RESEARCH ARTICLE

In Vitro Activity of Rifampicin and Verapamil Combination in Multidrug-Resistant Mycobacterium tuberculosis

Fernanda de Oliveira Demitto¹, Renata Claro Ribeiro do Amaral², Flaviane Granero Maltempe², Vera Lúcia Dias Siqueira³, Regiane Bertin de Lima Scodro³, Mariana Aparecida Lopes², Katiany R. Caleffi-Ferracioli³, Pedro Henrique Canezín¹, Rosilene Fressatti Cardoso³

¹ Postgraduation in Health Sciences, State University of Maringa, Avenida Colombo, 5790, Maringa, Parana, 87020–900, Brazil, ² Postgraduation in Bioscience and Pathophysiology, State University of Maringa, Avenida Colombo, 5790, Maringa, Parana, 87020–900, Brazil, ³ Laboratory of Medical Bacteriology, Department of Clinical Analysis and Biomedicine, State University of Maringa, Avenida Colombo, 5790, Maringa, Parana, 87020–900, Brazil

Abstract

The aim of the present study was to evaluate the effect of the combination of rifampicin (RIF) and verapamil (VP) against the Mycobacterium tuberculosis H37Rv reference strain and six multidrug-resistant (MDR) M. tuberculosis clinical isolates by determining Time-Kill Curves and the ability to efflux drug by fluorometry. The RIF+VP combination showed synergism in one MDR clinical isolate. For the other five MDR clinical isolates, the drug combination showed no interaction. The MDR clinical isolate had lower ethidium bromide (EtBr) accumulation when exposed to the RIF+VP combination, compared with RIF and VP exposure alone. The other MDR clinical isolates showed no significant difference in EtBr accumulation. These results suggest greater efflux action in one of the MDR clinical isolates compared with the M. tuberculosis H37Rv reference strain. The other five MDR isolates may have additional mechanisms of drug resistance to RIF. The use of the RIF+VP combination made one MDR bacillus more susceptible to RIF probably by inhibiting efflux pumps, and this combination therapy, in some cases, may contribute to a reduction of resistance to RIF in M. tuberculosis.

Introduction

The World Health Organization estimated that 8.6 million people developed tuberculosis (TB) in 2012, and 1.3 million died, maintaining the disease as a major global health problem [1].

One of the problems in combating TB is the intrinsic and acquired resistance of Mycobacterium tuberculosis to therapeutic agents, hindering the development of new drugs and therapeutic approaches [2]. Researchers have sought to understand the mechanisms of drug resistance, mainly in multidrug-resistant (MDR) and extensively drug-resistant (XDR) Mycobacterium
tuberculosis. The former is defined as resistance to at least the two first-line drugs, isoniazid (INH) and rifampicin (RIF). The latter is defined as resistance to INH and RIF, with additional resistance to fluoroquinolones and at least one of the three injectable second-line drugs (kanamycin, amikacin, and capreomycin) [1–3].

Rifampicin is well known to be the backbone of modern anti-TB chemotherapy. Bacilli that are resistant to RIF have become a serious health problem worldwide. Resistance to anti-TB drugs has been attributed to mutational alterations of the drug biotarget [4]. For RIF, mutations in the \textit{rpoB} gene have been shown to cause resistance in 95–98\% of RIF-resistant \textit{M. tuberculosis} isolates [5]. However, evidence suggests that efflux systems also play an important role in drug resistance in \textit{M. tuberculosis} [6–8].

Bacterial efflux pumps (EPs) are membrane proteins that are able to actively extrude a broad range of substrates, including drugs, from the cytoplasm to the external bacterial environment and can be inhibited by EP inhibitors (EPIs) [9].

One well-known EPI is verapamil (VP), an inhibitor of MDR P-glycoprotein, which is an adenosine triphosphate (ATP)-binding cassette transporter that influences the cellular accumulation of antiretroviral and anticancer drugs, and its inhibitory activity against mycobacterial EPs has been previously demonstrated [10–11].

The aim of the present study was to evaluate the synergism of a RIF+VP combination by determining time-kill curves and drug efflux activity in the \textit{M. tuberculosis} H\textsubscript{37}Rv reference strain and MDR \textit{M. tuberculosis} clinical isolates.

