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Aloe Emodin Reduces Phthiodiolone Dimycocerosate Potentiating Vancomycin Susceptibility on Mycobacteria

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21 ABSTRACT

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23 Multi- and extensively-drug resistant (MDR and XDR) *Mycobacterium tuberculosis* strains
24 represent an international health issue requiring immediate solutions. Here, we studied the
25 susceptibility of *M. tuberculosis* H37Rv as well as MDR and XDR strains to a combination of
26 a glycopeptide with a plant secondary metabolite, aloe emodine (AE). This combination
27 showed a partial synergic effect. Further analyses of the mycobacterial envelope revealed a
28 quite selective reduction of phthiodiolone dimycocerosate by AE concordant with the
29 observed partial synergic effect.

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41 *Mycobacterium tuberculosis* is the main etiological agent of tuberculosis (TB). This disease is
42 an international health issue, affecting one third of worldwide population and causing 1.4
43 million deaths annually. The emergence of multi- and extensively-drug-resistant strains
44 (MDR-TB and XDR-TB) makes current treatment less or barely not efficient on infected
45 patients (1). This emerging issue requires new drugs focusing on new targets. Medicinal plant
46 drug discovery has been useful strategy providing numerous new compounds against various
47 pathologies, including cancer and malaria (2-4). Here we studied a plant secondary
48 metabolite, the aloe emodin (AE), exhibiting among others potential anticancer activities.

49 The aloe emodin is an anthraquinone produced by several plants, among those the well-
50 known Aloe vera. Interestingly, Aloe vera extracts have been reported for their antibacterial
51 properties including an antimycobacterial effect (5, 6) and aloe emodin, one of its
52 constituents, has also been shown to possess antibacterial activities (7) and antimycobacterial
53 activities (8). *In silico* studies suggested that an acyl carrier protein (ACP) synthase could be
54 an AE target (9).

55 The potential action of AE on an ACP synthase led us to test if it could affect the
56 mycobacterial cell wall lipid synthesis and therefore could also synergize with vancomycin to
57 inhibit mycobacterial cell growth. We previously showed that vancomycin was efficient only
58 on mycobacteria possessing altered envelope, especially on those lacking phthiocerol and
59 phthiodiolone dimycocerosates, PDIM A and PDIM B, respectively (10). This envelope is
60 composed, from the outside, of a capsule containing selective complex waxy lipids (including
61 PDIM A and PDIM B), which may also be present in the outer leaflet of the mycomembrane,
62 whose inner leaflet is composed of long mycolic acids. The latter long chain fatty acids
63 esterified the heteropolysaccharide arabinogalactan, which in turn is covalently attached to
64 peptidoglycan (11). Enzymes specifically involved in this waxy wall synthesis belong to the
65 fatty acid synthases (FAS) I or II pathway. The FAS I system is a multi-domain protein

66 complex while the FAS II system is composed of multiple enzymes with separated functional
67 domains. Both FAS enzymes possess ACP domains. Well known anti-tuberculosis drugs are
68 targeting FAS II enzymes, such as isoniazid (INH) inhibiting InhA enoyl ACP reductase and
69 KasA ACP synthase, both involved in mycolic acid synthesis (12). The β -ketoacyl ACP
70 synthase docking model described by Ramesh *et al.* proposed a more potent effect of AE on
71 this enzymatic domain compared to cerulenine, a strong mycobacterial lipid synthesis
72 inhibitor (9). This potential protein interaction is interesting considering that cerulenine can
73 synergize with vancomycin to inhibit MDR and XDR strains growth (10). Consequently, we
74 investigated the action of the AE on the mycobacterial lipid cell wall integrity and its ability
75 to synergize with vancomycin to inhibit the growth of mycobacteria including different *M.*
76 *tuberculosis* strains.

77 Preliminary tests were performed on *M. bovis* BCG using the BacT/ALERT MP system. The
78 BacT/ALERT MP bottle (10 ml) completed with restoring fluid (0.5 ml) and drugs (0.1 ml)
79 were inoculated with a 0.5 McFarland diluted *M. bovis* BCG culture (0.5 ml) grown to mid-
80 log phase in a standard 7H9 medium, with 0.05% tween and 10% ADC (albumin dextrose
81 catalase). A dedicated bottle contained a 1/100 dilution of the initial inoculum, so that when
82 the mycobacterial growth in the other bottles (containing drugs) were flagged positive at the
83 same time or after the 1/100 inoculum control bottle, we could consider that the minimum
84 inhibitory concentration (MIC or 99% inhibition of the bacterial growth) was reached at the
85 drug concentration present in the bottles (13,14).

86 We investigated synergy with vancomycin calculating the $\Delta X/\Delta Y$ ratio. ΔX corresponds to
87 the growth index (GI) of both drugs in combination and ΔY to the lowest GI of one of these
88 drugs alone. Synergy is reached when $\Delta X/\Delta Y$ quotient is < 0.5 (10). Table 1A shows $\Delta X/\Delta Y$
89 quotients obtained for *M. bovis* BCG treated with a combination of 10 $\mu\text{g/ml}$ vancomycin
90 with 3 $\mu\text{g/ml}$ AE. Quotients obtained varied from 0.3 to > 0.5 .

