



UNIVERSIDADE ESTADUAL DE MARINGÁ  
CENTRO DE CIÊNCIAS DA SAÚDE  
PROGRAMA DE PÓS-GRADUAÇÃO  
EM CIÊNCIAS DA SAÚDE

KATIANY RIZZIERI CALEFFI FERRACIOLI

Efeitos da combinação de antimicrobianos e inibidores de bomba de efluxo na  
morfologia e expressão gênica em *Mycobacterium tuberculosis*

Maringá

2014

KATIANY RIZZIERI CALEFFI FERRACIOLI

Efeitos da combinação de antimicrobianos e inibidores de bomba de efluxo na  
morfologia e expressão gênica em *Mycobacterium tuberculosis*

Tese apresentada ao Programa de Pós-Graduação em  
Ciências da Saúde do Centro de Ciências da Saúde da  
Universidade Estadual de Maringá, como requisito para  
obtenção de título de Doutor em Ciências da Saúde.

Área de concentração: Doenças Infecciosas e Parasitárias  
Orientadora: Profa. Dra. Rosilene Fressatti Cardoso

Maringá

2014

## FOLHA DE APROVAÇÃO

KATIANY RIZZIERI CALEFFI FERRACIOLI

Efeitos da combinação de antimicrobianos e inibidores de bomba de efluxo na  
morfologia e expressão gênica em *Mycobacterium tuberculosis*

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito para obtenção do título de Doutor em Ciências da Saúde pela Comissão Julgadora composta pelos membros:

### COMISSÃO JULGADORA

Profa. Dra. Rosilene Fressatti Cardoso  
Universidade Estadual de Maringá

Prof. Dr. Fernando Rogério Pavan  
UNESP/Faculdade de Ciências Farmacêuticas

Profa. Dra. Jane Martha Graton Mikcha  
Universidade Estadual de Maringá

Profa. Dra. Regiane Bertin de Lima Scodro  
Universidade Estadual de Maringá

Profa. Dra. Eliana Valeria Patussi  
Universidade Estadual de Maringá

Aprovada em: 27 de Junho de 2014.

Local de defesa: Anfiteatro, Bloco 02, Universidade Estadual de Maringá.

## AGRADECIMENTOS

Em primeiro lugar, agradeço a Deus, pelo dom da vida e da sabedoria, e pela oportunidade do conhecimento.

Agradeço ao meu esposo, Camilo Junior, pelo apoio, paciência e compreensão durante todos os momentos.

Aos meus pais, José Alécio e Maria Élide, e a minha irmã, Edilainy, pelo carinho, orações, incentivo e apoio durante todo o meu trabalho.

À Profa. Dra. Rosilene Fressatti Cardoso pela orientação, amizade, paciência e confiança em todas as horas.

Também à Profa. Dra. Vera Lúcia Dias Siqueira pela amizade, auxílio e incentivo durante o desenvolvimento do meu trabalho.

À Profa. Dra. Regiane B. Scodro e aos funcionários e estagiários do Laboratório de Bacteriologia Médica da UEM, em particular, às alunas Mariana e Vanessa pelo auxílio e compreensão.

Agradeço em especial aos alunos Flaviane Granero, Fernanda Demitto, Renata Amaral e Pedro Henrique Canezin pela participação no trabalho e companheirismo.

Ao Prof. Dr. Celso Vataru Nakamura, por tornar possível a realização de parte deste trabalho, e às alunas Vânia e Kátia, do COMCAP, por toda colaboração dispensada.

À Fundação Araucária e Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), por financiar parte do projeto de doutorado.

Às professoras que compuseram a banca de minha qualificação: Profa. Dra. Rosilene Fressatti Cardoso, Profa. Dra. Vera Lúcia Dias Siqueira e Profa. Dra. Regiane Bertin de Lima Scodro. Obrigada por toda atenção, colaboração e tempo dedicado.

Enfim, a todos que, mesmo não mencionados, participaram, direta ou indiretamente, desta minha conquista, meus sinceros agradecimentos.

## EPÍGRAFE

*“Não desanimeis nunca, embora venham ventos contrários”*

**Santa Paulina**

Caleffi-Ferracioli, K.R. Efeitos da combinação de antimicrobianos e inibidores de bomba de efluxo na morfologia e expressão gênica em *Mycobacterium tuberculosis*. 2014. 80 f. Tese (Doutorado) – Universidade Estadual de Maringá, Brasil, 2014.