### Materials and Methods

#### Bacterial samples

Six MDR \textit{M. tuberculosis} clinical isolates (18, 19, 64A, 71A, 109, and 3614) were previously genotypically differentiated according to Mycobacterial Interspersed Repetitive Units (MIRU) [12] and spoligotyping [13] (Table 1). The RIF+VP combination was shown to have a synergic effect (fractional inhibitory concentration: 0.25–0.37) in a modified checkerboard assay (i.e., the Resazurin Drugs Combination Microtiter Assay [REDCA]) [14]. All of the studied MDR clinical isolates belong to the reference center for TB diagnosis (Laboratory of Teaching and Research in Clinical Analysis, State University of Maringa, Parana, Brazil), and drug resistance to RIF was determined by the Lowenstein-Jensen proportion method [15]. The wildtype reference strain \textit{M. tuberculosis} H\textsubscript{37}Rv (ATCC 27294) was used as a control.

#### Antimicrobial and efflux pump inhibitor agents

Rifampicin (Sigma, St. Louis, MO, USA) and VP (Sigma, St. Louis, MO, USA) stock solutions were freshly prepared at concentrations of 2,000 and 20,000 μg/ml, respectively. Verapamil was prepared in distilled water, and RIF was prepared in methanol:water (1:10, v/v). The drug solutions were sterilized by filtration through 0.22 μm filters (Millipore, Billerica, MA, USA). Additional dilutions were performed in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) supplemented with oleic acid, bovine serum albumin, dextrose, and catalase (OADC) enrichment (BBL/Becton-Dickinson, Sparks, MD, USA) to reach RIF concentrations of 0.002–500 μg/ml and VP concentrations of 15.62–1,000 μg/ml. The RIF and VP concentrations were determined according to the minimum inhibitory concentrations (MICs) for the \textit{M. tuberculosis} H\textsubscript{37}Rv reference strain and MDR clinical isolates that were previously determined by the Resazurin Microtiter Plate Assay (REMA) [16].
Time-kill studies

The MDR clinical isolates and \textit{M. tuberculosis} H$_{37}$Rv reference strain were first grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) supplemented with oleic acid, bovine serum albumin, dextrose, and catalase (OADC) enrichment (BBL/Becton-Dickinson, Sparks, MD, USA) with 0.2\% glycerol (v/v) and 0.025\% Tween 80 (v/v) to 1 McFarland standard turbidity (3 \times 10^8 colony-forming units [CFU]/ml) for 15 days at 35–37°C. The cell suspensions were then adjusted to a final concentration of 10^6 CFU/ml in OADC-supplemented Middlebrook 7H9 medium. Individual RIF and VP and RIF+VP combination drug solutions were added to each mycobacterial suspension to achieve 0.5 \times MIC of the drugs.

The cultures were incubated at 35–37°C with shaking at 96 rotations per minute (rpm) for 7 days. Aliquots (0.1 ml) were removed on the initial day of the experiment and then on the first, third, fifth, and seventh days of incubation and serially diluted (10^{-1}, 10^{-3}, and 10^{-5}) in OADC-supplemented Middlebrook 7H9 medium to avoid drug carry-over. An aliquot (20 \mu l) of each dilution was seeded on OADC-supplemented Middlebrook 7H11. The plates were incubated at 35–37°C for 21 days, and the colonies were counted. Time-Kill Curve assays were performed three times on different days. The results are expressed as the mean of the three assays. Synergism was defined as a decrease of 2 or more log$_{10}$ CFU/ml compared with the single agent. A decrease in CFU/ml between 2 log$_{10}$ and 1 log$_{10}$ was indicative of an additive interaction [17, 18].

Efflux assay

Ethidium bromide (EtBr) accumulation was assessed by fluorometry for the MDR \textit{M. tuberculosis} isolates and H$_{37}$Rv reference strain [19, 20]. MDR \textit{M. tuberculosis} isolates and the H$_{37}$Rv reference strain were grown in OADC-supplemented Middlebrook 7H9 medium at 35–37°C until an optical density at 600 nm (OD$_{600}$) of 0.6–0.8 was reached. The cultures were exposed to the 0.5 \times MIC of VP, RIF, and RIF+VP that was previously determined by REMA [16] (Table 1) and incubated at 35–37°C for 7 days. Aliquots (900 \mu l) on the initial, first, third, fifth, and seventh days of incubation were removed and centrifuged at 12,880 \times g for 3 min. The pellet was rinsed in phosphate-buffered saline (PBS; pH 7.4) with 0.05\% Tween 80 (Synth, Diadema, SP, Brazil), and the OD$_{600}$ was adjusted to 0.4 with PBS. Aliquots (100 \mu l) of the bacillus suspension were transferred to a 96-well plate that contained 0.25 \mu g/ml EtBr (0.5 \times MIC; Sigma-Aldrich Química SA; Table 1). Fluorescence was determined for the bacterial

### Table 1. Molecular characterization, drug susceptibility profile, minimum inhibitory concentration, and drug interaction in the \textit{M. tuberculosis} H$_{37}$Rv reference strain and multidrug-resistant clinical isolates.

| Clinical isolate | MIC RIF (\mu g/ml) | MIC VP (\mu g/ml) | MIRU-VNTR | Spoligotyping | Drug susceptibility profile | MIC EtBr (\mu g/ml) | REDCA FICI RIF/VP |
|------------------|------------------|------------------|-----------|----------------|-----------------------------|------------------|-----------------|
| H$_{37}$Rv       | 0.004            | 125              | NP        |                | Susceptible                 | 1                | 0.75            |
| 64A              | 25               | 125              | 124325163322 | 6777376077607771 | (INH, Rif)$^R$          | 0.5              | 0.37            |
| 71A              | 50               | 125              | 225313153323 | 77777777720771   | (INH, Rif, PZA)$^R$      | 1                | 0.25            |
| 19               | 25               | 62.5             | 224327153324 | 7761776077607771 | (INH, Rif, EMB)$^R$      | 0.5              | 0.37            |
| 18               | 50               | 125              | 224326153324 | 7761776077607771 | (INH, Rif, EMB)$^R$      | 0.5              | 0.25            |
| 109              | 25               | 125              | 224326153325 | 7761776077607771 | (INH, Rif)$^R$          | 0.5              | 0.37            |
| 3614             | 12.5             | 62.5             | 224222153321 | 6777376077607771 | (INH, Rif, EMB)$^R$      | 0.5              | 0.37            |

R, resistant; NP, not performed; INH, isoniazid; Rif, rifampicin; EMB, ethambutol; PZA, pirazinamid; FICI, fractional inhibitory concentration index; REDCA: Resazurin Drugs Combination Microtiter Assay. VP, verapamil; EtBr, ethidium bromide. Numbers in bold represent synergism.

doi:10.1371/journal.pone.0116545.t001
suspension in the absence of drug as a reference assay. Fluorescence relative to EtBr-loaded cells was acquired every 51 s for 60 min at 35–37°C for each drug exposure time using a VICTOR² D fluorometer (PerkinElmer, Santa Clara, CA, USA). The excitation wavelengths were 530/25 nm, and the detection wavelengths were 590/20 nm [18]. The relative fluorescence values were obtained by normalizing the data against the background fluorescence of EtBr [21, 22].

Results

In contrast to the REDCA performed previously, a synergistic effect of the RIF+VP combination was observed for only one of the MDR M. tuberculosis isolates (71A) in the time-kill curve assay, reflected by a decrease of more than two log₁₀ CFU/ml on the seventh day of exposure to the drug combination. For the other five MDR clinical isolates and M. tuberculosis H₃₇Rv reference strain, the effect of the drug combination did not show an interaction (Fig. 1).

The results of the EtBr efflux analysis of the M. tuberculosis H₃₇Rv reference strain and MDR clinical isolates on the initial, first, third, fifth, and seventh days of incubation with VP, RIF, and the RIF+VP combination are shown in Fig. 2. The MDR M. tuberculosis clinical isolate 71A exhibited a different EtBr accumulation profile during the incubation time compared with the other five MDR isolates and M. tuberculosis H₃₇Rv reference strain.

Discussion

Mutations in drug resistance-associated genes can partially explain the molecular development of drug resistance to some drugs in M. tuberculosis. For RIF, specific mutations in the rpoB gene that encodes the β subunit of DNA-dependent RNA polymerase, which is the main biotarget of RIF, may result in resistant bacilli. Approximately 2–5% of RIF-resistant M. tuberculosis isolates do not harbor mutations in this target gene. Therefore, the modulatory actions of drug EPs have been shown to be relevant in the resistance to RIF in this bacillus.

The present study evaluated the synergistic effects of the RIF+VP combination by determining time-kill curves and the activity of EPs in MDR M. tuberculosis clinical isolates, which previously showed synergism based on the checkerboard method. Time-kill curves reflect the bactericidal effect of a drug and can be used to evaluate synergism between two or more drugs [20], and the fluorometry assay evaluates the inhibitory action of EPs on EPs based on the accumulation of EtBr, a fluorescent substrate, inside the cell. When inside the cell, EtBr can bind to numerous targets, and the balance between influx and efflux can be estimated in real time in the presence or absence of EPs [22].

In the present study, the RIF+VP combination did not improve the activity of RIF in the susceptible M. tuberculosis H₃₇Rv reference strain. In this strain, RIF exhibited time-dependent killing activity, which was clearly observed after the fifth day of RIF exposure and consistent with previous studies [23]. The time-kill curve assays of the six MDR isolates revealed the growth of bacilli after the fifth day of exposure to RIF alone or the RIF+VP combination, which was not observed with the susceptible M. tuberculosis H₃₇Rv reference strain. The reason why MDR isolates begin to grow after that exposure time is still unclear, but the EtBr accumulation assay suggests the involvement of EPs.

Variations in the EtBr accumulation profile were observed throughout the incubation time among the MDR M. tuberculosis clinical isolates, demonstrated by the fluorometry assay. The M. tuberculosis H₃₇Rv reference strain, which is a well-known susceptible reference strain, had higher EtBr accumulation compared with the MDR M. tuberculosis isolates. This result led us to infer the presence of EP actions in the MDR isolates, in which the addition of the RIF+VP combination increased EtBr accumulation. In general, for all of the MDR isolates, EtBr
accumulation was lower and occurred time-independently compared with the susceptible strain (H37Rv).

For the MDR isolate 71A, the RIF+VP combination had a synergistic effect, indicated by the time-kill curve assay, and a very different EtBr accumulation profile was observed after the third day of drug exposure. The fluorometry assay showed that the exposure of this MDR...
Fig 2. Fluorometry assay. Accumulation of EtBr in the Mycobacterium tuberculosis H37Rv reference strain and multidrug-resistant clinical isolates 71A, 18, 19, 109, 3614, and 64A. The mycobacteria were loaded with 0.25 μg/ml EtBr in the presence of 0.5 × MIC of verapamil (VP), rifampicin (RIF), and RIF+VP combination for 7 days at 35–37°C.

doi:10.1371/journal.pone.0116545.g002
isolate to VP alone caused high EtBr accumulation. This effect may be attributable to the blockade of EP action and interference with the efflux system. In the cultures that were exposed to the RIF+VP combination, lower EtBr accumulation was observed with the same incubation time. Lower EtBr accumulation may be attributable to fewer EPs that were still available to the action of VP because some EPs were performing RIF extrusion. Another possibility is that the expression of EPs may be increased by the stress caused by RIF exposure. The present findings are consistent with Jiang [7], in which the ABC superfamily ATP-binding cassette EP, encoded by the \textit{pstB} gene, was overexpressed in the presence of RIF in \textit{M. tuberculosis}.

The present results corroborate previous studies [24–26], in which the constitutive or inducible expression of efflux systems in response to treatment with RIF may contribute to a decrease in the intracellular concentration of RIF and consequently the development of \textit{M. tuberculosis} drug resistance. Additionally, the efflux-mediated response may provide an early stress response that creates an opportunity for other resistance mechanisms to arise [27].