91 This combination was further studied on *M. tuberculosis* by the CLSI approved agar
92 proportion method (13). Synergy was investigated following the checkerboard process.
93 Minimal inhibitory concentration (MIC) of each drug alone (MIC_a or MIC_b) or in
94 combination (MIC_{ab} or MIC_{ba}) allowed us to calculate a fractional inhibitory index (FIC),
95 FIC_a : MIC_{ab}/ MIC_a and FIC_b: MIC_{ba}/ MIC_b. These FIC would allow to calculate a
96 fractional inhibitory concentration index (FICI) : FIC_a + FIC_b. A FICI ≤ 0.5 indicates synergy
97 of drugs “a” and “b” together (15). As shown in Table 1B, we obtained a MIC for
98 vancomycin of 50-100 µg/ml, a MIC for AE of 12.5-25 µg/ml, a MIC for vancomycin with a
99 fixed 2.5 µg/ml AE of 12.5-25 µg/ml and a MIC for AE with a fixed 5 µg/ml vancomycin of
100 6.25- 12.5 µg/ml. These MICs allowed us to calculate a FICI of 0.375-1. The effect of the
101 combination of both drugs is therefore just between a synergy and an additive effect, both on
102 *M. bovis* BCG and on the H37Rv *M. tuberculosis* strain.

103 The lipid composition of H37Rv *M. tuberculosis* exposed to AE was analyzed in order to
104 better assess the potential effect of AE on the mycobacterial cell wall lipid synthesis. Total
105 lipid extracts were analyzed by high performance thin layer chromatography (HPTLC)
106 following the procedure already described by Simeone *et al.* (16). The HPTLC plates were
107 scanned to measure the absorption of different spots in order to compare, in percentage, AE-
108 treated lipid extracts to drug-free extracts.

109 Interestingly we observed a decrease of the PDIM B and triacylglycerol (TAG) and a slight
110 decrease of PDIM A in AE-treated *M. tuberculosis* (Fig. 1). On the opposite, no variation in
111 mycolic acid methyl esters (MAMES) were detected in AE-treated samples compared to the
112 control condition (data not shown). As most of the compounds able to synergize with
113 vancomycin against mycobacterial cell growth act by both reducing PDIM A and PDIM B
114 lipids in the mycobacterial cell wall (10, 17), it is tempting to assume that the weak synergy

115 observed by the combination of AE with vancomycin could result from a less pronounced
116 effect of AE on PDIM A.

117 Considering that vancomycin combined with cerulenin is efficient on most MDR and XDR
118 strains (10), we tested AE (3 $\mu\text{g}/\text{mL}$) and vancomycin (10 $\mu\text{g}/\text{mL}$), both separate and
119 combined on a set of MDR and XDR strains by using the MGIT960/TBeXIST system
120 according to the manufacturer's instructions. Only one strain out of 6 showed a clear synergy
121 to the combination of AE with vancomycin, giving a $\Delta X/\Delta Y$ ratio of 0.099 (Table 1C). These
122 results were confirmed by agar proportion method, showing synergy for the same XDR strain
123 (ID W_14614) termed isolate 3 in Table 1C. AE, alone, was previously shown to be inactive
124 on MDR and XDR strains (8). Here, we showed that AE can also rarely synergize with
125 vancomycin on such strains.

126 In conclusion, the combination of AE and vancomycin shows an intriguing result between a
127 synergy and an additive effect on *M. bovis* BCG, *M. tuberculosis* H37Rv and only one out of
128 6 MDR strains was susceptible to AE in combination with vancomycin. Lipid analysis
129 revealed a slight decrease of PDIM A compared to PDIM B that could explain this
130 intermediate effect of AE in combination vancomycin.

131 Although AE is not showing a strong synergy with vancomycin, this drug should not be
132 discarded as it could still inhibit some MDR strain. Moreover, AE is a good drug candidate
133 acting against parasites, fungi and viruses with potential action against HIV-1 (18).
134 Considering the frequent co-infection of *M. tuberculosis* with HIV, the use of a drug efficient
135 on various pathogens would be of great interest. Obviously, the action of AE on *M.*
136 *tuberculosis* co-infected HIV patients should be further investigated.

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195

196 **FIG 1** Lipid profile of 25 µg/ml AE-treated *M. tuberculosis*, expressed in percent compared
197 to a DMSO-treated control condition. A. Lipid quantification of triacylglycerol (TAG),
198 phthiocerol dimycocerosates (PDIM A) and phthiodiolone dimycocerosates (PDIM B) based
199 on fluorometric recording of primuline-treated TLC. B. Corresponding HPTLC of TAG,
200 PDIM A and PDIM B separated with petroleum ether/diethylether (9:1, v/v) and visualized by
201 primuline.

202

203 **TABLE 1** Drug susceptibility on (A) *M. bovis* BCG analyzed by the BaCT/ALERT system
204 using 3 µg/ml AE and 10 µg/ml vancomycin, on (B) *M. tuberculosis* H37Rv by the agar
205 proportion method and on (C) MDR- and XDR-TB strains using the MGIT method and 3
206 µg/ml AE and 10 µg/ml vancomycin.

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