Indian J Microbiol
https://doi.org/10.1007/s12088-018-0734-0

SHORT COMMUNICATIONS

Aloe Emodin Reduces Phthiodiolone Dimycocerosate Potentiating Vancomycin Susceptibility on Mycobacteria

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Received: 30 January 2018 / Accepted: 22 April 2018

Abstract Treatment of tuberculosis still represent a major public health issue. The emergence of multi-and extensively-drug resistant (MDR and XDR) *Mycobacterium tuberculosis* clinical strains further pinpoint the urgent need for new anti-tuberculous drugs. We previously showed that vancomycin can target mycobacteria lacking cell wall integrity, especially those lacking related phthiocerol and phthiodolone dimycocerosates, PDIM A and PDIM B, respectively. As aloe emodin was previously hypothesized to be able to target the synthesis of mycobacterial cell wall lipids, we tested its ability to potentiate glycopeptides antimycobacterial activity. The aloe emodin with the vancomycin induced a combination effect beyond simple addition, close to synergism, at a concentration lower to reported IC_{50} cytotoxic value, on *M. bovis* BCG and on H37Rv *M. tuberculosis.* Interestingly, out of six MDR and pre-XDR clinical strains, one showed a strong synergic susceptibility to the drug combination. Mycobacterial cell wall lipid analyses highlighted a selective reduction of PDIM B by aloe emodin.

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Keywords *Mycobacterium tuberculosis* · Aloe emodin · PDIM · Vancomycin · MDR

Mycobacterium tuberculosis is the main etiological agent of tuberculosis (TB), affecting one-third of worldwide population and causing 1.3 million deaths annually in 2016. There is a growing concern on the emergence of multi- and extensively-drug-resistant tuberculosis (MDR-TB and XDR-TB) as their current treatment is only approximatively successful in 54% [1]. This emerging issue requires new drugs focusing on new targets. Here we studied a plant secondary metabolite, the aloe emodin, exhibiting, among others, potential anticancer activities.

The aloe emodin is an anthraquinone produced by several plants, among others, the Aloe vera. Interestingly, Aloe vera extracts have been reported for their antibacterial properties including for an antimycobacterial effect [2]. The aloe emodin, one of its compounds, has also been shown to possess antibacterial and antimycobacterial activities [3, 4]. In silico studies suggested that an acyl carrier protein (ACP) synthase could be the target of the aloe emodin [5].

The potential action of the aloe emodin on an ACP synthase allowed us to hypothesized that this compound could synergize with vancomycin by targeting the synthesis of mycobacterial cell wall lipids. Indeed, we previously showed that vancomycin could target mycobacteria lacking phthiocerol and phthiodiolone dimycocerosates, PDIM A and PDIM B, respectively, present in the outermost layer of their cell wall [6]. The inner leaflet of this membrane is composed of long mycolic acids, esterified to heteropolysaccharide arabinogalactan which is in turn covalently attached to peptidoglycans [7]. The enzymes specifically involved in this waxy wall synthesis belong to

Published online: 04 May 2018

the fatty acid synthases (FAS) I or II pathway. The FAS I system is a multi-domain protein complex while the FAS II system is composed of multiple enzymes with separated functional domains. Both FAS enzymes possess ACP domains. Some anti-tuberculosis drugs are targeting FAS II enzymes involved in mycolic acid synthesis, such as isoniazid (INH), inhibiting the InhA enoyl ACP reductase and KasA inhibiting the ACP synthase [7]. The β-ketoacyl ACP synthase docking model described by Ramesh et al. proposed a more potent effect of the aloe emodin on the ACP enzymatic domain, compared to cerulenine, a strong mycobacterial lipid synthesis inhibitor able to synergize with vancomycin to inhibit MDR and XDR strains [5, 6]. Consequently, we investigated the ability of the aloe emodin to synergize with vancomycin to inhibit mycobacterial cell growth and its impact on mycobacteria lipid cell wall composition.