## RESUMO

Este trabalho teve por objetivo primeiramente propor um ensaio para facilitar a avaliação da combinação de fármacos contra *Mycobacterium tuberculosis* (*Mtb*) e, posteriormente, avaliar os efeitos da combinação de rifampicina (RIF), isoniazida (INH) ou etambutol (EMB) com inibidores de bomba de efluxo (IBEs), a morfologia e o perfil transcrional de genes que codificam para bombas de efluxo (BEs) em *Mtb* H<sub>37</sub>Rv exposto a uma específica combinação de fármacos. Os resultados estão apresentados em dois artigos: “Fast detection of drug interaction in *Mycobacterium tuberculosis* by a checkerboard resazurin method” e “Morphological changes and differentially expressed efflux pump genes in *Mycobacterium tuberculosis* exposed to rifampicin and verapamil combination”. No primeiro artigo, o ensaio *Resazurin drugs combination microtiter assay* (REDCA) foi proposto para avaliar o sinergismo em *Mtb* usando como modelo a combinação INH ou EMB com levofloxacino. Os resultados foram promissores para a combinação de EMB com levofloxacino o que a torna uma opção terapêutica com vista a reduzir os efeitos adversos do EMB. O segundo artigo avaliou a atividade *in vitro* de RIF, INH ou EMB com IBEs (verapamil (VP) e carbonil cianeto *m*-clorofenil-hidrazona) isoladamente por *Resazurin Microtiter Assay Plate* (REMA) e em combinação por REDCA em *Mtb* H<sub>37</sub>Rv. A inibição das BEs foi avaliada pelo ensaio de acumulação de brometo de etídeo. O crescimento, a morfologia e a expressão de genes que codificam para BEs foram avaliados para a combinação RIF+VP que apresentou o menor índice de fração inibitória por REDCA. No ensaio de curva de tempo de morte bacteriana, a contagem de células viáveis após exposição à RIF+VP foi similar a obtida pela exposição à RIF. Enrugamento e arredondamento celular foram as principais alterações morfológicas observadas pela microscopia eletrônica de varredura após exposição à RIF e VP, respectivamente. Os efeitos da combinação RIF+VP no bacilo parecem ser uma somatória das mudanças morfológicas citadas anteriormente. Foi observada expressão aumentada de alguns genes que codificam para BEs na presença de RIF e diminuição dos mesmos na presença da combinação RIF+VP, após 72h de exposição, no ensaio de PCR em tempo real. O presente estudo evidenciou que o melhor efeito da combinação de fármacos em *Mtb* H<sub>37</sub>Rv ocorreu com RIF+VP, a qual sugere vantagens sobre a RIF isoladamente que compõe a terapia convencional para tuberculose, incluindo a diminuição na resistência mediada por BEs.

**Palavras-chave:** *M. tuberculosis*, REDCA, microscopia eletrônica de varredura, expressão gênica, combinação de medicamentos, verapamil, proteínas de membrana transportadoras.

Caleffi-Ferracioli, K.R. Efeitos da combinação de antimicrobianos e inibidores de bomba de efluxo na morfologia e expressão gênica em *Mycobacterium tuberculosis*. 2014. 80 f. Tese (Doutorado) – Universidade Estadual de Maringá, Brasil, 2014.

## ABSTRACT

This work aimed in a first moment to propose an assay to easily evaluate drug combination action against *Mycobacterium tuberculosis* (*Mtb*) and second, evaluate the effects of rifampicin (RIF), isoniazid (INH) and ethambutol (EMB) with efflux pump inhibitors (EPIs), the morphology and transcriptional profile of genes encoding efflux pumps (EPs) in *Mtb* H<sub>37</sub>Rv exposed to specific drug combination. The results are presented in two articles: combination in *Mtb* H<sub>37</sub>Rv "Fast detection of drug interaction in *Mycobacterium tuberculosis* by the checkerboard resazurin method" and "Morphological changes and efflux pump genes differentially expressed in *Mycobacterium tuberculosis* exposed to rifampicin and verapamil combination." At first article, Resazurin combination drugs microtiter assay (REDCA) was proposed to evaluate the drug synergism in *Mtb* using as model the INH or EMB with levofloxacin drugs combination. The results were promising for levofloxacin and EMB combination, which turn it a therapeutic option to reduce EMB adverse effects. The second article evaluated the *in vitro* activity of individual RIF, INH, EMB and EPIs (verapamil (VP) and carbonyl cyanide *m*-chlorophenyl-hydrazone) by Resazurin Microtiter Plate Assay (REMA) and the combinations of anti-tuberculosis drugs with EPI by REDCA in *Mtb* H<sub>37</sub>Rv. EPs inhibition was assessed by the accumulation of ethidium bromide assay. The growth, morphology and expression of genes encoding for EPs were evaluated for RIF+VP combination that had the lowest fractional inhibitory concentration index by REDCA. In the bacterial time kill curve assay, a viable cell count after exposure to RIF+VP combination was similar to RIF assay. Wrinkled and rounding cells were the main morphological changes observed by scanning electron microscopy after RIF and VP exposure, respectively. The effects of RIF+VP combination appeared to be a summation of the above mentioned changes. Overexpression of some EP genes to RIF and decrease to RIF+VP, after 72 h of exposure, was observed by Real-time PCR. The present study demonstrated that the best drug combination effect in *Mtb* H<sub>37</sub>Rv occurred with RIF+VP, which suggest it to provide advantages over the individual RIF that compose the conventional therapy for tuberculosis, including a decrease in resistance mediated by EPs.

**Keywords:** *M. tuberculosis*, REDCA, scanning electron microscopy, gene expression, combination of drugs, verapamil, membrane transport proteins.

## LISTA DE ILUSTRAÇÕES

<b>Figura 1</b>	Estrutura química dos principais fármacos anti-tuberculose e de inibidores de bomba de efluxo.....	18
<b>Table 1</b>	MICs values alone and FICI obtained by REDCA and classical checkerboard assay with isoniazid or ethambutol vs. levofloxacin combinations in <i>Mycobacterium tuberculosis</i> H <sub>37</sub> Rv and drug-susceptible and multidrug-resistant (MDR) <i>Mycobacterium tuberculosis</i> clinical isolates.....	49
<b>Table 1</b>	Primers used to assess relative efflux pump gene expression by qPCR.....	74
<b>Table 2</b>	MIC and FICI values for the classical drugs, efflux pump inhibitors, and drug combinations in <i>Mycobacterium tuberculosis</i> H <sub>37</sub> Rv.....	75
<b>Fig. 1</b>	Efflux of ethidium bromide (EtBr) by <i>Mycobacterium tuberculosis</i> H <sub>37</sub> Rv by fluorometry. The assays were conducted at 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 <i>m</i> -chlorophenyl-hydrazone (CCCP) at 0.5×MIC. Relative final fluorescence (RFF) was calculated for each EPI.....	75
<b>Fig. 2</b>	Time-kill curve results of <i>Mycobacterium tuberculosis</i> 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.....	76
<b>Fig. 3</b>	Scanning electron micrograph of <i>Mycobacterium tuberculosis</i> ( <i>Mtb</i> ) 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 <i>Mtb</i> cells without drugs is also shown (1). The arrow (1a) shows <i>Mtb</i> cell division. Inset magnification: 1a = 18,000×; 3b and 4a-b = 10,000×.....	77
<b>Fig. 4</b>	Relative expression of 12 efflux pump genes in <i>Mycobacterium tuberculosis</i> ( <i>Mtb</i> ) 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 <sup>-ΔΔCT</sup> method. * <i>p</i> < 0.001, compared with <i>Mtb</i> control growth in the absence of drugs.....	78

