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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) [16]. Ten microliters 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 × 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

and photographed. SEM was performed in duplicate with different cultures to ensure reproducibility of the obtained data.

2.7. Efflux pump gene expression

Mtb H_{37}Rv was exposed to VP, RIF, and RIF + VP (0.5 × MIC) for 16 and 72 h at 35–37 °C. Total RNA from growth was extracted in two time-independent experiments and purified using RNeasy Mini Kit Plus (Qiagen Biotechnology, Valencia, CA, USA) according to the manufacturer's instructions. Quantification/purity and quality assessments were performed with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). RNA quality (RIN > Qubit 2.0 fluorometer9.0) was assessed using an Agilent BioAnalyzer. Contaminating DNA was removed by prior treatment with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA).

First-strand cDNA was synthesized with random primer (Invitrogen, Carlsbad, CA, USA) and qPCR was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA). EPs specific primers (Promega, Madison, WI, USA) are listed in Table 1. Melting curves for reactions were assessed, and samples were run in triplicate. The 16S RNA (*rrs*) gene was used to normalize all reactions. Negative control was performed in all experiments. A reference assay was conducted in the absence of drugs. The relative quantification of target gene expression was calculated by the $2^{-\Delta\Delta CT}$ method [18]. The data analysis was performed using a one-way test with SAS 9.0 software (SAS OnlineDoc 9, SAS Institute, Cary, NC, USA), followed by the Tukey *post hoc* test ($p < 0.01$ were considered significant).

3. Results

The MICs for RIF, EMB, INH, CCCP, and VP were 0.004, 2.0, 0.03, 3.125, and 125 μg/mL, respectively. The FICIs observed by REDCA were 0.75 and 1.5 for RIF + VP and RIF + CCCP, respectively. For the other drug combinations, the FICI was 2 (Table 2).

The 0.25 μg/mL (0.5 × MIC) of EtBr did not affect cell viability (influx-efflux in equilibrium). EtBr efflux was inhibited by VP and CCCP (Figure 1). The RFF was 0.544 for VP and 0.154 for CCCP.

Time–kill curves were performed for VP (EPI that showed the largest intracellular EtBr accumulation), RIF + VP (combination that showed the lowest FICI by REDCA), and RIF (drug of choice) (Figure 2). No decrease ≥ 2 $\log_{10}$ CFU/mL was observed with *Mtb* H_{37}Rv exposed to RIF + VP compared with RIF alone.

Figure 3.1a and b shows the appearance of drug-unexposed cells at 16 and 72 h, respectively, with dimorphic rod-shaped cells, a smooth surface, and typical cord formation. Figure 3.2, 3.3, and 3.4 show the aspects of treated cells. Wrinkled and rounding cells were observed in *Mtb* H_{37}Rv exposed to RIF and VP, respectively, mainly at 72 h. The effect of RIF on cell morphology was less evident (Figure 3.2a and b) compared with cells exposed to VP (Figure 3.3a and b). A summation of effects caused by RIF and VP exposure was observed with the RIF + VP, with a larger intensity at 72 h (Figure 3.4a and b).

Figure 4 shows the relative quantification of the transcript levels for 12 selected EP genes in *Mtb* H_{37}Rv exposed to RIF, VP, and RIF + VP for 16 h (Figure 4a) and 72 h (Figure 4b). After 16 h of drug exposure, a significant difference in relative expression ($p \leq 0.01$) was detected in six EP genes (*Rv1458*, *Rv1218*, *Rv1819*, *Rv2846*, *Rv1258* and *Rv1410*) for RIF, nine (*Rv1456*, *Rv1458*, *Rv1218*, *Rv1457*, *Rv1819*, *Rv2846*, *Rv1258*, *Rv2942* and *Rv1410*) for VP, and seven (*Rv1458*, *Rv1218*, *Rv1457*, *Rv1819*, *Rv1258*, *Rv1217* and *Rv2459*) for RIF + VP. At 72 h, a significant difference in the relative expression ($p \leq 0.01$) for the EP genes was also observed in ten (*Rv1456*, *Rv3065*, *Rv1458*, *Rv1457*, *Rv2846*, *Rv1258*, *Rv2942*, *Rv1217*, *Rv2459* and *Rv1410*) for RIF, three (*Rv3065*, *Rv1457*, and *Rv1410*) for VP, and four (*Rv1457*, *Rv2846*, *Rv1258* and *Rv2942*) for RIF + VP.

