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1 **I3-Ag85 effect on phthiodiolone dimycocerosate synthesis**

2 Céline Rens<sup>a</sup>, Françoise Laval<sup>b</sup>, Ruddy Wattiez<sup>c</sup>, Philippe Lefèvre<sup>a</sup>, François Dufrasne<sup>d</sup>,  
3 Mamadou Daffé<sup>b</sup> and Véronique Fontaine<sup>a</sup>

4

5 <sup>a</sup>Université Libre de Bruxelles (ULB), Unit of Pharmaceutical Microbiology and Hygiene,  
6 CP205/2, Boulevard du Triomphe, 1050 Brussels, Belgium

7 <sup>b</sup>Institute of Pharmacology and Structural Biology, University of Toulouse, CNRS, University  
8 Paul Sabatier (UMR 5089), Department of “Tuberculosis and Infection Biology” 205 route de  
9 Narbonne, BP64182, 31077 Toulouse cedex 04, France.

10 <sup>c</sup>Department of Proteomics and Microbiology, University of Mons, 20, place du Parc, B-7000  
11 Mons, Belgium

12 <sup>d</sup>Université Libre de Bruxelles (ULB), Therapeutic Chemistry, CP205/05, Boulevard du  
13 Triomphe, 1050 Brussels, Belgium

14

15 # Address correspondence to Véronique Fontaine, [vfontain@ulb.ac.be](mailto:vfontain@ulb.ac.be)

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

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

27

28 ABSTRACT

29

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

39

40 The emergence of multi- and extensively drug resistant (MDR and XDR) *Mycobacterium*  
41 *tuberculosis* (Mtb) strains emphasized the urgent need for new antitubercular drug development  
42 [1]. In this perspective, we focused on drugs targeting the external lipid envelope of these  
43 bacteria. Mtb, the main causative agent of tuberculosis, has a particular waxy cell wall outward  
44 its peptidoglycan layer. The very long chain fatty acids, up to C100 and called mycolic acids,  
45 are attached to arabinogalactan, which in turn is covalently bound to peptidoglycan. The giant  
46 complex macromolecule interact with extractable waxy lipids, forming a hydrophobic wall [2].  
47 Among these complex lipids, trehalose dimycolate (TDM), sulfolipids (SL), diacyltrehalose  
48 (DAT), penta- or poly-acyltrehalose (PAT) and phthiocerol- or phthiodiolone dimycocerosate  
49 (PDIM A and PDIM B) are virulence factors important for host interaction. Additionally, both  
50 TDM and PDIM play an important structural role. TDM, known as “cord factor”, is involved  
51 in the host’s immune system modulation during granuloma formation, but it is also involved in  
52 the mycobacteria wall impermeability, conferring protection against drug entrance [3, 4]. PDIM  
53 A and PDIM B have been shown to be involved in mycobacterial wall impermeability against  
54 drugs, oxidative stresses and SDS [5-9].

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

63 Based on Warriar *et al.* results, we tested the susceptibility of Mtb to vancomycin in the  
64 presence of this inhibitor, I3-Ag85, in order to investigate a potential synergistic effect of this  
65 combination [11]. The I3-Ag85 was synthesized as previously described [13]. We performed  
66 drug susceptibility assay following the agar proportion method on the Mtb H37Rv strain [14].  
67 Vancomycin and I3-Ag85 were serially diluted alone or in combination in 24-well plates and  
68 inoculated with 10  $\mu$ l 10<sup>-1</sup> to 10<sup>-4</sup> dilutions of McFarland No. 1 turbidity culture. The obtained  
69 minimal inhibitory concentration (MIC) were used to calculate the fractional inhibitory  
70 concentration index (FICI) following the Checkerboard method:  $FICI = MIC_{ab}/MIC_a +$   
71  $MIC_{ba}/MIC_b$  [15]. MIC<sub>a</sub> were 50  $\mu$ g/ml for vancomycin and MIC<sub>b</sub> was 44  $\mu$ g/ml for I3-Ag85.  
72 Vancomycin serially diluted with 4.4  $\mu$ g/ml I3-Ag85 fix concentration still gave an MIC<sub>ab</sub> of

73 50 µg/ml vancomycin. Similarly, I3-Ag85 serially diluted with 10 µg/ml vancomycin fixed  
74 concentration gave a MIC<sub>ba</sub> of 44 µg/ml I3-Ag85. A FICI of 2 was obtained, showing no  
75 synergistic effect of the two drugs.