## LISTA DE SIGLAS

AIDS	<i>Acquired Immunodeficiency Syndrome</i>
ABC	<i>ATP binding cassette</i>
Anti-TB	Anti-tuberculose
BEs	Bombas de Efluxo
CCCP	Carbonil cianeto <i>m</i> -clorofenil hidrazona
CIM	Concentração Inibitória Mínima
DOTS	<i>Directly Observed Therapy Short</i>
EMB	Etambutol
EPIs	<i>Eflux Pump Inhibitors</i>
EPs	<i>Eflux Pumps</i>
EtBr	<i>Ethidium Bromide</i>
HIV	<i>Human Immunodeficiency Virus</i>
IBEs	Inibidores de Bombas de Efluxo
IGRAs	<i>Interferon Gamma Release Assays</i>
INH	Isoniazida
LEVO	Levofloxacino
MATE	<i>Multidrug and Toxic Compound Extrusion</i>
MFS	<i>Major Facilitator Superfamily</i>
MIC	<i>Minimal Inhibitory Concentration</i>
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
<i>Mtb</i> -MDR	<i>M. tuberculosis</i> multirresistente
<i>Mtb</i> -XRD	<i>M. tuberculosis</i> extensivamente resistente
OADC	<i>Oleic Acid Albumin Dextrose Catalase</i>
OMS	Organização Mundial da Saúde

PCR	<i>Polymerase Chain Reaction</i>
PZA	Pirazinamida
qPCR	<i>Quantitative real-time PCR</i>
REDCA	<i>Resazurin Drugs Combination Microtiter Assay</i>
REMA	<i>Resazurin Microtiter Assay Plate</i>
RIF	Rifampicina
RND	<i>Resistance Nodulation Division</i>
RT-qPCR	<i>Reverse Transcription quantitative real-time PCR</i>
SEM	<i>Scanning Electron Microscopy</i>
SMR	<i>Small Multidrug Resistance</i>
TB	Tuberculose
TEM	<i>Transmission Electron Microscopy</i>
WHO	<i>World Health Organization</i>

Tese elaborada e formatada conforme as normas da ABNT (Capítulo I e II) e das publicações científicas (Capítulo II): *Tuberculosis* (artigo 1) disponível em: <<http://www.journals.elsevier.com/tuberculosis/>> e *Antimicrobial Agents and Chemotherapy* (artigo 2) disponível em: <<http://aac.asm.org/>>

## SUMÁRIO

CAPÍTULO I .....	13
1. INTRODUÇÃO .....	13
1.1 Tuberculose: Aspectos Gerais e Epidemiologia.....	13
1.2 Patogênese e Manifestações Clínicas .....	14
1.3 Diagnóstico e Tratamento da Tuberculose.....	15
1.4 Mecanismo de Ação e Resistência aos Principais Fármacos Utilizados no Tratamento da TB.....	17
1.5 Bombas de Efluxo e Inibidores .....	19
1.6 Metodologias Empregadas no Estudo da Atividade e Ação Combinada de Fármacos Anti-TB .....	22
1.6.1 REMA, <i>chequerboard</i> e curva de tempo de morte .....	22
1.6.2 Ensaios de acúmulo de brometo de etídio em presença de IBEs .....	24
1.6.3 Microscopia eletrônica.....	24
1.6.4 Avaliação da expressão gênica relativa por PCR em tempo real .....	25
2. JUSTIFICATIVA .....	27
3. OBJETIVOS .....	28
3.1 Objetivo Geral .....	28
3.2 Objetivos Específicos .....	28
REFERÊNCIAS .....	29
CAPÍTULO II .....	36
Artigo 1: “FAST DETECTION OF DRUG INTERACTION IN <i>Mycobacterium tuberculosis</i> BY A CHECKERBOARD RESAZURIN METHOD” .....	36
Artigo 2: “MORPHOLOGICAL CHANGES AND DIFFERENTIALLY EXPRESSED EFFLUX PUMP GENES IN <i>Mycobacterium tuberculosis</i> EXPOSED TO RIFAMPICIN AND VERAPAMIL COMBINATION” .....	51
CAPÍTULO III .....	79
CONCLUSÕES .....	79
PERSPECTIVAS FUTURAS.....	79
ANEXOS .....	80
Artigo Publicado na revista <i>Tuberculosis</i> .....	80

## CAPÍTULO I

### 1. INTRODUÇÃO

#### 1.1 Tuberculose: Aspectos Gerais e Epidemiologia

A tuberculose (TB) é uma doença infectocontagiosa causada principalmente pelo bacilo *Mycobacterium tuberculosis* (*Mtb*) descrito por Robert Koch em 1882. É uma doença conhecida há séculos, no entanto, as altas taxas de mortalidade e morbidade associadas à TB são aspectos que ainda a caracterizam como um importante problema de saúde pública em todo o mundo (WHO, 2013).