The contributory role of EPs in drug resistance in \textit{M. tuberculosis} may be extended to other anti-TB drugs, including combinations of INH and other EPIs (e.g., INH and EMB) [27, 28] and ofloxacin+VP [28–31], which showed promising results in killing \textit{M. tuberculosis in vitro}. Sharma et al. [8] studied a combination of RIF and piperine (i.e., an EPI) and reported a reduction of the MIC values of RIF in the \textit{M. tuberculosis} H37Rv reference strain and MDR clinical isolates. The results obtained by these authors corroborate a previous study by Piddock et al. [32], who observed an increase in the intracellular concentration of RIF in \textit{M. tuberculosis} and other mycobacteria using a RIF+reserpine combination, suggesting a role for EPIs in enhancing the activity of RIF in some species of mycobacteria. However, to our knowledge, no \textit{in vitro} study with a RIF+VP combination has been performed in MDR \textit{M. tuberculosis} clinical isolates.

Studies of nontuberculous mycobacteria (NTM) have shown a significant impact of EPIs in reducing the resistance to drugs that are used to treat this kind of infection. Rodrigues et al. [33] performed an \textit{in vitro} study using a microdilution method and fluorometry and found a significant reduction of resistance to clarithromycin and erythromycin in \textit{M. avium} ATCC 25291 in the presence of VP. Jin et al. [34] found that the EPIs farnesol, carbonyl cyanide-m-chlorophenyl-hydrazone, reserpine, chlorpromazine, and VP caused significant EtBr accumulation in \textit{M. smegmatis}, and the most pronounced effect was induced by VP.

The \textit{in vitro} findings of the present study corroborate previous studies that evaluated anti-TB drug and EPI combinations \textit{in vivo}, showing promising results in the treatment of TB. Louw et al. [35] observed a significant reduction of lung bacillus loads in BALB/c mice that were infected with an MDR strain after 1–2 months of treatment using the combination of VP and first-line anti-TB drugs (RIF, INH, and pyrazinamide). Additionally, Gupta et al. [36] evaluated the pharmacokinetic interactions between RIF and VP in mice infected with \textit{M. tuberculosis} H37Rv and concluded that standard TB chemotherapy combined with VP accelerated bacillus clearance, with near sterilization and significantly lower relapse rates in just 4 months.

Finally, coadjutant therapy with VP inhibited mycobacterial EPs. This renders the bacillus more susceptible to RIF and may reduce the probability of the selection of spontaneously arising mutants. Additional studies are required to elucidate the mechanism of action of the RIF +VP combination and evaluate the safety and efficacy of the combination for patients infected with MDR \textit{M. tuberculosis}.

\section*{Acknowledgments}

We are grateful to Vânia Cristina Desoti for her assistance with fluorometry, Complexo de Centrais de Apoio a Pesquisa (COMCAP), Laboratório de Ensino e Pesquisa em Análises.
Clínicas (LEPAC), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

Author Contributions
Conceived and designed the experiments: RFC RCRA KRCF VLDS. Performed the experiments: FOD RCRA PHC MAL FGM. Analyzed the data: RFC RBLS VLDS KRCF. Contributed reagents/materials/analysis tools: RFC RBLS VLDS KRCF. Wrote the paper: RFC FOD RBLS VLDS KRCF.

References
1. World Health Organization (2013) Global tuberculosis report 2013. World Health Organization, Geneva, Switzerland. Available: http://www.who.int/tb/publications/global_report/2012/en/. Accessed 2014 January 2.
2. Haydel SE (2010) Extensively drug-resistant tuberculosis: a sign of the times and an impetus for antimicrobial discovery. Pharmaceuticals 3: 2268–90. doi: 10.3390/ph3072268 PMID: 21170297
3. Rodrigues L, Villellas C, Baiio R, Viveiros M, Aínsa JA (2013) Role of the Mmr efflux pump in drug resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 57: 751–7. doi: 10.1128/AAC.01482-12 PMID: 23165464
4. Shi R, Itagaki N, Sugawara I (2007) Overview of anti-tuberculosis (TB) drugs and their resistance mechanisms. Mini Rev Med Chem 7: 1177–85. doi: 10.2174/138955707782331740 PMID: 18045221
5. Louw GE, Warren RM, Gay van Pittius NC, McEvory CRE, Van Helden PD, et al. (2009) A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrob Agents Chemother 53: 3181–9. doi: 10.1128/AAC.01577-08 PMID: 19451293
6. Putman M, Van Veen HW, Konings WN (2000) Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev 64: 672–93. doi: 10.1128/MMBR.64.4.672-693.2000 PMID: 11104814
7. Jiang X, Zhang W, Zhang Y, Gao F, Lu C, et al. (2008) Assessment of efflux pump gene expression in a clinical isolate Mycobacterium tuberculosis by real-time reverse transcription PCR. Microb Drug Resist 14: 7–11. doi: 10.1089/mdr.2008.0772 PMID: 18321205
8. Sharma S, Kumar M, Sharma S, Nargotra A, Koul S, et al. (2010) Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of Mycobacterium tuberculosis. J Antimicrob Chemother 65: 1694–701. doi: 10.1093/jac/dkq186 PMID: 20529733
9. Piddock LJ (2006) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev 19: 382–402. doi: 10.1128/CMR.19.2.382-402.2006 PMID: 16614254
10. Jones K, Bray PG, Khoo SH, Davey RA, Meaden ER, et al. (2001) P-Glycoprotein and transporter MRP1 reduce HIV protease inhibitor uptake in CD4 cells: potential for accelerated viral drug resistance? AIDS 15: 1353–8. doi: 10.1097/00002030-200107270-00004 PMID: 11504956
11. Van Veen HW, Callaghan R, Soceneantu L, Sardini A, Konings WN, et al. (1998) A bacterial antibiotic-resistance gene that complements the human multidrug-resistance P-glycoprotein gene. Nature 391: 291–5. doi: 10.1038/34669 PMID: 9440694
12. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdies S, et al. (2006) Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. J Clin Microbiol 44: 4498–510. doi: 10.1128/JCM.01392-06 PMID: 17005759
13. Molhuizen HOF, Bunschoeten AE, Schouls LM, Van Embden JDA (1998) Rapid detection and simultaneous strain differentiation of Mycobacterium tuberculosis complex bacteria by spoligotyping. Methods Mol Biol 101: 381–94.
14. Caleffi-Ferracioli KR, Maltempe FG, Siqueira VL, Cardoso RF (2013) Fast detection of drug interaction in Mycobacterium tuberculosis by a checkerboard resazurin method. Tuberculosis 93: 660–3. doi: 10.1016/j.tube.2013.09.001 PMID: 24083948
15. Canetti G, Rist N, Grosset J (1963) Mesure de la sensibilité du bacilli tuberculeux aux drogues antibactériaux par la méthode des proportions. Rev Tuberc Pneumol 27: 217–72.
16. Palomino JC, Martín A, Camacho M, Guerra H, Swings J et al. (2002) Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 46: 2720–2. doi: 10.1128/AAC.46.8.2720-2722.2002 PMID: 12121966
17. Pillai SK, Moellering RC, Eliopoulos GM (2005) Antimicrobial combinations in antibiotics in laboratory medicine. In: Lorian V, editor. Antibiotics in laboratory medicine. Lippincott Williams & Wilkins, Philadelphia, pp. 365–400.

18. Luna-Herrera J, Reddy MV, Gangadharma PR (1995) In vitro activity of the benzoxazinonifamyacin KRM-1648 against drug-susceptible and multidrug-resistant tubercle bacilli. Antimicrob Agents Chemother 39: 440–4. doi: 10.1128/AAC.39.2.440 PMID: 7726512

19. Germann UA (1996) P-glycoprotein: a mediator of multidrug resistance in tumour cells. Eur J Cancer 32A: 927–44. doi: 10.1016/0959-849(96)90057-3 PMID: 8763334

20. Chan EL, Zabransky RJ (1987) Determination of synergy by two methods with eight antimicrobial combinations against tobramycin-resistant and tobramycin-resistant strains of Pseudomonas. Diagn Microbiol Infect Dis 6: 157–64. doi: 10.1016/0732-8893(87)90101-5 PMID: 3102156

21. Machado L, Spengler G, Evaristo M, Handzlik J, Molnár J, et al. (2011). Biological activity of twenty-three hydantoin derivatives on intrinsic efflux pump system of Salmonella enterica serovar Enteritidis NCTC 13349. In Vivo: 25: 769–72.