Downregulation of relative expression was observed in most of the studied EP genes at 16 h of VP exposure. At 72 h, upregulation of most of the studied EP genes was observed with RIF. The RIF + VP showed lower EP gene expression (with the exception of *Rv2846*) at 72 h compared with RIF.

4. Discussion

Evidence suggests the involvement of EPs to contribute to resistance to anti-TB drugs in *Mtb* and the use of EPIs may restore

Table 1
Primers used to assess relative efflux pump gene expression by qPCR.

Efflux pump gene	Transporter family	Sequences (5′–3′)	Amplicon size (bp)	Reference
Rv2942	RND	Fw- TACCCAAGCTGGAAACAA Rv- CCGTCAGAATAGAGGAACAG	214	[28]
Rv3065	SMR	Fw- AACCAGCCTGCTCAAAAG Rv- CAACCACCTTCATCACAGA	221	[28]
Rv2846	MFS	F- ATGGTAATGCCTGACATCC Rv- CTACGGGAAACCAACAAAG	131	[28]
Rv1410c	MFS	Fw- AGTGGGAAATAAGCCAGTAA R- TGGTTGATGTCGAGCTGT	198	[28]
Rv1258c	MFS	Fw- AGTTATAGATCG GCTGGATG Rv- GTGCTGTTCCCGAAATAC	268	[28]
Rv2459	MFS	Fw- CATCTTCATGGTGTT CGT G Rv- CGGTAGCACACAGACAATAG	232	[27]
Rv1456c	ABC	Fw- GAGTCGCACCAGAATCGC Rv- TCGCTGTTGGTTGCCTAC	90	[25]
Rv1457c	ABC	Fw- GTAGCACCGAGTCGTTTG Rv- ATCTCCACCGCATTCACC	80	[25]
Rv1458c	ABC	Fw- CAGTCCAAGTACCTCAATG Rv- GCGATACGGGTCAATAAC	163	[25]
Rv1218c	ABC	Fw- CCGCAAGGCGTCTAGTGAA Rv- TGGACCCGTTGATGGAAAA	173	[23]
Rv1217c	ABC	Fw- CGGTGAGGTTGGCGTAG Rv- CGGTCGGAATCTGGAAA	150	[23]
Rv1819c	ABC	Fw- CGGTGATTTCTTTCACAGC Rv- CCGACAGATTCCATCCATT	351	[23]
16s RNA		Fw- CAAGGCTAAAACTCAAAGGA Rv- GGACTTAACCCAACATCTCA	197	[28]

F, forward. R, reverse.

Table 2
MIC and FICI values for the classical drugs, efflux pump inhibitors and drug combinations in *Mycobacterium tuberculosis* $H_{37}Rv$.

Drugs combination	MIC (μg/mL)					REDCA	
	INH	EMB	RIF	CCCP	VP	MICs (μg/mL) drug/inhibitor	FICI
RIF + VP	–	–	0.004	–	125	0.001/62.5	0.75
RIF + CCCP	–	–	0.004	3.125	–	0.004/1.56	1.5
EMB + VP	–	2	–	–	125	2/125	2
EMB + CCCP	–	2	–	3.125	–	2/3.125	2
INH + VP	0.03	–	–	–	125	0.03/125	2
INH + CCCP	0.03	–	–	3.125	–	0.03/3.125	2

RIF, rifampicin; EMB, ethambutol; INH, isoniazid; VP, verapamil and CCCP, *m*-chlorophenyl-hydrazone; MIC, minimal inhibitory concentration; FICI, fractional inhibitory concentration index; REDCA, resazurin drugs combination microtiter assay; –, not performed.

