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Intramacrophage *Mycobacterium tuberculosis* efflux pump gene regulation after rifampicin and verapamil exposure

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Objectives: Since resistance of *Mycobacterium tuberculosis* (*Mtb*) partially derives from efflux pumps (EPs) in the plasma membrane, the current study evaluates EPs in *Mtb* exposed to rifampicin in the presence of the EP inhibitor verapamil, within a macrophage environment.

Methods: Human acute monocytic leukaemia cell line THP-1 was infected with *Mtb* H_{37}Rv and exposed to rifampicin and verapamil alone and in combination for 24 and 72 h. After RNA extraction, quantitative PCR was carried out for 11 EP genes using SYBR green PCR master mix in the StepOne™ Real-Time PCR System.

Results: After 24 h of exposure to rifampicin, *Mtb* H_{37}Rv showed that 10 EP genes were up-regulated when compared with the control. The rifampicin/verapamil combination induced down-regulation of 54.5% (6/11) of the EP genes. At 72 h, rifampicin exposure induced up-regulation of 10 EP genes and rifampicin/verapamil induced down-regulation of 8 EP genes, which suggests effective EP-inhibitory activity of verapamil against *Mtb* H_{37}Rv in an intramacrophage environment.

Conclusions: The current study demonstrated that rifampicin/verapamil caused down-regulation of several EP genes in *Mtb* inside the macrophage environment. *In vivo* trials may show that rifampicin/verapamil therapy could be of value in enhancing anti-TB treatment.

Introduction

TB, an infectious disease caused by members of the *Mycobacterium tuberculosis* (*Mtb*) complex, caused 1.4 million deaths worldwide in 2015, with estimates of 10.4 million new cases. In 2015, there were 480 000 new cases of MDR-TB and an additional 100 000 people with rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment.[1]

Current TB treatment consists of an initial intensive phase with isoniazid, rifampicin, ethambutol and pyrazinamide for 2 months followed by 4 months of isoniazid and rifampicin. In MDR-TB cases, a treatment consisting of other drugs, such as streptomycin, terizidone and a fluoroquinolone (levofloxacin or ofloxacin) has been proposed.[2] Patients with XDR-TB (i.e MDR-TB with additional resistance to any fluoroquinolone and an injectable second-line drug) should be sent to a reference centre for individualized regimens with reserved drugs, which comprise capreomycin, moxifloxacin, para-aminosalicylic acid and ethionamide.[3,4]

The MDR and XDR *Mtb* phenotypes are mainly caused by sequential mutations in specific chromosomal genes, related to the mechanisms of rifampicin and isoniazid and other drugs. However, the genetic basis of these two and other anti-TB resistant drugs are not fully understood. In the case of some resistant bacilli, the classic mutations related to specific drug resistance are not present suggesting other mechanisms are causing the resistance phenotype.[5]

Sequence analysis of the *Mtb* genome showed multiple efflux pumps (EPs)[6,7] which encode membrane proteins capable of actively transporting a broad range of compounds, including drugs. EP activities have been linked with the efflux of anti-TB drugs, such as isoniazid, rifampicin and ethambutol.[8,9] Among the leading families of EPs, five are the most frequently mentioned in relation to mycobacteria: the resistance nodulation division (RND) family, the small drug multiresistance (SMR) family, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family and the ATP binding cassette (ABC) family.[10]

Combined with anti-TB drugs, EP inhibitors have been tested to increase the effectiveness of anti-TB drugs by inhibiting their efflux from the intrabacillary environment. Antagonists of transmembrane electrochemical potential and calcium channels, such as verapamil, have been tested *in vitro* and they proved to be good synergistic drug candidates for TB treatment.[9,11]

A relationship between EPs and the ability of *Mtb* to overcome oxidative stress within macrophages has been reported.[12] As an EP inhibitor, verapamil may reduce tolerance to anti-TB drugs (isoniazid and rifampicin) and promote the death of mycobacteria in a macrophage environment, because it retains the drug for a longer time inside the bacilli.[6,11,12]

The macrophage model, which attempts to mimic the conditions in humans at the cell level, proved to be useful for studying the effect of drug combinations on *Mtb*. The model provides more data than *in vitro* models based on culture assays, such as drug penetration and the interaction between drugs inside macrophages.[13,14] Moreover, studies involving *Mtb* macrophage infection may be of great help in understanding the mechanisms of bacillus resistance and immune response. The current analysis evaluates the regulation of EP genes in *Mtb* exposed to the rifampicin/verapamil combination, inside the macrophage environment.