A TB é um dos agravos fortemente influenciados pela condição social e demonstra relação direta com a pobreza e a exclusão social. A história tem mostrado que a melhoria das condições sócio-econômicas pode facilitar o controle da TB (GLAZIOU et al., 2013). O desenvolvimento e a evolução da doença são dependentes de fatores como aglomerados humanos, a desnutrição e a baixa resistência imunológica, como em casos de infecção provocada pelo vírus da imunodeficiência humana (HIV, sigla do inglês, *Human Immunodeficiency Virus*). Quando a eliminação da TB parecia possível no início de 1980, a pandemia de infecção pelo HIV resultou em um ressurgimento da TB (SHARMA; MOHAN, 2013). A co-infecção TB/HIV representa mais de 10% dos casos anuais de TB, sendo que os indivíduos HIV-positivos possuem até 37 vezes maior probabilidade de desenvolver TB do que aqueles HIV-negativos (WHO, 2010a).

A pandemia da Síndrome da Imunodeficiência Adquirida (AIDS, sigla do inglês, *Acquired Immunodeficiency Syndrome*) modificou não apenas a tendência epidemiológica da TB e as infecções causadas por outras micobactérias, mas também a apresentação clínica da doença, duração do tratamento, resistência aos fármacos anti-TB, intolerância medicamentosa e possivelmente a susceptibilidade dos comunicantes (LIBERATO et al., 2004).

Estima-se que um terço da população mundial está infectada pelo bacilo, mas não apresentam a doença, quadro conhecido como TB latente. Apesar de todos os países já terem relatado casos de TB, a maioria (85%) ocorre na África (30%) e Ásia (55%), com a Índia e a China sozinhas respondendo por 35% de todos os casos (WHO, 2010a). O Brasil ocupa o 17º lugar entre os 22 países responsáveis por 80% do total de casos notificados de TB no mundo. Em 2012, um número estimado de 8,6 milhões de indivíduos, dos quais 1,1 milhões HIV-positivos desenvolveram TB e 1,3 milhões de mortes ocorreram. Segundo a Organização

Mundial da Saúde (OMS), este número de mortes por TB é inaceitável, dado que a maioria dos casos poderia ter sido evitada (WHO, 2013).

Entre os principais obstáculos para o controle global da doença estão o surgimento de isolados multirresistentes aos medicamentos e a recalcitrância de infecções persistentes ao tratamento com fármacos anti-TB convencionais (SMITH; SHARMA; SACCHETTINI, 2004). Os maiores índices de isolados multirresistentes são encontrados na Europa Oriental e Ásia Central, sendo que em alguns países, mais de 20% dos casos novos e mais de 50% daqueles tratados anteriormente já apresentam multirresistência (WHO, 2013).

## **1.2 Patogênese e Manifestações Clínicas**

O bacilo é transmitido pessoa a pessoa por meio de contato com gotículas dispersas no ar durante a fala, espirro e tosse de indivíduos com doença pulmonar ou laríngea. O contato com o indivíduo bacilífero aumenta a chance de infecção, principalmente em ambiente fechado, com pouca ventilação e na ausência de luz solar (VERONESI, FOCACCIA, 2010). Devido a sua forma de transmissão e por ser um bacilo aeróbio estrito, ele infecta principalmente os pulmões, onde encontra condições favoráveis de oxigênio para sua multiplicação, e a doença, neste caso, é definida como TB pulmonar. O bacilo pode, por disseminação linfohematogênica, atingir outras partes do corpo como as meninges, rins, ossos e linfonodos, e neste caso a doença é definida como TB extrapulmonar (BRASIL, 2002).

Os sintomas da TB pulmonar são semelhantes aos de várias outras doenças, como uma simples gripe, o que torna o seu diagnóstico complexo. Os principais sintomas incluem tosse, febre vespertina, sudorese noturna, dor torácica, anorexia, perda de peso e adinamia (LAWN; ZUMLA, 2011b). Quando a TB é extrapulmonar, os sinais e sintomas dependem dos órgãos e/ou sistemas acometidos e mais frequentemente atingem indivíduos que vivem com HIV, especialmente aqueles com grave comprometimento imunológico (BRASIL, 2002).

A infecção pelo bacilo da TB pode ocorrer em qualquer idade, mas nem todos os indivíduos expostos se tornam doentes. A transmissão depende de alguns fatores: da contagiosidade do indivíduo bacilífero que é fonte da infecção; do tipo de ambiente em que a exposição ocorreu e da duração da exposição. Uma vez infectado, o indivíduo pode desenvolver a doença em qualquer fase da vida. Isto acontece quando o sistema imune não pode mais manter o equilíbrio para o controle da multiplicação bacilar (BRASIL, 2002).

Uma lesão tuberculosa é composta por populações micobacterianas de diferentes perfis metabólicos, os quais incluem: (a) bacilos que se multiplicam continuamente com

velocidade diferente (crescimento rápido ou lento); (b) bacilos que permanecem latentes e com períodos esporádicos de atividade metabólica (CONDE; FITERMAN; LIMA, 2011).

Neste sentido, o tratamento da TB exige uma combinação de medicamentos, levando em conta as diferentes características da população bacilar e tendo em vista três objetivos principais: (1) a ação bactericida precoce, que é a capacidade de matar o maior número de bacilos possíveis, responsável pela diminuição da transmissibilidade da TB no início do tratamento; (2) a prevenção da seleção de bacilos resistentes, visto que cada população micobacteriana possui diferentes proporções de bacilos com resistência natural aos diferentes fármacos anti-TB e (3) a esterilização da lesão, ou seja, a capacidade de eliminar todos os bacilos de uma lesão evitando assim a recidiva da doença depois de finalizado o tratamento (DONALD; SCHAAF, 2007; CONDE; FITERMAN; LIMA, 2011).