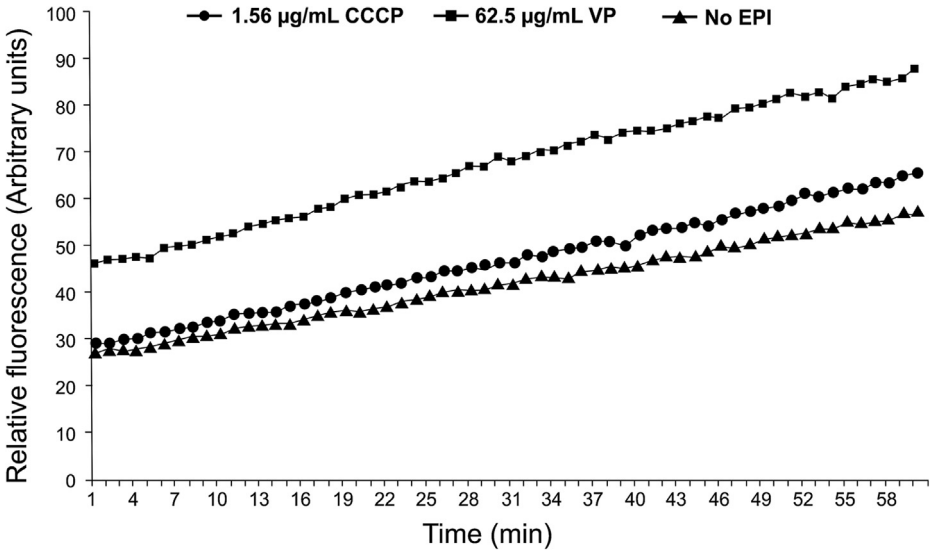

Figure 1. Efflux of ethidium bromide (EtBr) by *Mycobacterium tuberculosis* $H_{37}Rv$ by fluorometry. The assays were conducted at 35–37 °C, with or without an efflux pump inhibitor (EPI). Relative fluorescence was obtained by normalizing the data to the background fluorescence of EtBr. The efflux of EtBr was inhibited by verapamil (VP) and *m*-chlorophenyl-hydrazone (CCCP) at 0.5 × MIC. Relative final fluorescence (RFF) was calculated for each EPI.

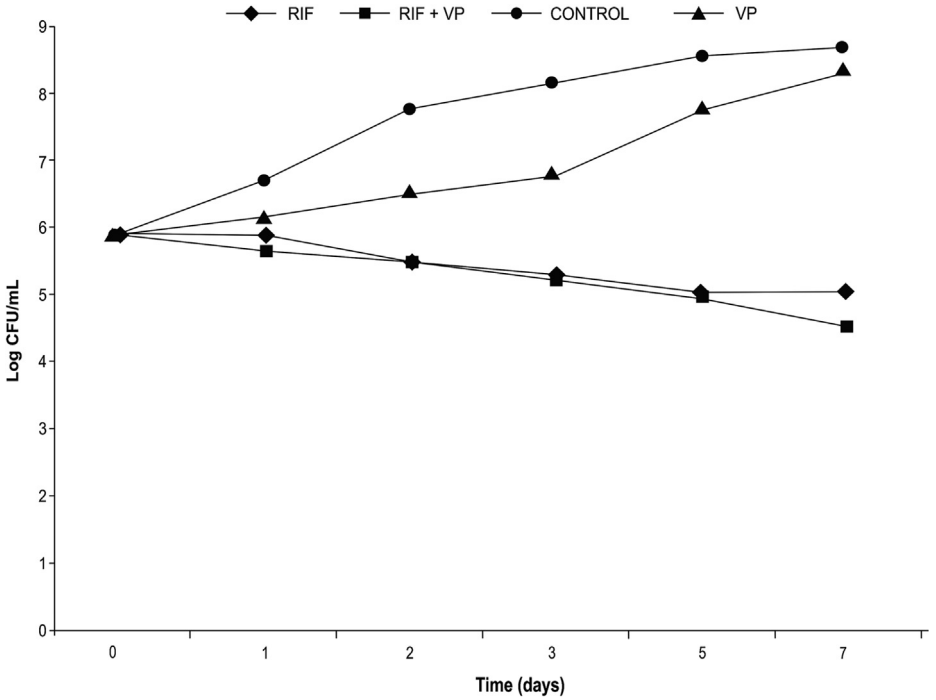

Figure 2. Time–kill curve results of *Mycobacterium tuberculosis* using 0.5 × MIC of rifampicin (RIF) and verapamil (VP) alone and in combination (RIF + VP) for 7 days. Each data point (days 0, 1, 2, 3, 5, and 7) represents the mean number of viable bacterial cell counts in duplicate experiments.