Materials and methods

Mtb

Mtb H_{37}Rv (ATCC 27294) was grown at 35°C for 15 days in a Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA), supplemented with 10% (v/v) OADC (BBL/Becton and Dickinson, Sparks, MD, USA), with the addition of 0.2% glycerol (v/v) and 0.025% Tween 80 (v/v).

MICs

MICs of rifampicin and verapamil alone were determined using a resazurin microtitre assay plate method[15] and the activity of rifampicin/verapamil was evaluated by Caleffi-Ferracioli *et al.*[11] using a resazurin drug combination microtitre assay.

THP-1 cell culture

Human acute monocytic leukaemia cell line THP-1 (ATCC TIB-202) was maintained in DMEM-6429 (Sigma–Aldrich, St Louis, MO, USA) containing a 10% (v/v) FBS and 100 U/mL penicillin/streptomycin mixture (Sigma–Aldrich). Cells were incubated at 37°C in 5% CO_2. THP-1 cells were calculated by trypan blue (Sigma–Aldrich) exclusion. When exponential cell growth was achieved, a concentration of 5×10^5 cells/mL was seeded into two 24-well plates (one for samples and one for the *Mtb* growth control) and 100 nM phorbol-12-myristate-13-acetate (PMA) (Sigma–Aldrich) was added. The plates were incubated until they reached macrophage differentiation (12 h at 37°C in 5% CO_2). The supernatant was then discarded and PMA was added again at the same concentration (100 nM). Incubation was continued for an additional 4 days at the same temperature and atmosphere conditions. Macrophage differentiation was checked by microscopy and macrophages were washed once with 1 mL of supplemented DMEM, without the penicillin/streptomycin mixture.

Infection of THP-1 cells with Mtb

Mtb H_{37}Rv growth was centrifuged at 2880 **g** for 10 min and the bacterial pellet was shaken for 45 s with beads and suspended in DMEM. Single-cell suspension was checked by Ziehl–Neelsen stain.

The bacterial concentration was adjusted to $OD_{600} = 0.8–1.0$ in a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo), which corresponds to a concentration of 10^7 cfu/mL. The sample was diluted until a concentration of 5×10^5 cfu/mL was obtained.

The THP-1 macrophages in the two 24-well plates (assay and control) were infected with 5×10^5 cfu/mL of *Mtb*, which corresponds to a model of infection (MOI) of 1:1, and incubated for 3 h at 37°C in 5% CO_2. Infected macrophages were washed three times with PBS to remove excess bacteria.

Two wells of the control plate containing the infected THP-1 macrophages at an MOI of 1:1 were lysed with 0.1% Triton (Sigma–Aldrich) for infection control. Lysed wells were plated onto OADC-supplemented Middlebrook 7H11 agar (Difco Laboratories) and incubated for 15 days at 37°C to determine cfu/mL. The other wells of both plates containing the infected macrophages were incubated with DMEM containing 10% (v/v) FBS without antibiotics, at 37°C in 5% CO_2 for 72 h, so that *Mtb* would grow inside the macrophages.

Drug exposure

After incubation of *Mtb* growth inside the macrophages (corresponding to time 0 h), the infected macrophages were rinsed with PBS, lysed with 0.1% Triton and plated on OADC-supplemented Middlebrook 7H11 agar. At the same time, rifampicin and verapamil alone and combined were added at $0.25\times$ MIC for verapamil (31.25 mg/L) and $0.5\times$ MIC for rifampicin (0.0015 mg/L)[11,16] in a 24-well plate and incubated at 37°C for 24 and 72 h.

Further, each well was washed three times with 1.0 mL of PBS (pH 7.4) and 0.1% Triton was added. Lysed macrophages of two control wells were plated on OADC-supplemented Middlebrook 7H11 agar and infected macrophages from the other wells were used for the study of EP gene regulation. All experiments were carried out in triplicate, on different days.

Gene regulation study

Total RNA was extracted in two time-independent experiments and purified using the RNeasy Mini Kit Plus (QIAGEN Biotechnology, Valencia, CA, USA), following the manufacturer's instructions. Quantity assessment was performed using a Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, CA, USA). Synthesis of the first cDNA was carried out by random primer total RNA using SuperScript III Reverse Transcriptase (Invitrogen), with modifications by Bowler *et al.*[17]

Quantitative PCR was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) in the StepOne™ Real-Time PCR System. The EP-specific primers are listed in Table 1.