Preliminary tests were performed on *M. bovis* BCG using the BacT/ALERT MP system. As previously described, the BacT/ALERT MP bottle with or without drugs were inoculated with a 0.5 McFarland diluted *M. bovis* BCG culture grown to mid-log phase in a standard 7H9 medium, in the presence of 0.05% Tween 80 and 10% albumin dextrose catalase [8]. We considered that the minimum inhibitory concentration (MIC or 99% inhibition of the bacterial growth) was reached in the drug containing bottles flagged positive in the meantime as the 1/100 inoculum control bottle [8]. The bacterial growth was measured by a colorimetric system given in reflectance units (= growth index, GI).

We investigated synergy with vancomycin calculating the ΔX/ΔY ratio. When the 1/100 vial's GI differentiated from the lag phase GI, GI measures were also taken after one additional day. A ΔGI between the two consecutive days was calculated for the vials containing drug combination (ΔX) and containing only one drug. The lowest ΔGI from the two vials containing only one drug consisted in the ΔY. A ΔX/ΔY quotient was calculated, indicating a synergy between drugs if < 0.5 [6]. The ΔX/ΔY quotient obtained for *M. bovis* BCG treated with a combination of 10 μg/ml vancomycin (Sigma Aldrich) with 3 μg/ml aloe emodin (TCI chemicals) was $0{,}83 \pm 0.48$.

This combination was further studied on *M. tuberculosis* by the Clinical & Laboratory Standards Institute (CLSI) approved agar proportion method [8]. Synergy was investigated following the checkerboard process. Minimal inhibitory concentration (MIC) of each drug alone (MICa or MICb) or in combination (MICab or MICba) allowed us to calculate a fractional inhibitory index (FIC), FICa = MICab/MICa and FICb = MICba/MICb. These FIC would allow to calculate a fractional inhibitory concentration index (FICI) = FICa + FICb. A FICI ≤ 0.5 indicates synergy of drugs "a" and "b" together [8]. We obtained a MIC for vancomycin of 50–100 μg/ml, a MIC for aloe emodin of 12.5–25 μg/ml, a MIC for vancomycin with a fixed 2.5 μg/ml aloe emodin of 12.5–25 μg/ml (p value = 0.0191) and a MIC for aloe emodin with a fixed 5 μg/ml vancomycin of 6.25–12.5 μg/ml. The FICI was 0.73 ± 0.16, just between a synergy and an additive effect, both on *M. bovis* BCG and on the H37Rv *M. tuberculosis* strain.

The lipid composition of H37Rv *M. tuberculosis* exposed to aloe emodin was analyzed to better assess its potential effect on mycobacterial cell wall lipid synthesis. Total lipid extracts were analyzed by high performance thin layer chromatography (HPTLC) following the procedure described by Simeone et al. [9]. Their identifications were based on their relative mobilities compared to structurally well-characterized external standards. Lipid quantification were performed based on fluorometric analysis of TLC to be converted to a percentage, with untreated samples normalized to 100%.

Interestingly we observed a decrease of the PDIM B and triacylglycerol (TAG) and a slight decrease of PDIM A in aloe emodin-treated *M. tuberculosis* (Fig. 1) and no variation in mycolic acid methyl esters (MAMES) (data not shown). As vancomycin can target mycobacteria lacking PDIM A and PDIM B lipids in their cell wall [6], it is tempting to assume that the weak synergy observed by the combination of aloe emodin with vancomycin result from the PDIM B reduction attenuated by a mild impact on PDIM A.

Considering that vancomycin combined with cerulenin is efficient on most MDR and XDR strains [6], we tested aloe emodin (3 μg/mL) and vancomycin (10 μg/mL), both separate and combined on a set of MDR and pre-XDR clinical isolates using the MGIT960/TBeXIST system. The aloe emodin alone was inactive on all the isolates (Table 1), as previously described [4]. Only one out of 6 vancomycin resistant isolates showed a clear synergy to the combination of aloe emodin with vancomycin, giving a ΔX/ΔY ratio of 0.099 (Table 1). These results were confirmed by agar proportion method.

In conclusion, the combination of aloe emodin and vancomycin showed a result between a synergy and an additive effect on *M. bovis* BCG and on *M. tuberculosis* H37Rv and a strong synergy on one out of 6 MDR/pre-XDR strains. Although the amount of PDIM B is decreased by approximately 50% using the aloe emodin, its slight impact on PDIM A could explain the intermediate effect of the combination.