### **1.3 Diagnóstico e Tratamento da Tuberculose**

Baciloscopia, cultura (padrão ouro) e teste de susceptibilidade aos antimicobacterianos são atualmente métodos tradicionais recomendados para o diagnóstico da TB ativa (BAMMANN et al., 2010). A baciloscopia se dá pela pesquisa microscópica de *Mtb*, um bacilo álcool-ácido resistente (BAAR), corado pelo método de Ziehl-Neelsen em materiais de prováveis sítios da doença, tal como o escarro. A cultura, mais sensível e específica que a baciloscopia, é realizada por semeadura da amostra em meios de cultura sólidos (Löwenstein-Jensen, Ogawa, Middlebrook 7H10 e 7H11) ou líquidos (Middlebrook 7H9). Os meios Löwenstein-Jensen e Ogawa têm a vantagem de apresentar menor custo e um menor índice de contaminação, porém com a desvantagem do tempo de detecção do crescimento bacteriano variar de 14 a 30 dias, podendo se estender por até 60 dias (BRASIL, 2002).

Para amostras biológicas proveniente de sítios anatômicos que apresentam microbiota envolvida, uma descontaminação do material é previamente requerida à realização da cultura (SAÚDE, 2002). O teste de susceptibilidade às drogas pode ser realizado por diferentes metodologias, porém o método das proporções é o recomendado pelo Ministério da Saúde (CONDE; FITERMAN; LIMA, 2011).

Ensaios com *Interferon Gamma Release Assays* (IGRAs) e Teste Cutâneo de Mantoux (Prova Tuberculínica) auxiliam principalmente no diagnóstico de infecção latente. Testes que amplificam ácidos nucléicos, exames de imagens e histopatológico de biópsia são ferramentas que complementam o diagnóstico da TB (BRASIL, 2002).

Um teste molecular para o diagnóstico da TB, Xpert MTB/RIF detecta bacilos do complexo *Mtb* dentro de 2 horas diretamente do escarro, com uma sensibilidade maior do que a baciloscopia (BOEHME et al., 2010). Além da detecção da presença de *Mtb*, este teste também detecta resistência a rifampicina (RIF) auxiliando assim no diagnóstico de *Mtb* multirresistente. Entre os infectados com o HIV, este teste aumentou em 45% a taxa de detecção de casos de TB comparado com a baciloscopia (LAWN et al., 2011a). Este ensaio molecular, recomendado pela OMS tem o potencial de melhorar o desempenho de programas nacionais de combate a TB e está atualmente sendo implantado em laboratórios/países com alta prevalência da doença (WHO, 2013).

Após a confirmação do diagnóstico, o tratamento adequado pode ser iniciado rapidamente. A OMS recomenda que o tratamento da TB ativa com *Mtb*, suscetível aos fármacos anti-TB, seja realizado pela administração diária de RIF, isoniazida (INH), pirazinamida (PZA) e etambutol (EMB) durante dois meses, seguido de RIF e INH durante quatro meses (WHO, 2010a).

A poliquimioterapia é usada para potencializar a ação contra *Mtb* e prevenir o desenvolvimento de resistência. A elaboração do comprimido 4 em 1, dose fixa combinada, trouxe vantagens, como a diminuição de erros de prescrição e número de comprimidos diários a serem tomados, o que facilita a adesão do doente à terapêutica (BLOMBERG et al., 2001; WHO, 2010b). Outra estratégia desenvolvida, em meados dos anos 1990, para o controle da TB foi o tratamento diretamente supervisionado (DOTS, sigla do inglês, *Directly Observed Treatment Short*), uma abordagem recomendada internacionalmente a fim de reduzir a irregular e descontínua ingestão do medicamento durante o tratamento (WHO, 2010a).

Mesmo com os avanços diagnósticos e terapêuticos no decorrer das últimas décadas, a almejada erradicação da TB mostrou-se difícil de ser alcançada. Isolados de *Mtb* resistentes aos fármacos surgiram como uma grande ameaça para o controle global da doença. Apesar da disponibilidade de terapia anti-TB curativa por quase meio século, o tratamento inadequado permitiu ao bacilo adquirir resistência aos fármacos anti-TB (DALEY; CAMINERO, 2013). Em 2012, cerca de 450.000 indivíduos desenvolveram TB por *Mtb* multirresistente (*Mtb* MDR), considerado resistente a pelo menos à INH e RIF e, houve cerca de 170.000 mortes por cepas extensivamente resistentes aos medicamentos (*Mtb* XDR), as quais são classificadas como MDR com resistência adicional às fluoroquinolonas e pelo menos um fármaco injetável de segunda linha (canamicina, capreomicina e amicacina) (WHO, 2013).

A toxicidade evidenciada pelos fármacos disponíveis, aliada à necessidade de seu uso diário por no mínimo seis meses, tem despertado, a necessidade da busca por novos

medicamentos ou combinações destes, a fim de diminuir os efeitos colaterais, encurtar o tempo de tratamento da forma ativa da doença e eliminar o bacilo em estado latente ou resistente aos fármacos disponíveis (CONDE; FITERMAN; LIMA, 2011).

