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

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

19

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 μ l 10⁻¹ to 10⁻⁴ 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_a were 50 μ g/ml for vancomycin and MIC_b was 44 μ g/ml for I3-Ag85.
72 Vancomycin serially diluted with 4.4 μ g/ml I3-Ag85 fix concentration still gave an MIC_{ab} of

73 50 µg/ml vancomycin. Similarly, I3-Ag85 serially diluted with 10 µg/ml vancomycin fixed
74 concentration gave a MIC_{ba} 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 (Ac₂PIM₆) 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 CHCl₃/CH₃OH/H₂O (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