Further studies should be carried out with other drugs to reduce its concentration below its reported IC_{50} value. This molecule has an in vitro cytotoxic effect on different eukaryotic cell lines with IC_{50} values ranging between 2.4 and 10.8 μg/ml [10, 11]. As aloe emodin is also a drug

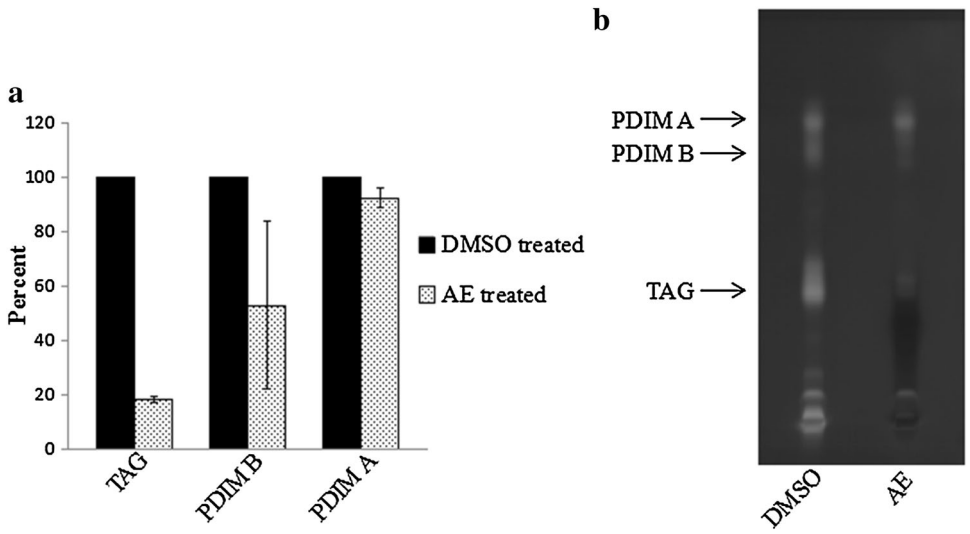

Fig. 1 Lipid profile of 25 µg/ml aloe emodin (AE)-treated *M. tuberculosis*, expressed in percent compared to a DMSO-treated control condition. **a** Lipid quantification of triacylglycerol (TAG), phthiocerol dimycocerosates (PDIM A) and phthiodiolone dimycocerosates (PDIM B) based on fluorometric recording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

Table 1 Drug susceptibility on MDR- and pre-XDR-TB strains using the MGIT method and 3 µg/ml AE and 10 µg/ml vancomycin

Isolate[a]	FP[b]	TN no.[c]	Resistance profile		ΔX/ΔY
			First-line drugs	Second-line drugs	
1	OO1_16054	16054	INH, RIF, EMB, STR	RMC, PAS	> 0.5
2	P_16442	16442	INH, RIF, PZA, STR	RMC, PAS	> 0.5
3	W_14614	14614	INH, RIF, EMB, STR	ETH, AMI, KAN, RFB, RIP	0.099
4	W12_15183	15183	INH, RIF, EMB, STR	ETH, AMI, KAN	> 0.5
5	W_14003	14003	INH, RIF, EMB, STR	ETH, RFB, RMC, PAS	> 0.5
6	W148_13438	13438	INH, RIF, EMB, PZA, STR	KAN, AMI, RFB, RIP	> 0.5

AMI Amikacin; *EMB* ethambutol; *ETH* ethionamide; *INH* isoniazid; *KAN* kanamycin; *PAS para*-aminosalicylic acid; *PZA* pyrazinamide; *RFB* rifabutin; *RIF* rifampin; *RIP* rifapentine; *RMC* rifamycin; *STR* streptomycin

[a]Isolates 3, 4 and 6 are pre-XDR strains

[b]FP, fingerprint name based on IS*6110* typing and PHRI nomenclature

[c]TN no., tracking number, a PHRI unique identifier for each isolate

candidate to fight against parasites, fungi and viruses, considering the frequent co-infection of *M. tuberculosis* with HIV, the action of the aloe emodin should be further investigated in combination with other drugs [12].

Acknowledgements Céline Rens was supported by "Les amis des Instituts Pasteurs à Bruxelles" asbl. We thank Pr. Caroline Stevigny for providing us the first aloe emodin pure product and Dong Yang for his help in the manuscript layout.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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