22. Paixão L, Rodrigues L, Couto I, Martins M, Fernandes P, et al. (2009) Fluorometric determination of ethidium bromide efflux kinetics in Escherichia coli. J Biol Eng 3: 18. doi: 10.1186/1754-1611-3-18 PMID: 19835592

23. Steenwinkel JEM, de Knegt GJ, ten Kate MT, van Belkum A, Verbrugh HA, et al. (2010) Time-kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of Mycobacterium tuberculosis. J Antimicrob Chemother 65: 2582–9. doi: 10.1093/jac/dkq374 PMID: 20947621

24. Ramon-Garcia S, Martin C, Thompson CJ, Ainsa JA (2009) Role of the Mycobacterium tuberculosis PS5 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. Antimicrob Agents Chemother 53: 3675–82. doi: 10.1128/AAC.00550-09 PMID: 19564371

25. de Knegt GJ, Bruning O, ten Kate MT, de Jong M, van Belkum A, et al. (2013) Rifampicin-induced transcriptional response in rifampicin-resistant Mycobacterium tuberculosis. Tuberculosis (Edinb) 93: 96–101. doi: 10.1016/j.tube.2012.10.013

26. Danilchanka O, Mailaender C, Niederweis M (2008) Identification of a novel multidrug efflux pump of Mycobacterium tuberculosis. Antimicrob Agents Chemother 52: 2503–11. doi: 10.1128/AAC.00298-08 PMID: 18458127

27. Machado D, Couto I, Perdigão J, Rodrigues L, Portugal I, et al. (2012) Contribution of efflux to the emergence of isoniazid and multidrug resistance in Mycobacterium tuberculosis. PLoS One 7: e34538. doi: 10.1371/journal.pone.0034538 PMID: 22493700

28. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M (2012) Contribution of efflux activity to isoniazid resistance in the Mycobacterium tuberculosis complex. Infect Genet Evol 12: 695–700. doi: 10.1016/j.meegid.2011.08.009 PMID: 21871582

29. Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, et al. (2010) Microarray analysis of efflux pumps in drug-susceptible Mycobacterium tuberculosis during stress induced by common antituberculous drugs. Microbiol Drug Resist 16: 21–8. doi: 10.1089/mdr.2009.0054 PMID: 20001742

30. Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, et al. (2004) Mycobacterium tuberculosis isolate with a distinct genomic identity over-expresses a tap-like efflux pump. Infection 32: 109. doi: 10.1007/s15010-004-3097-x PMID: 15507575

31. Singh M, Jadaun GP, Ramdas, Srivastava K, Chauhan V, et al. (2011) Effect of efflux pump inhibitors on drug susceptibility of ofloxacin resistant Mycobacterium tuberculosis isolates. Indian J Med Res 133: 535–40. PMID: 21623040

32. Piddock LJ, Williams KJ, Ricci V (2000) Accumulation of rifampicin by Mycobacterium aurum, Mycobacterium smegmatis and Mycobacterium tuberculosis. J Antimicrob Chemother 45: 159–65. doi: 10.1093/jac/45.2.159 PMID: 10660497

33. Rodrigues L, Sampaio D, Couto I, Machado D, Kern WV, et al. (2009) The role of efflux pumps in macrolide resistance in Mycobacterium avium complex. Int J Antimicrob Agents 34: 529–33. doi: 10.1016/j.ijantimicag.2009.07.010 PMID: 19740629

34. Jin J, Zhang JY, Guo N, Sheng H, Li L, et al. (2010) Famesol, a potential efflux pump inhibitor in Mycobacterium smegmatis. Molecules 15: 7750–62. doi: 10.3390/molecules15117750 PMID: 21042264

35. Louw GE, Warren RM, Gey van Pittius NC, Leon R, Jimenez A, et al. (2011) Rifampicin reduces susceptibility to ofloxacin in rifampicin resistant Mycobacterium tuberculosis through efflux. Am J Respir Crit Care Med 184: 269–76. doi: 10.1164/rccm.201011-1924OC PMID: 2152166

36. Gupta S, Tyagi S, Almeida DV, Maiga MC, Ammerman NC, et al. (2013) Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. Am J Respir Crit Care Med 188: 600–7. doi: 10.1164/rccm.201304-0650OC PMID: 23805786