Melting curves were assessed; samples were run in triplicate and the 16S rRNA (*rrs*) gene was used to normalize all reactions. A negative control was included in all experiments. A reference assay was conducted in the absence of any drug. The relative quantification of target gene regulation was calculated by the $2^{-\Delta\Delta CT}$ method.[20] Data analysis was performed by one-way test with BioEstat 5.0 software,[21] followed by Tukey's *post hoc* test (significant at $P < 0.05$). Figure 1 displays the experiment.

Results

MOI 1:1 was performed for the two drugs alone (rifampicin and verapamil) and their combination to determine *Mtb* survival inside the macrophages at the time of infection (without drugs, time 0 h) and at 24 and 72 h of drug exposure. At time 0 h, the *Mtb* count on Middlebrook 7H11 agar was $\sim5.7\times10^5$ cfu/mL (Figure 2); at 24 and 72 h, the counts were 5.4×10^5 cfu/mL and 5.1×10^5 cfu/mL, respectively.

After 24 h of rifampicin exposure (Figure 3a), 10 EP genes were up-regulated when compared with the control, with the exception of *Rv1258c* (MFS family). After exposure to verapamil, eight EP

Table 1. Primers used in quantitative PCR for the EP gene regulation study in *Mtb*

EP gene	Transporter family	Sequences (5′–3′)	Amplicon size (bp)	Reference
Rv3065	SMR	Fw - AACCAGCCTGCTCAAAAG Rv - CAACCACCTTCATCACAGA	221	6
Rv2846	MFS	Fw - ATGGTAATGCCTGACATCC Rv - CTACGGGAAACCAACAAAG	131	6
Rv1410c	MFS	Fw - AGTGGGAAATAAGCCAGTAA Rv - TGGTTGATGTCGAGCTGT	198	6
Rv1258c	MFS	Fw - AGTTATAGATCGGCTGGATG Rv - GTGCTGTTCCCGAAATAC	268	6
Rv2459	MFS	Fw - CATCTTCATGGTGTTCGTG Rv - CGGTAGCACACAGACAATAG	232	18
Rv1456c	ABC	Fw - GAGTCGCACCAGAATCGC Rv - TCGCTGTTGGTTGCCTAC	90	7
Rv1457c	ABC	Fw - GTAGCACCGAGTCGTTTG Rv - ATCTCCACCGCATTCACC	80	7
Rv1458c	ABC	Fw - CAGTCCAAGTACCTCAATG Rv - GCGATACGGGTCAATAAC	163	7
Rv1218c	ABC	Fw - CCGCAAGGCGTCTAGTGAA Rv - TGGACCCGTTGATGGAAAA	173	19
Rv1217c	ABC	Fw - CGGTGAGGTTGGCGTAG Rv - CGGTCGGAATCTGGAAA	150	19
Rv1819c	ABC	Fw - CGGTGATTTCTTTCACAGC Rv - CCGACAGATTCCATCCATT	351	19
16S RNA	-	Fw - CAAGGCTAAAACTCAAAGGA Rv - GGACTTAACCCAACATCTCA	197	6

Fw, forward; Rv, reverse.

