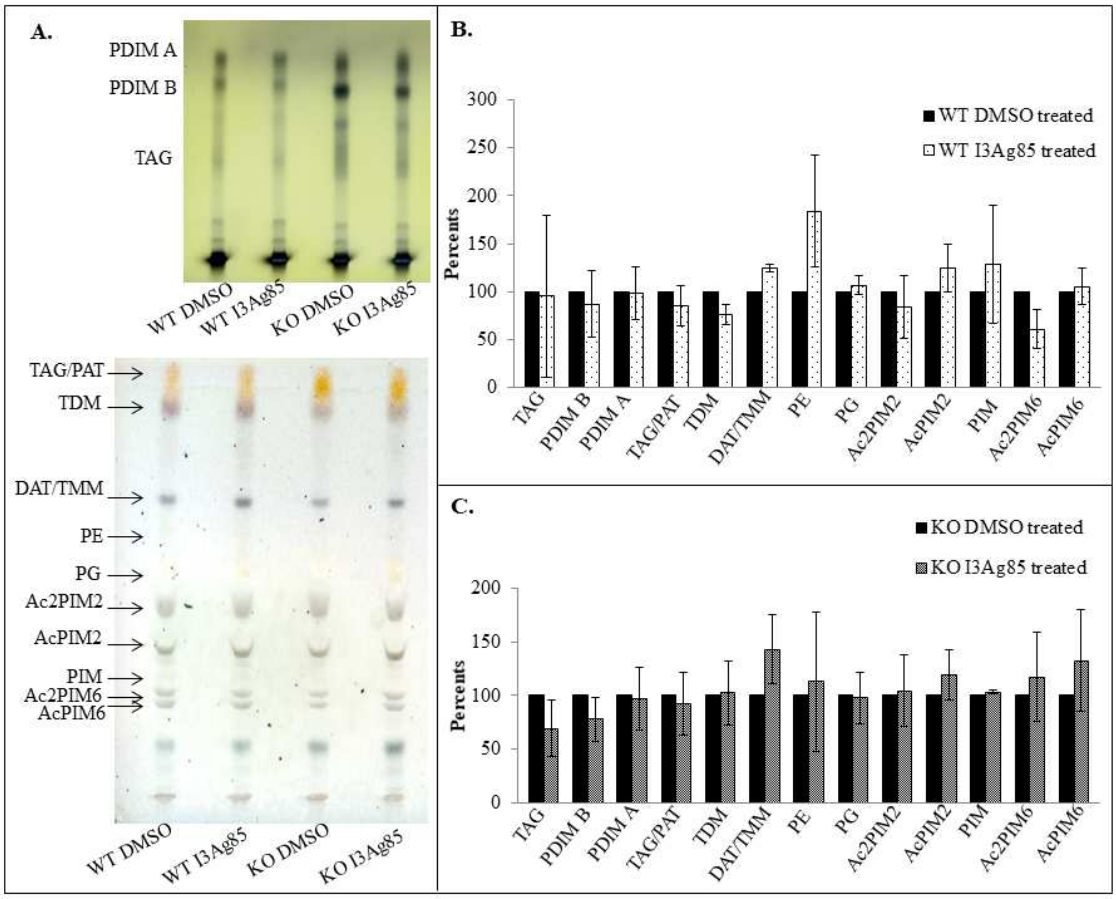
ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

FIGURE LEGEND

Fig. 1. HP-TLC analyses of the CDC1551 *wt* and *Δfbp*C strain lipids. Each experiment was performed at least three times using independent samples. **A.** HP-TLC migration profiles of lipids in petroleum ether/diethyl ether (9 :1) revealed with phosphomolybdic acid to visualise PDIM (upper panel) or migrated in $CHCl_3/CH_3OH/H_2O$ (60:35:8) revealed with anthrone to visualise more polar cell wall lipids (lower panel). **B.** Lipid spots quantification, performed on HP-TLC using primuline for the revelation, for the *wt* strain, normalized to the total amount of lipids in the I3-Ag85 treated condition compared to the DMSO control (set as 100%). **C.** Lipid spots quantification, performed on HP-TLC using primuline for the revelation, for the *Δfbp*C strain, normalized to the total amount of lipids in the I3-Ag85 treated condition compared to the DMSO control. The relative abundance of the different classes of lipids in B. and C. was determined by loading 5 µg of lipid mixture onto a HP-TLC silica gel 60 plate (Merck) with a Camag ATS4 apparatus. The plate was developed in the appropriate solvent mixture using a Camag ADC2 device and stained by the reagent with a Camag CID3 apparatus, followed by heating at 150°C for 20 min, when necessary. Lipids were quantified by absorption measurement at 400 nm with a Camag Scanner 3 device using Wincats software.

ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT