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DRUG DISCOVERY AND RESISTANCE

Morphological changes and differentially expressed efflux pump genes in *Mycobacterium tuberculosis* exposed to a rifampicin and verapamil combination

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SUMMARY

The aim of the present study was to (*i*) evaluate the *in vitro* action of rifampicin (RIF), ethambutol or isoniazid with efflux pumps inhibitors (EPIs) in *Mycobacterium tuberculosis* (*Mtb*) $H_{37}Rv$ and (*ii*) evaluate the morphological and efflux pumps (EPs) transcriptional changes by the action of rifampicin + verapamil combination (RIF + VP). The minimal inhibitory concentration and synergic effect of drug combinations were determined by Resazurin Microtiter Plate Assay and Resazurin Drugs Combination Microtiter Assay, respectively. VP showed greater capacity of ethidium bromide accumulation and RIF + VP had the lower fractional inhibitory concentration index. The RIF + VP exerted a similar reduction of viable cell counts to RIF by time–kill curve, but decreases in the expression of EPs genes were observed by Real time PCR at 72 h of RIF + VP exposure. Accumulative morphological changes (wrinkled and rounding) caused by each drug were observed by scanning electron microscopy after RIF + VP exposure. The downexpression of EPs related genes exposed to RIF + VP, suggest an effective inhibitory activity of VP in *Mtb* $H_{37}Rv$. The role of EPs and the use of EPIs open up a powerful approach and the RIF + VP combination should be studied in *Mtb* more thoroughly.

1. Introduction

Tuberculosis (TB) is responsible for 8.6 million new cases and accounted for 1.3 million deaths in 2012, especially in poor countries [1]. The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* (*Mtb*) has become a major public health concern worldwide [2].

Mtb are naturally resistant to commonly used drugs because of the slow uptake of drugs across the highly hydrophobic mycobacterial cell envelop [3]. In addition to the unique structure of the bacteria, mutations in target genes, which encode proteins involved in the drug metabolism, are known to be an important mechanism of resistance. However, explaining the resistance to anti-TB drugs, without mutations in known target genes, has not yet been possible. Recently, this resistance has been attributed to active drug efflux mechanism.

Bacterial efflux pumps (EPs) are membrane proteins that are capable of actively transporting a broad range of drugs [3,4]. The constitutive or inducible expression of EPs in response to treatment contributes to a decrease in the intracellular concentration of anti-TB drugs and thus resistance by the bacillus [5,6].

Mtb has a large number of putative drug EPs genes [2]. Some EPs have been described and well characterized in *Mtb* as belonging to the adenosine triphosphate binding cassette (ABC), major

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facilitator superfamily (MFS), resistance nodulation division (RND), and small multidrug resistance (SMR) families [2,3,7]. Some EPs have been reported to play a role in resistance to anti-TB drugs, such as rifampicin (RIF), ethambutol (EMB) and isoniazid (INH) [5,8,9].

Efflux pump inhibitors (EPIs) have been tested in combinations with anti-TB drugs to increase the intracellular concentration and restore the activity of the drugs. Some EPIs, affect transmembrane electrochemical potential and others are calcium channel antagonist such as carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) and verapamil (VP), respectively [10]. However, the mechanisms involved in the induction and regulation of EPs are not yet fully understood [2].

Studies *in vitro* [11] and *in vivo* [12] have shown the applicability of EPIs, used as antiarrhythmic, antihypertensive, antiulcer, and antiemetic [10], in restoring the susceptibility of *Mtb* MDR clinical isolates to anti-TB drugs.

EPs-mediated resistance has become important, once help the mycobacteria survive under presence of the anti-TB drugs, until relevant mutations emerge in the genome [11]. In this sense, the use of EPIs, as an adjunctive therapy, in attempt to restore the anti-TB drugs activity or accelerate the treatment, seems to be of interest [12]. For this, the knowledge of the effect of combination EPIs and anti-TB drugs, as well as, the influence of this combination in morphology and gene expression in the bacillus could help us to understand the advantages of this therapeutic approach.

The aim of the present study was to (*i*) evaluate the *in vitro* action of RIF, EMB or INH with EPIs (CCCP or VP) combinations in *Mtb* $H_{37}Rv$ and (*ii*) evaluate possible morphological and EPs transcriptional changes in *Mtb* $H_{37}Rv$ exposed to the anti-TB drug and EPI combination, which had the lower fractional inhibitory concentration index (FICI) against the bacillus.

2. Materials and methods

2.1. Bacterial culture

M. tuberculosis $H_{37}Rv$ (ATCC 27294), was used throughout the study. Bacterial cells were grown at 35–37 °C for 15 days in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) supplemented with 10% (v/v) oleic acid–bovine serum albumin–dextrose catalase enrichment (OADC, BBL/Becton–Dickinson, Sparks, MD, USA), with the addition of 0.2% glycerol (v/v) and 0.025% Tween 80 (v/v).

2.2. Anti-TB drugs and efflux pump inhibitors

All drugs were provided by Sigma (St. Louis, MO, USA). INH, EMB, and VP were prepared in distillate water, CCCP in dimethyl sulfoxide (DMSO; Synth, Diadema/SP, Brazil) and RIF in methanol:water (1:10, v/v). Further dilutions were prepared in OADC-supplemented Middlebrook 7H9 with the following concentration ranges: RIF (0.0005–0.25 μg/mL), INH (0.0009–0.25 μg/mL), EMB (0.125–32 μg/mL), CCCP (0.39–100 μg/mL), and VP (3.90–1000 μg/mL). The final DMSO and methanol concentrations had no effect on *Mtb* growth.

2.3. Determination of MIC and checkerboard assay

The MIC was determined in triplicate for anti-TB drugs and EPIs using the Resazurin Microtiter Assay Plate (REMA) as described by Palomino et al. [13].

The interactions between anti-TB drugs and EPIs were evaluated in triplicate using the Resazurin Drugs Combination Microtiter Assay (REDCA) as described previously by Caleffi-Ferracioli et al. [14]. The fractional inhibitory concentration index (FICI) was used to evaluate the drugs combination and the results were interpreted as synergism (FICI $\leq$ 0.50), indifference/additive (FICI > 0.50–4) or antagonism (FICI > 4) [15].

2.4. Accumulation of ethidium bromide

The ethidium bromide (EtBr) MIC was determined by the REMA [13] and EtBr accumulation in *Mtb* cells was assessed by fluorometry [6,16]. *Mtb* $H_{37}Rv$ was grown in 7H9-OADC medium at 35–37 °C until an optical density at 600 nm (OD_{600}) of 0.6–0.8. The culture was centrifuged at 2880 × g for 10 min. The pellet was washed and resuspended in phosphate-buffered saline (PBS; pH 7.4). After adjusting the OD_{600} to 0.4 with PBS (with 0.05% Tween 80, Synth, Diadema/SP, Brazil), 100 μL aliquots of bacterial suspension were transferred to microplate wells that contained 0.25 μg/mL EtBr (0.5 × MIC) (26). Ten microlitres of CCCP and VP (0.5 × MIC) were added to the corresponding well in the microplate and incubated at 25 °C for 15 min. Fluorescence was determined in the absence of CCCP and VP as a reference assay. Relative fluorescence to EtBr-loaded cells was acquired every 51 s for 60 min at 37 °C in a VICTOR^2 D fluorometer (PerkinElmer, Santa Clara, CA, USA) using 530/25 nm as the excitation wavelengths and 590/20 nm as the detection wavelengths, respectively [6]. The relative fluorescence values were obtained by normalizing the data against the background fluorescence of EtBr. The relative final fluorescence (RFF) was determined using the formula $(RF_{assay} - RF_{ref})/RF_{ref}$, where RF_{assay} is the relative fluorescence at the last time point (minute 60) of the EtBr accumulation assay with EPIs, and RF_{ref} is the relative fluorescence at the last time point of the EtBr accumulation assay without EPIs [6].

2.5. Time–kill curve assay

The time–kill curve was performed with the drug combination that showed the lowest FICI by REDCA using the 0.5 × MIC of each drug. Five millilitres of *Mtb* $H_{37}Rv$ (7.5×10^6 CFU (colony-forming unit)/mL, range $6–8 \times 10^6$) in OADC-supplemented Middlebrook 7H9 was exposed to RIF, VP and to RIF + VP combination, in shaking at 96 rpm at 35–37 °C. A growth control without drugs was included. Aliquots (0.1 mL) were removed at 0, 1, 2, 3, 5, 7 days, provided that mycobacterial suspensions did not show visible aggregation, and serially diluted (10^{-1}, 10^{-3} and 10^{-5}) in sterile saline to avoid RIF and VP carry-over. Afterward, 20 μL of each dilution was seeded on OADC-supplemented Middlebrook 7H11 (Difco Laboratories, Detroit, MI, USA). The plates were incubated at 35–37 °C for 21 days, and the colonies were counted. The time–kill curves were performed in two time independent experiments. Synergy was defined as a decrease of $\geq$2 $\log_{10}$ CFU/mL compared with the most active single drug [17].

2.6. Scanning electron microscopy

The RIF and VP combination that showed the lowest FICI, by REDCA, was selected for scanning electron microscopy (SEM). *Mtb* $H_{37}Rv$ was exposed to the 0.5 × MIC of VP, RIF, and RIF + VP for 16 and 72 h at 35–37 °C. After drug exposure, the cells were fixed with 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (Electron Microscopy Science, Hatfield, PA, USA) for at least 2 h at 4 °C. The treated cells were placed on a glass support with poly-L-lysine (Sigma), dehydrated in graded ethanol, critical-point-dried in CO_2, coated with gold, and observed in a Shimadzu SS-550 (Kyoto, Japan) scanning electron microscope. An average of 20–30 microscopic fields, in each sample, were selected by random scanning

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