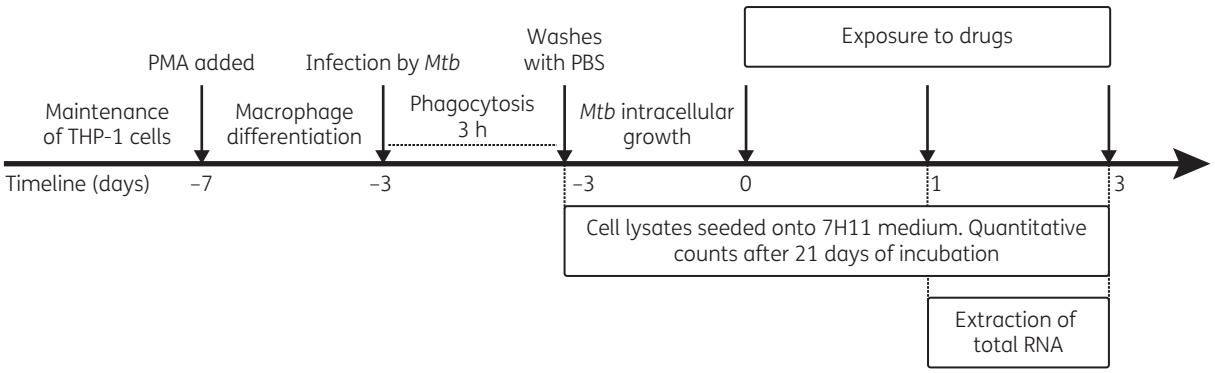

Figure 1. Experiment design of antimicrobial drug activity in *Mtb*-infected macrophage model. Each drug condition (rifampicin, verapamil and rifampicin/verapamil) was tested in triplicate. Drug-free infected macrophages were added as a control for bacterial growth, per triplicate, for each time-point. RIF, rifampicin; VP, verapamil.

genes were up-regulated, with the exception of *Rv1258c*, *Rv2459* and *Rv1217c* (MFS, MFS and ABC family, respectively). Rifampicin/verapamil induced down-regulation of 54.5% (6/11) of the EP genes. However, in the case of the *Rv1456c*, *Rv3065*, *Rv1217c* and *Rv2459* genes (ABC, SMR, ABC and MFS family, respectively), a significant up-regulation ($P < 0.01$ for *Rv1456c* and $P < 0.05$ for *Rv3065*, *Rv1217c* and *Rv2459*) was observed.

At 72 h (Figure 3b), most of the studied EP genes (10/11) were up-regulated after exposure to rifampicin, with the exception of *Rv1457c* (ABC family). After exposure to verapamil, down-regulation of most genes (8/11) was observed, with the exception of *Rv1456c*, *Rv2459* and *Rv1217c* (ABC, MFS and ABC families, respectively). Finally, after exposure to rifampicin/verapamil, down-regulation of the majority of genes (8/11) was observed, with the exception of *Rv1218c*, *Rv1457c* and *Rv1819c* (ABC family).

Discussion

Intramacrophage growth is a hallmark of *Mtb* during TB development. The bacilli face the most varied adversities inside the host

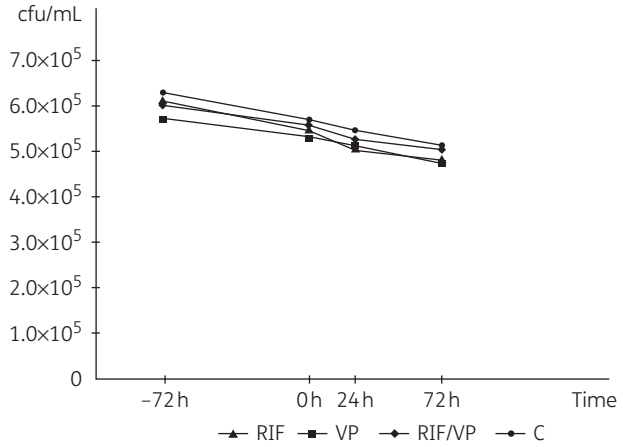

Figure 2. *Mtb* cfu/mL, from infection of THP-1 cells until 24 and 72 h of drug exposure. RIF, rifampicin; VP, verapamil; C, control.

cell, such as nutritional deficiency, acidic environment, hypoxia, oxidative stress and others, which influence TB development. Similarly, different alterations in the bacilli cause different consequences, mainly affecting susceptibility to anti-TB drugs.[22,23] Owing to the difference in environments of *Mtb* during the development of the disease, it is highly relevant to not only investigate the resistance mediated by EP expression in the *in vitro* extracellular (extramacrophage) environment, but also in the bacilli within the intramacrophage environment.

Recently, our research group reported extramacrophage studies involving EP gene regulation[11] and fluorimetry assays[24] to evaluate the *Mtb* inhibitory activity of rifampicin, when combined with verapamil, on EP-mediated resistance. These studies showed rifampicin MIC reduction and down-regulation of some EP genes when *Mtb* was exposed to rifampicin/verapamil in an extramacrophage environment, suggesting an increase in rifampicin activity when combined with verapamil. Previous results motivated us to continue studying rifampicin/verapamil in *Mtb* and compare EP gene regulation in *Mtb* in intramacrophage and extramacrophage environments.