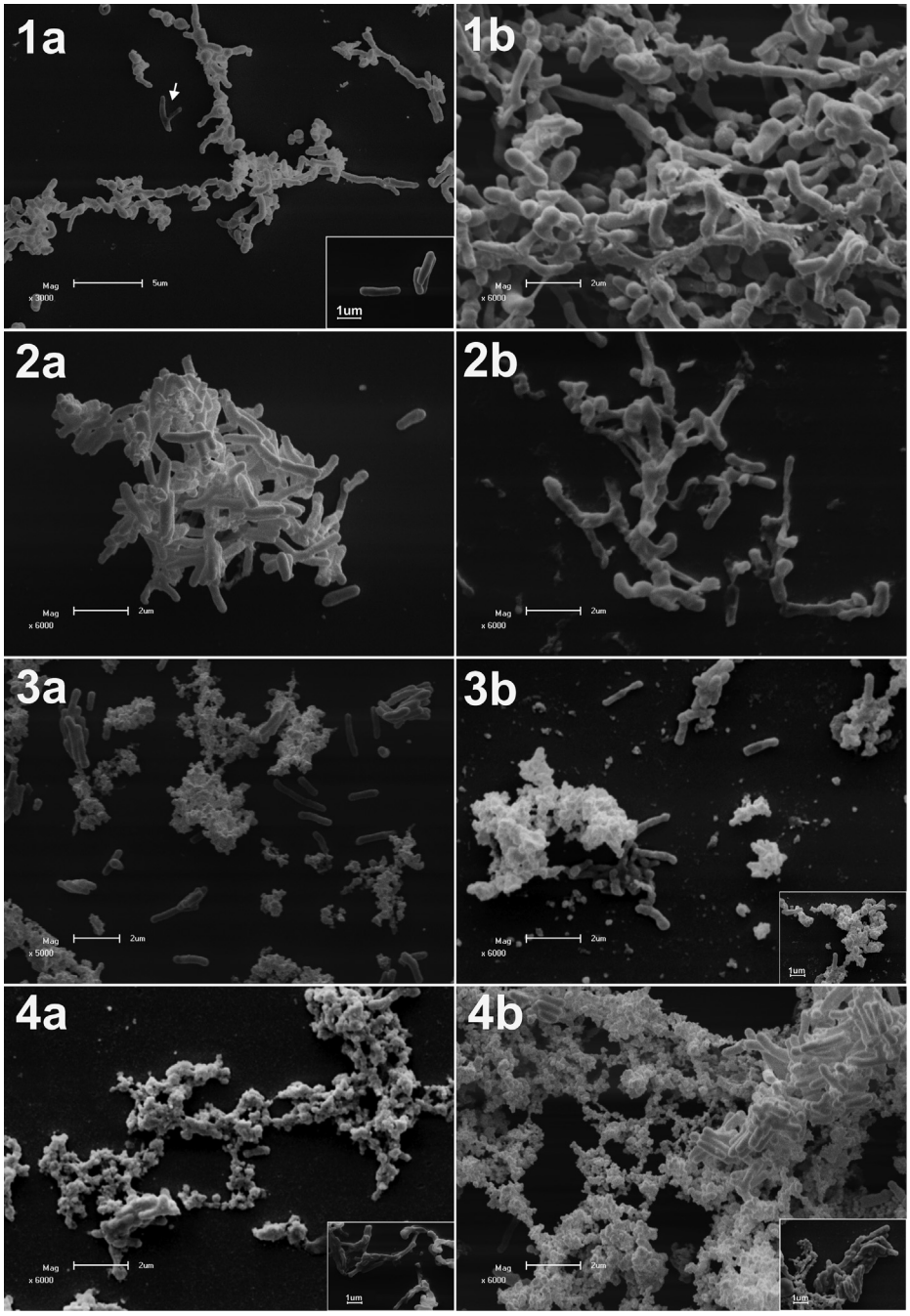

Figure 3. Scanning electron micrograph of *Mycobacterium tuberculosis* (*Mtb*) after 16 h (a) and 72 h (b) of exposure to a sub-inhibitory concentration (0.5 × MIC) of rifampicin (2) or verapamil (3) alone and in combination (4). A control of *Mtb* cells without drugs is also shown (1). The arrow (1a) shows *Mtb* cell division. Inset magnification: 1a = 18,000×; 3b and 4a–b = 10,000×.