#### **1.4 Mecanismo de Ação e Resistência aos Principais Fármacos Utilizados no Tratamento da TB**

A INH é um dos principais fármacos usados no tratamento da TB, apresenta efeito bactericida sobre os bacilos ativos. Este medicamento apresenta fórmula estrutural simples (Figura 1), e sua ação primária consiste em inibir a síntese dos ácidos micólicos presentes na parede celular micobacteriana. INH é um pró-fármaco que exige ativação pelo sistema enzimático catalase/peroxidase micobacteriano, o qual é codificado pelo gene *katG* (ZHANG et al., 1992). A resistência à INH é um processo complexo, pois mutações em vários genes, incluindo *katG*, *ahpC*, *inhA*, *kasA*, *ndh* e região intergênica *oxyR-ahpC*, têm sido observadas, sendo mais freqüente a presença de mutações na fase aberta de leitura dos genes *katG* e *inhA*, bem como na região promotora de *inhA* (CARDOSO et al., 2004; SILVA et al., 2003).

Devido à ação antimicrobiana eficiente da RIF (Figura 1), considera-se, em conjunto com INH, a base do esquema de tratamento de curta duração para TB. Mais de 90% dos isolados resistentes à RIF também são resistentes à INH, fazendo da resistência a RIF um marcador de *Mtb* MDR (PANG et al., 2013). A RIF atua inibindo a subunidade beta da RNA polimerase, pela formação de um complexo fármaco-enzimático estável, o que leva à supressão do início da transcrição para a síntese de RNAm (LEWIS, 2013). Este fármaco atua em bacilos ativos e bacilos metabolicamente lentos (GUMBO et al., 2007). Cerca de 95% dos casos de resistência à RIF são causados por mutações no gene *rpoB* que codifica a enzima RNA polimerase em *Mtb* (TELENTI et al., 1993).

Ao contrário da RIF e INH, que são fármacos bactericidas, o EMB é bacteriostático (RAMASWAMY et al., 2000). O EMB (Figura 1) atua na fase intensiva do tratamento inibindo as arabinosil transferases envolvidas na biossíntese da parede celular, bloqueando assim a síntese de arabinogalactano. A resistência bacteriana a este fármaco desenvolve-se por mutações no operon *embCAB* que codifica arabinosil transferases (COLL, 2003). Mutações em *embB* são identificadas em mais de 65% dos isolados clínicos, sendo associadas com alto nível de resistência. No entanto, uma porcentagem importante dos isolados resistentes ao EMB não apresentam mutações em *embB*, sugerindo assim um outro mecanismo para a resistência ainda não descrito (ZHANG; YEW, 2009).

A PZA (Figura 1) é um análogo estrutural da nicotinamida, um pró-fármaco convertido em ácido pirazinóico pela enzima pirazinamidase bacteriana, a qual é codificada pelo gene *pncA* (SCORPIO; ZHANG, 1996). Este fármaco apresenta atividade bactericida sobre bacilos semi-adormecidos residentes em ambientes ácidos, como dentro dos macrófagos. Mutações no gene *pncA*, é o principal mecanismo de resistência a este fármaco. A ação específica da PZA em *Mtb*, pode ser explicada pelo fato do gene *pncA* apresentar polimorfismos que levam a não ativação da PZA em ácido pirazinóico conforme a espécie de micobactéria (SUN; ZHANG, 1999).

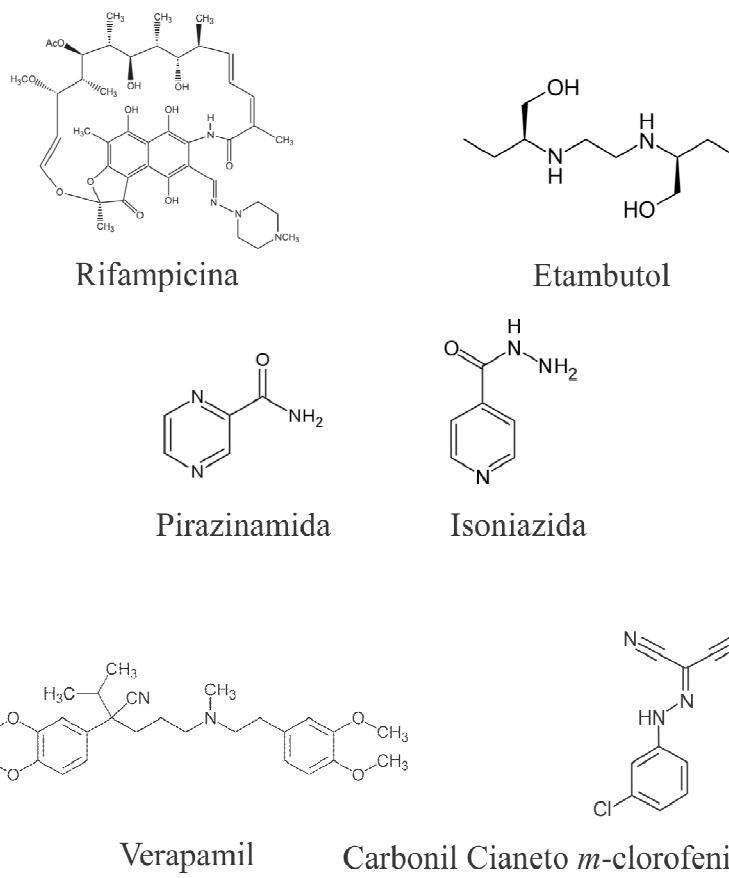


Figura 1- Estrutura química dos principais fármacos anti-tuberculose e de inibidores de bomba de efluxo.

Fonte: Adaptado de Viveiros et al. (2012, p. 989); Souza e Vasconcelos et al. (2005, p. 679).