First, the number of viable intramacrophage bacilli was evaluated for gene regulation studies in internalized *Mtb*. We observed a non-significant decrease in cfu/mL at 24 h, after 72 h of drug exposure. The above demonstrated that the number of live bacilli was consistent with the MOI for gene regulation studies of *Mtb* internalized in macrophages.[13] Helguera-Repetto *et al.*[25] observed that, after 6 h of macrophage exposure to *Mtb*, 100% of THP-1 cells were infected; after 48 h, the *Mtb* population remained the same inside the macrophage; a fact consistent with our results. Since, in the current study, 72 h of *Mtb* internalization did not reveal multiplication or significant bacillus death in the macrophage environment, the experiment was carried out.

Eleven EP genes from the ABC, MFS and SMR families, associated with resistance to rifampicin and other drugs,[7,9,10] were chosen to analyse the profile of EP gene regulation in intramacrophage *Mtb*. *Mtb* gene regulation was previously studied by Caleffi-Ferracioli *et al.*,[11] at the same laboratory, under extramacrophage conditions. It should be emphasized that the two studies had one different time of drug exposure. Caleffi-Ferracioli *et al.*[11] carried out EP regulation analyses at 16 and 72 h of drug exposure, whilst, in the current study, times of 24 and 72 h were chosen according to previous studies by our group, when times of 16 and 24 h failed to present any significant difference based on time–kill curves, since this interval of time encompasses the bacilli's first cellular division. Further, we believe that comparative studies should be performed.

Rifampicin penetrates *Mtb* and macrophage cells, including phagolysosomes, passively. It binds to the bacterial DNA-dependent RNA polymerase, blocks the protein synthesis and causes bacillus death.[26] We could expect bacillus EP up-regulation in the presence of rifampicin alone, in the first hours, once the bacilli try to expel the rifampicin.

In the current study, after 24 h of exposure to rifampicin alone, most of the *Mtb* EP genes were up-regulated, with the exception of *Rv1258c* (MFS family). We could observe different EP regulation patterns by the bacilli exposed to rifampicin alone in intramacrophage and extramacrophage studies,[11] although the *Rv1258c* gene was down-regulated in both environments, extramacrophage and intramacrophage. In contrast, Jiang *et al.*[19] reported up-regulation of *Rv1258c* in *Mtb* H_{37}Rv exposed to rifampicin alone, in an *in vitro* extramacrophage environment. However, comparing the above with the current study is rather difficult, since time exposure was not specified by the authors.

Down-regulation of three EP genes, *Rv1258c*, *Rv1217c* and *Rv2459*, from the MFS, ABC and MFS families, respectively, was observed at 24 h of exposure to verapamil alone. Caleffi-Ferracioli *et al.*[11] showed down-regulation of all EPs studied, revealing a marked difference between *Mtb* EP regulation in extramacrophage and intramacrophage environments.

The 24 h rifampicin/verapamil *Mtb* exposure results showed variable EP gene regulation patterns, with up-regulation of *Rv1456c*, *Rv3065*, *Rv1458c*, *Rv1217c* and *Rv2459* genes and down-regulation of *Rv1218c*, *Rv1457c*, *Rv1819c*, *Rv2846*, *Rv1258c* and *Rv1410c* genes. The only similarity between extramacrophage and intramacrophage studies was observed with three EP genes, *Rv1217c*, *Rv2459* and *Rv1410c*. Genes *Rv1217c* (ABC family) and *Rv2459* (MFS family) were statistically up-regulated, whereas the *Rv1410c* gene (MFS family) was down-regulated in both studies.

According to Ramon-Garcia *et al.*,[27] the *Rv1410c* gene is involved in the maintenance of normal growth characteristics and in the oxidative stress response. Further, Martinot *et al.*[28] corroborated the above result by reporting that deficiency of the protein coded by the *Rv1410c* gene caused a high degree of bacterial attenuation in a mouse model infection. These two studies may demonstrate that *Rv1410c* down-regulation by the bacilli, observed in a macrophage environment, may induce regulation of other genes, such as *Rv1217c* and *Rv2459* (at 24 h of exposure), in an attempt to survive in the intramacrophage environment.

An initial EP gene down-regulation in *Mtb* exposed to rifampicin/verapamil for 24 h has been reported in an intramacrophage environment, when compared with rifampicin alone. The difference in EP regulation requires further studies to better understand the true activity of verapamil in this condition.

The 72 h drug exposure results revealed a different *Mtb* EP gene regulation pattern. Exposure to rifampicin alone induced up-regulation of 10 *Mtb* EP genes in the macrophage environment. However, five of them (*Rv1458c*, *Rv1218c*, *Rv1819c*, *Rv2846* and *Rv1217c*), four from the ABC family and one from the MFS family, had significant up-regulation. In previous studies, carried out in an extramacrophage environment, the up-regulation of EP genes

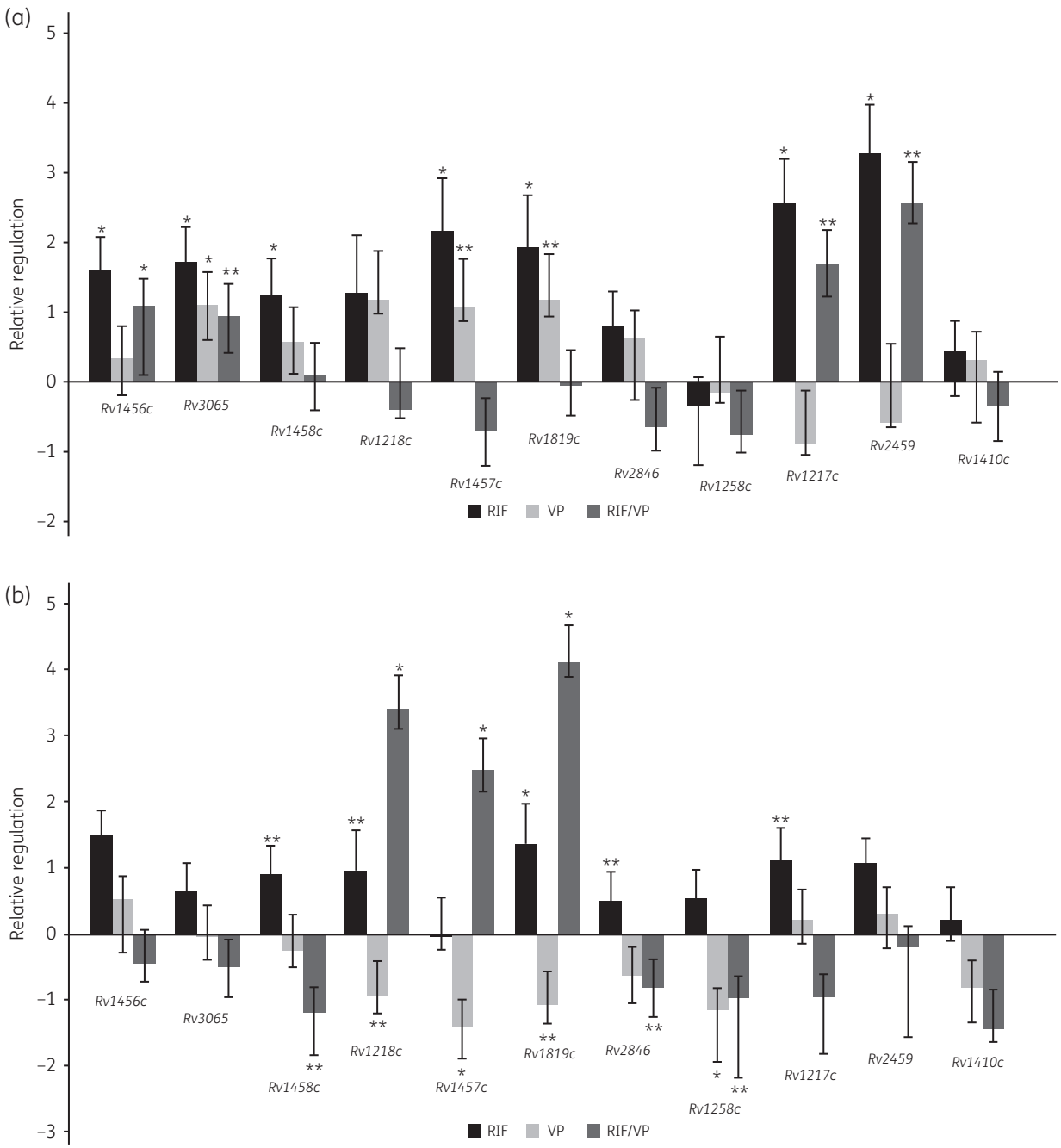

Figure 3. Relative differential regulation of 11 EP genes in *Mtb* assessed by quantitative PCR after 24 h (a) and 72 h (b) of exposure to 0.5 × MIC rifampicin (0.0015 mg/L), 0.25 × MIC verapamil (31.25 mg/L) and rifampicin/verapamil (logarithmic scale). Error bars indicate standard deviations. Results were normalized to 16S rRNA and the relative regulation was calculated using the $2^{-\Delta\Delta CT}$ method. $^{*}P < 0.01$ and $^{**}P < 0.05$ compared with *Mtb* control growth in the absence of drugs. RIF, rifampicin; VP, verapamil; C, control.