drug susceptibility [11]. However, the effect of these combinations on the morphology and expression of particular genes is unclear. In the present study, the activity of RIF, INH, or EMB combined with CCCP or VP were evaluated in *Mtb* H_{37}Rv.

Although no synergistic effect was revealed between anti-TB drugs and CCCP or VP, the RIF + VP combination (FICI = 0.75) showed a considerable reduction on the MIC. By EtBr accumulation assay, VP showed a greater capacity of accumulation (RFF = 0.544), indicating better EPs inhibition compared to CCCP (RFF = 0.154).

The above result encouraged us to carry out time–kill curve using RIF + VP at subinhibitory concentration. Although, synergistic effect of the RIF + VP combination was not observed (<2 $\log_{10}$ CFU/mL), an effect on MIC decreases was confirmed by time kill curve, mainly with seven days of exposure. The activity of RIF in reducing viable cell counts was time-dependent, which is consistent with the de Steenwinkel et al. [19].

Most SEM studies in *Mtb*, were performed in the 1950s and 1960s, with lack of high resolution compared to modern standards [20]. To our knowledge, no morphological study, by SEM, of *Mtb* after RIF and VP exposure has been published to date. The exposure times (16 and 72 h) were based on the *Mtb* generation time and activity in reducing viable cell counts in time–kill curve. Sub-inhibitory drug concentrations were used to evaluate the primary morphological changes and a true transcriptional profile related to