As micobactérias são naturalmente resistentes à maioria dos antimicrobianos tradicionalmente usados na clínica, devido à presença de um envelope celular contendo ácido micólico altamente hidrofóbico que dificulta a entrada dos antibióticos no bacilo, e de alguns

receptores e enzimas micobacterianas que impedem a ação de antimicrobianos como betalactâmicos e aminoglicosídeos (BRENNAN; NIKAIDO, 1995; CALGIN et al., 2013). Além da estrutura original das micobactérias, as mutações nos genes que codificam enzimas alvo são importantes razões da resistência aos fármacos anti-TB (LOUW et al., 2009). No entanto, em algumas situações, a resistência não pode, ainda, ser explicada pela presença dessas mutações gênicas, sugerindo a existência de outros mecanismos de resistência (SILVA et al., 2001).

### **1.5 Bombas de Efluxo e Inibidores**

Evidências recentes sugerem que a resistência de *Mtb* a múltiplos antimicrobianos pode ser atribuída a uma combinação de uma parede celular altamente impermeável com um mecanismo ativo de efluxo do fármaco. Expressões constitutivas ou induzíveis de sistemas de efluxo podem ajudar o bacilo a escapar dos efeitos nocivos dos fármacos (DANILCHANKA; MAILAENDER; NIEDERWEIS, 2008; DE KNEGT et al., 2013). O papel de mecanismos de efluxo tem sido também reconhecido como um importante fator para a resistência natural de espécies de micobactérias a alguns antimicrobianos, como tetraciclinas, fluoroquinolonas, aminoglicosídeos, entre outras (DE ROSSI; AINSA; RICCARDI, 2006; DA SILVA et al., 2011).

As bombas de efluxo (BEs) são proteínas transportadoras envolvidas na eliminação de substâncias tóxicas da célula. Esta função de proteção permite às células bacterianas sobreviverem em ambientes hostis, incluindo aqueles que contêm antimicrobianos, o que as tornam assim tolerantes. A atividade dos sistemas de efluxo pode diminuir significativamente a concentração intracelular de diversos antimicrobianos, reduzindo a sua eficácia clínica e contribuindo para a seleção de bactérias resistentes (DE ROSSI et al., 2002). Além disso, o efluxo de fármacos também tem sido associado à virulência, formação de biofilme e patogenicidade, incluindo a sobrevida no interior do macrófago (DA SILVA et al., 2011; SZUMOWSKI et al., 2013).

Recentes estudos têm mostrado que o estado dormente de *Mtb* no hospedeiro pode ser devido à tolerância mediada pela atividade dos sistemas de efluxo que foram estimulados com a entrada do bacilo no macrófago (ADAMS et al., 2011).

As BEs bacterianas são classificadas em cinco famílias: sendo duas delas superfamílias, *ATP Binding Cassete* (ABC) e *Major Facilitator Superfamily* (MFS). As outras três são famílias menores e evolutivamente mais recentes: *Small Multidrug Resistance*

(SMR), *Resistance Nodulation Cell Division* (RND) e *Multidrug and Toxic Compounds Extrusion* (MATE) (DE ROSSI; AINSA; RICCARDI, 2006; ZECHINI; VERSACE, 2009; DA SILVA et al., 2011). A família ABC pertence à classe de transportadores ativos primários, que utilizam energia derivada da hidrólise do ATP para transportar compostos contra um gradiente de concentração. Já os membros das famílias MFS, SMR, RND e MATE são transportadores secundários, e utilizam o gradiente eletroquímico de prótons para expulsar os fármacos das células (ZECHINI; VERSACE, 2009; DA SILVA et al., 2011).

Enquanto membros das famílias ABC, MFS, MATE e SMR são observados em bactérias Gram-positivas, BEs pertencentes às famílias ABC, RND e MFS são frequentemente encontrados em bactérias Gram-negativas (OKANDEJI et al., 2011). Nos últimos anos, algumas BEs pertencentes às famílias ABC, MFS, RND e SMR têm sido descritas em *Mtb* (DE ROSSI et al., 1998; DANILCHANKA; MAILAENDER; NIEDERWEIS, 2008; GUPTA et al., 2010b; DA SILVA et al., 2011; BALGANESH et al., 2012; RODRIGUES et al., 2012; CALGIN et al., 2013; RODRIGUES et al., 2013). O papel dos transportadores MATE em procariotos incluindo *Mtb* ainda não é bem caracterizado (MISHRA; DANIELS, 2013).

*Mtb* apresenta um dos maiores números de BEs de fármacos em comparação com o tamanho do seu genoma, representando de 6 a 18% de todos os transportadores encontrados na célula bacteriana (DA SILVA et al., 2011). Resistência aos fármacos, mediada por efluxo, pode ser devido a uma ou mais BEs atuando isoladamente ou em combinação (BALGANESH et al., 2012). Algumas BEs são específicas para determinados fármacos, ao passo que outras fazem o efluxo de vários compostos denominadas de BEs de resistência a múltiplos compostos (CALGIN et al., 2013), como por exemplo, a proteína *Mmr*, da família SMR, que confere resistência a acriflavina, brometo de etídeo e eritromicina em *M. smegmatis* (DE ROSSI et al., 1998).

A atividade das BEs parecem também induzir resistência cruzada a compostos diferentes, tal como observado por Louw et al. (2011), onde o tratamento com RIF de isolados resistentes a este fármaco induziu resistência a ofloxacina, a qual pode ser revertida com o uso de inibidores de bomba de efluxo (IBEs). Os mecanismos de indução e regulação dessas BEs ainda não são totalmente compreendidos (DA SILVA et al., 2011) e nem todos os fármacos anti-TB disponíveis atualmente são considerados substratos de mecanismos de efluxo (PALOMINO; RAMOS; DA SILVA, 2009).