Rv1258c,[11,19] *Rv1457c*,[7] *Rv1819c*,[11,29] *Rv2846*,[11] *Rv1258c*,[11,19] *Rv1456c*,[11] *Rv3065*,[11] *Rv1458c*,[7,11] *Rv2459*,[11] *Rv1410c*[11,27] and *Rv1217c*[11,30] was also observed in the bacilli exposed to rifampicin alone.

Inside phagolysosomes, *Mtb* is exposed to reactive oxygen species, acidity and other chemical agents produced by macrophages,[23] which may direct the regulation of other genes, including other EP genes, in order to survive inside the macrophages. This seems to happen when we compare EP gene regulation at 24 and 72 h, with a decrease in regulation levels as time passes for exposure to rifampicin alone. A similar interpretation can be made for low *Mtb* EP regulation inside the macrophage when compared with extramacrophage studies by Caleffi-Ferracioli *et al.*[11] and others.[31,32]

Exposure to verapamil alone induced the bacilli to lower EP regulation at 72 h when compared with 24 h. Verapamil acts on

two pathways that directly affect the survival of *Mtb* inside the macrophage. The first one involves the inhibition of Ca^{2+} pumps located at the phagolysosome membrane, increasing acidity and inducing better activity of hydrolases inside the phagolysosome, leading to mycobacterial death.[31] The second pathway is related to the inhibition of EPs of the bacillus membrane, which increases the concentration of rifampicin inside the bacilli and improves the efficacy of the drug.[32] Although other authors have demonstrated the inhibitory activity of verapamil against EPs, mainly of the ABC family, the present study demonstrated the inhibition of EPs from other families too, such as MFS and SMR, to a greater or lesser extent, according to the exposure time and the EP gene studied.

The evaluation of EP gene regulation at 72 h of exposure to rifampicin/verapamil showed down-regulation of most EPs, with the exception of *Rv1218c*, *Rv1457c* and *Rv1819c*. It was expected that for rifampicin/verapamil the bacilli were at risk of death, which would result in an up-regulation of EP genes in an attempt to survive by expelling rifampicin. This effect was observed with *Rv1218c*, *Rv1457c* and *Rv1819c* at 72 h of exposure. However, it appears that rifampicin/verapamil was effective, to a point, in causing down-regulation of most EPs when compared with rifampicin alone. The above was also reported in an extramacrophage environment by Caleffi-Ferracioli *et al.*[11]

It should be emphasized that, at 72 h of exposure to rifampicin/verapamil, the *Mtb* showed down-regulation of most EP genes (eight) when compared with exposure to rifampicin alone, including statistically significant results for *Rv2846*, *Rv1258c* and *Rv1458c*. However, the EPs *Rv1218c*, *Rv1457c* and *Rv1819c* (all from the ABC family) were statistically up-regulated. Studies in the extramacrophage environment[11] showed the same pattern of gene regulation for most EPs under analysis, with the exception of *Rv1218c*, *Rv1457c*, *Rv1819c* and *Rv2846*.

EP down-regulation was reported after exposure to rifampicin/verapamil at 72 h when compared with EP gene regulation at 24 h. Results agreed for five EPs (*Rv3065*, *Rv1458c*, *Rv2846*, *Rv1258c* and *Rv1410c*) in the extramacrophage study.[11]

The above results, associated with other research on reduction of MIC when *Mtb* is exposed to rifampicin/verapamil,[11,24] reinforce the fact that verapamil may enhance rifampicin activity, ensuring more effectiveness in therapy for TB caused by *Mtb* involving resistance mediated by EPs.

Conclusions

The current study demonstrated that rifampicin/verapamil decreased the regulation of several EP genes in *Mtb* inside the macrophage environment. This combination would undoubtedly aid in the therapy against *Mtb* MDR. Additional *in vivo* studies would highlight the activity of rifampicin/verapamil against the bacilli in *in vitro* extramacrophage and intramacrophage environments.

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Transparency declarations

None to declare.

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