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

88 I3-Ag85 inhibitory effect should therefore rely on the inhibition of additional targets, including  
89 potentially orthologous Ag85A or B proteins, given that the KO strain is devoid of Ag85C but  
90 shows the same MIC to the inhibitor as the WT strain. We therefore analyzed their lipid  
91 composition by high-pressure thin-layer chromatography (HPTLC) as previously described  
92 [10], comparing midlog-phase growing Mtb CDC1551 WT and KO cultures (with inoculum  
93 size 100 fold higher compared to drug susceptibility assays), treated or untreated 24 h with 44  
94 µg/ml I3-Ag85 [17]. As described by Warriar *et al.*, we observed a slight decrease of TDM and  
95 an increase of DAT+TMM in the treated WT strain (Fig. 1A and B) [11]. Additionally, we  
96 observed a decrease of acylated phosphatidylinositol hexamannoside (Ac<sub>2</sub>PIM<sub>6</sub>) and an  
97 increase of phosphatidyl ethanolamine (PE) (Fig. 1A and B). Although both the I3-Ag85 treated  
98 KO and the WT strains showed an increase of DAT+TMM, the mutant additionally exhibited  
99 a decrease of the triacylglycerol (TAG) and PDIM B (Fig. 1A and C). This decrease of PDIM  
100 B by the I3-Ag85 treatment in the KO strain is highlighted by the stronger PDIM B HPTLC  
101 signal intensity compared to the untreated WT strain (Fig. 1A).

102 As reported by Warriar et al, mycolic acid methyl esters (MAME) were not notably changed  
103 by the I3-Ag85 treatment, suggesting that other enzymes, including the orthologous Ag85A or  
104 B proteins could rescue mycolic acid transfer on arabinogalactan (data not shown). Considering

105 that Warriar et al. also reported a free mycolic acid change, these authors suggested that a  
106 specific effect on the TDM synthesis by this inhibitor [11].

107 Our lipid analyses, especially on the KO strain lacking Ag85C, suggest that the I3-Ag85 has an  
108 additional effect by reducing the PDIM B production, either directly or indirectly . Indeed, a  
109 change in the balance between an acetyl-CoA derived lipid (e.g. TMM) and a propionyl-CoA  
110 derived lipids (e.g. PDIM B), as observed by the I3-Ag85 treatment, has been already reported  
111 in a  $\Delta mce1$  KO mutant strain [18]. Propionyl-CoA derived lipid synthesis could protect bacteria  
112 against propionate induced toxicity [2]. The inhibition of PDIM synthesis could therefore be  
113 harmful for Mtb and explain the I3-Ag85 susceptibility of both WT and MT0137  $\Delta fbpC$  strains.

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117

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169

170 FIGURE LEGEND

171

172 Fig. 1. HP-TLC analyses of the CDC1551 *wt* and *ΔfbpC* strain lipids. Each experiment was  
173 performed at least three times using independent samples. **A.** HP-TLC migration profiles of  
174 lipids in petroleum ether/diethyl ether (9 :1) revealed with phosphomolybdic acid to visualise  
175 PDIM (upper panel) or migrated in CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O (60:35:8) revealed with anthrone to  
176 visualise more polar cell wall lipids (lower panel). **B.** Lipid spots quantification, performed on  
177 HP-TLC using primuline for the revelation, for the *wt* strain, normalized to the total amount of  
178 lipids in the I3-Ag85 treated condition compared to the DMSO control (set as 100%). **C.** Lipid  
179 spots quantification, performed on HP-TLC using primuline for the revelation, for the *ΔfbpC*  
180 strain, normalized to the total amount of lipids in the I3-Ag85 treated condition compared to  
181 the DMSO control. The relative abundance of the different classes of lipids in B. and C. was  
182 determined by loading 5 μg of lipid mixture onto a HP-TLC silica gel 60 plate (Merck) with a  
183 Camag ATS4 apparatus. The plate was developed in the appropriate solvent mixture using a  
184 Camag ADC2 device and stained by the reagent with a Camag CID3 apparatus, followed by  
185 heating at 150°C for 20 min, when necessary. Lipids were quantified by absorption  
186 measurement at 400 nm with a Camag Scanner 3 device using Wincats software.

187