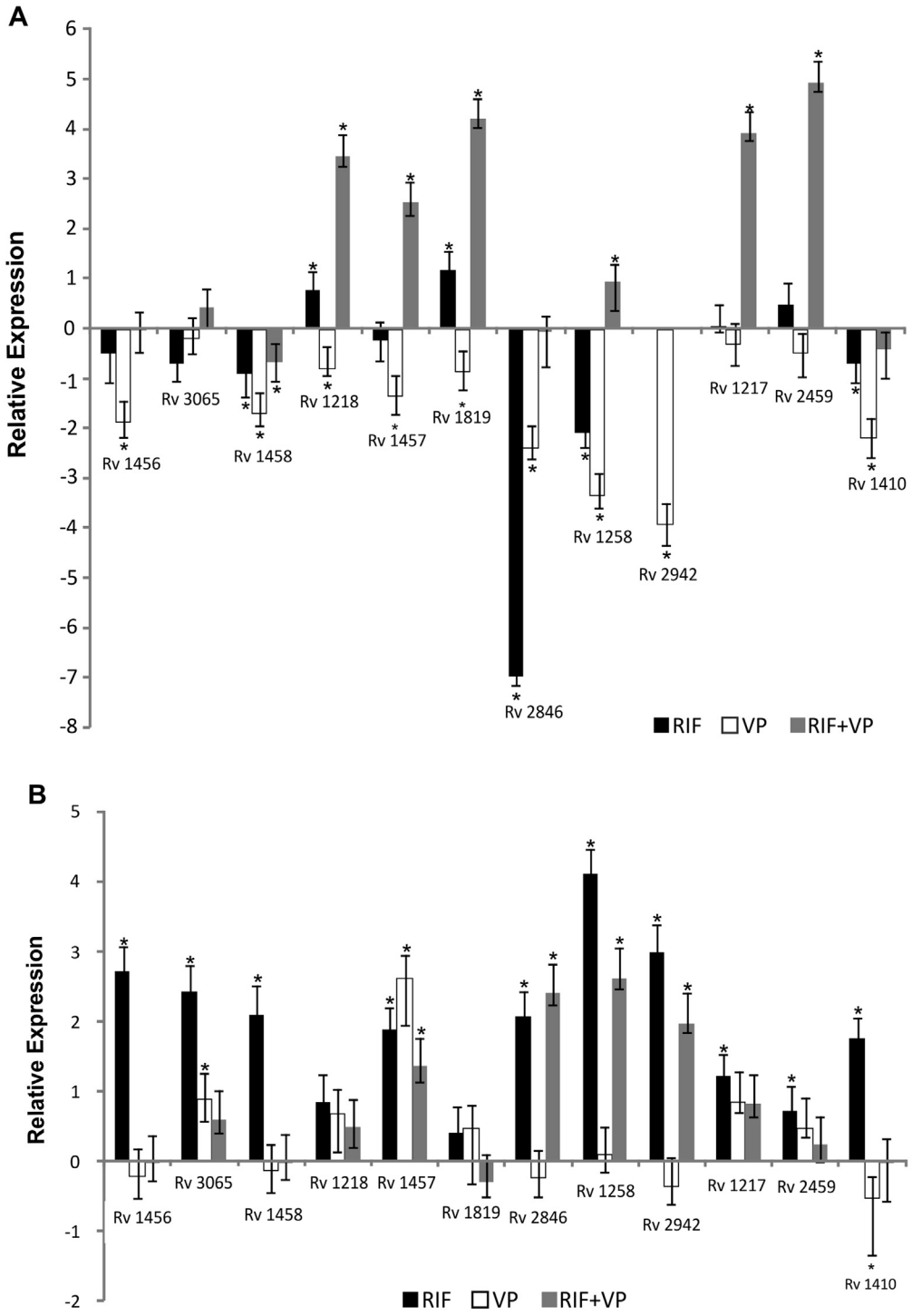

Figure 4. Relative expression of 12 efflux pump genes in *Mycobacterium tuberculosis* (*Mtb*) assessed by qPCR after 16 h (A) and 72 h (B) of exposure to 0.5 × MIC rifampicin (RIF), verapamil (VP), and RIF + VP combination in logarithmic scale. The error bars indicate SD. The results were normalized to 16s RNA and the relative expression calculated by $2^{-\Delta\Delta CT}$ method. *$p < 0.001$, compared with *Mtb* control growth in the absence of the drugs.

exposure to the RIF + VP and reducing the effect of stress on the bacteria.

The morphology of *Mtb* H_{37}Rv, not exposed to drugs, showed transient branching structures of cell division (Figure 3.1a) as observed by Dahl [21]. The effect of RIF on the morphology was more evident at 72 h, which is consistent with lower viable cell counts at 16 h by time–kill curve. Interestingly, with VP exposure, induction of cell rounding was observed, but viable cell counts were not appreciably affected at 72 h.

Cell wrinkling was observed in RIF exposure, that is consistent with the presently known mechanism of action of the drug. The main biotarget of RIF is the β subunit of DNA-dependent RNA polymerase, encoded by the *rpoB* gene, which suppresses the transcription process [8]. The morphological changes (i.e., cell rounding) observed in VP exposure agree with its action on the cell wall. Verapamil is a well-known calcium channel antagonist and inhibitor of human membrane protein P-glycoprotein (P-gp) that is responsible for the efflux of a wide range of drugs [10].

Twelve EPs genes from the ABC [10,22], MFS [4,23], RND [7], and SMR [6] families, which have been associated with resistance in *Mtb*, were chosen for conducting the study.

The best EPs inhibitory effect by VP was observed at 16 h. This is consistent with the time–kill curve, in which an initial bacillus growth inhibition was observed. After this time, bacillus restored the growth, notably VP exhibited bacteriostatic effect.

For RIF, overexpression of most of the studied EPs genes was observed at 72 h. A parallel may be observed with the time–kill curve where an adaptation of the bacillus to RIF started at this time in an attempt to restore their growth. Notably, RIF is a hydrophobic drug and may enter the cell by diffusion through the hydrophobic bilayer; thus, the active EPs extrude the drug out of the cell, allowing for the typical drug tolerance phenomenon.

The observed expression changes over RIF exposure time are consistent with Calgin et al. [3], who postulated that an increase in EPs expression in *Mtb* clinical isolates during treatment can drive constitutive or inducible EPs expression and lead to an increase in the MICs of anti-TB drugs and render bacillus resistant.

Resistance in *Mtb* has long been assumed to arise mainly by spontaneous mutations in specific genes that are related to the drugs target. In addition, increased efflux pump activity may contribute to unexplained resistance to anti-mycobacterial drugs in the absence of gene mutations known to confer resistance [24].

Although eight of the studied genes, *Rv1410c* [4], *Rv1456c* [25], *Rv1457c* [25], *Rv1458c* [25], *Rv1258c* [23], *Rv1217c* [5,22], *Rv1819c* [26], and *Rv1218c* [5,23], have already been reported to be overexpressed in mycobacteria exposed to RIF, the overexpression of *Rv3065*, *Rv2846*, *Rv2942*, and *Rv2459* EPs genes were demonstrated in the present study at 72 h. Overexpression of the *Rv3065* (*mmr*) [6,27,28], *Rv1258c* (*tap*-like gene) [23,27,28], *Rv1410c* (*p55*) [23,27,28], *Rv1819* [26], *Rv2459* (*jefA*) [9,26,27], *Rv2942* (*mmpL7*) [27,28], and *Rv2846c* (*efpA*) [28] genes has also been reported previously in mycobacteria exposed to INH and EMB.

Gupta et al. [29] and Pang et al. [8] did not find significant expression of the *Rv3065* in resistant *Mtb* clinical isolates exposed to RIF, as observed in the present study in *Mtb* $H_{37}Rv$ at 72 h of exposure to 0.5 × MIC of RIF. One possible explanation for the difference may be related to the use of clinical isolates, the exposure time and RIF concentration, which were not specified by Gupta et al. [26] or Pang et al. [8].

At 16 and 72 h of exposure to the RIF + VP combination, six EPs genes (*Rv1218*, *Rv1457*, *Rv1819*, *Rv1217*, *Rv2459*, and *Rv1258*) and four (*Rv1457*, *Rv1258*, *Rv2846*, and *Rv2942*) exhibited significant overexpression ($p \leq 0.01$), respectively. Notably, at 72 h a smaller number of genes and lower EPs expression, with the exception of *Rv2846*, were observed compared with RIF exposure.

Although, the time–kill curve showed decrease in viable cell counts in *Mtb* $H_{37}Rv$ exposed to the RIF + VP combination, similar to RIF, the expression study showed an EPs inhibitory effect in the combination exposure, which was more pronounced throughout the time compared to RIF. Based on the obtained results with *Mtb* $H_{37}Rv$, it would be of interest to conduct a study with RIF + VP combination on RIF resistant clinical isolates. Clearly, this study would clarify the possibility of reducing the resistance mediated by EPs in resistant *Mtb*. This theory is shared by Ramon-Garcia et al. [30] who studied the effect of other EPIs in *Mycobacterium fortuitum*.

Additionally, it has been observed that EPIs may also block anti-TB drug efflux in *Mtb*-infected macrophages, leading to an increase in intracellular drug levels and in drug actions on the bacillus [31]. According to Adams et al. [31], VP is perhaps the most promising inhibitor for further evaluation as an adjunctive anti-TB agent, given its ability to reverse macrophage-induced tolerance to RIF. This combination therapy could be a promising alternative for the treatment of patients with MDR-TB, including in TB/HIV co-infection, which is associated with difficult therapeutic management [12].

5. Conclusions

The best drug combination against *Mtb* $H_{37}Rv$, in the present study, occurred with RIF + VP, which showed changes in the morphology of bacillus consistent with the sum of the changes observed in each drug individually. The downexpression of some EPs related genes by RIF + VP exposure suggests an effective inhibitory activity of VP in *Mtb* $H_{37}Rv$ as observed by EtBr accumulation. Thus, the role of EPs in promoting drug tolerance and the use of EPIs open up a potentially powerful approach, making necessary the continuation more thoroughly of the study of the RIF + VP combination in *Mtb* using additional concentration and time of exposition.

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Competing interests

None declared.

Ethical approval

Not required.

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