Várias BEs em micobactéria têm sido associadas à resistência a fármacos anti-TB, tais como: *jefA* (*Rv2459*), que confere resistência a INH e EMB (GUPTA et al., 2010b); *Mmr*

(*Rv3065*) resistência a INH (RODRIGUES et al., 2013); *P55* (*Rv1410c*) resistência a aminoglicosídeos e tetraciclina (SILVA et al., 2001); *Rv1217c* e *Rv1218c* resistência a RIF (WANG et al., 2013); *Rv1258c* e *Rv1410c* resistência a INH e RIF (JIANG et al., 2008); *Rv1634* resistência a fluoroquinolona (DE ROSSI et al., 2002); entre outras.

Em geral, as BEs de antimicrobianos conferem baixos níveis de resistência, em contraste com os altos níveis de resistência conferidos por mutações em genes que codificam os alvos primários desses agentes (SZUMOWSKI, et al. 2013). De fato, mesmo a superexpressão de uma bomba de efluxo com espectro ampliado para múltiplos fármacos pode não conferir alto nível de resistência. Entretanto, embora o significado clínico imediato desta resistência seja pequeno, a diminuição da concentração intracelular do antimicrobiano permite à bactéria sobreviver até que sejam selecionados mutantes com alterações moleculares que podem determinar níveis de resistência clinicamente significativos (MARTINEZ; BAQUERO, 2000; VIVEIROS, et. al., 2012; WEBBER; PIDDOCK, 2003). Neste sentido, a atividade de uma única BE pode não resultar em um alto nível de resistência, porém a superexpressão associada de múltiplos genes que codificam para BEs em isolados clínicos resistentes não pode ser ignorada (WEBBER; PIDDOCK, 2003).

Alguns trabalhos têm focado na identificação de IBEs em bactérias Gram-negativas e Gram-positivas que podem potencialmente ser utilizados em combinação com antimicrobianos para melhorar a eficácia clínica destes e evitar a resistência (ZECHINI; VERSACE, 2009; JIN et al., 2010). Esses inibidores, ao bloquear o efluxo dos antimicrobianos, aumentam a concentração intracelular dos mesmos, favorecendo assim a morte da célula bacteriana (ZECHINI; VERSACE, 2009).

Vários compostos IBEs tem sido descrito, tais como verapamil (VP), carbonil cianeto *m*-clorofenil-hidrazona (CCCP), nigericina, valinomicina, *o*-vanadato, reserpina, clorpromazina, haloperidol, farnesol, omeprazol, piperina, tioridazina, entre outros (VIVEIROS et al., 2012; ZECHINI; VERSACE, 2009). Dentre estes fármacos, o VP e o CCCP estão entre os mais estudados (RODRIGUES et al., 2013).

O VP (Figura 1) tem sido reconhecido como o mais promissor IBE, pois inibe a tolerância de *Mtb* a múltiplos fármacos; é geralmente bem tolerado pelo paciente; tem sua farmacocinética bem caracterizada e em modelos animais está significativamente concentrado nos pulmões (ADAMS; SZUMOWSKI; RAMAKRISHNAN, 2014). Seu uso clínico é relativamente seguro como um bloqueador de canais de cálcio no tratamento de hipertensão, arritmias cardíacas e angina. No entanto, recentemente foi observado que a atividade inibitória do VP em *Mtb* independe de sua atividade como antagonista de cálcio (ADAMS;

SZUMOWSKI; RAMAKRISHNAN, 2014; GUPTA et al., 2013). Estudos têm mostrado que o VP pode inibir algumas BEs tipo ABC, tal como o transportador *p*-glicoproteína (*gpP*) humano, que funciona como uma bomba ATP-dependente e está relacionada com a resistência a múltiplos fármacos (CHOUDHURI et al., 2002; ZECHINI; VERSACE, 2009).

Por outro lado, o CCCP (Figura 1) é um inibidor químico da fosforilação oxidativa que vem sendo utilizado nos estudos por dispersar o gradiente próton-motor das membranas e por despolarizar a membrana energizada, inibindo assim as BEs que utilizam o gradiente eletroquímico de prótons para expulsar os fármacos das células (VIVEIROS et al., 2012). Entretanto, o mecanismo de ação da maioria dos IBEs ainda não está totalmente elucidado (ZECHINI; VERSACE, 2009).

O aparecimento de isolados de *Mtb* resistentes aos antimicrobianos disponíveis contribui para a falta de opções terapêuticas (WANG et al., 2013; ZUMLA et al., 2014). Assim, os IBEs podem ser usados para tornar o bacilo mais suscetível aos agentes antimicrobianos, principalmente em *Mtb* com resistência mediada por BEs (RAMON-GARCIA et al., 2006). Neste sentido, alguns trabalhos *in vitro* têm obtido bons resultados em *Mtb*, tais como: diminuição da resistência a INH em presença de CCCP, reserpina (PASCA et al., 2005) e VP (RODRIGUES et al., 2012); e redução da concentração inibitória mínima (CIM) de RIF quando associada ao inibidor piperina (SHARMA et al., 2010). Recentemente um estudo *in vivo* com camundongos, realizado por Gupta et al. (2013), demonstrou a redução do tempo de tratamento da TB quando associado com VP.

O surgimento de *Mtb* MDR e XDR se tornou um problema na gestão da TB, levando a uma necessidade urgente de compreender os mecanismos de resistência aos fármacos, bem como desenvolver pesquisas relacionadas a novos antimicrobianos ou esquemas terapêuticos, incluindo a combinação de IBEs com fármacos atualmente disponíveis (REY-JURADO et al., 2013).

## **1.6 Metodologias Empregadas no Estudo da Atividade e Ação Combinada de Fármacos Anti-TB**

### **1.6.1 REMA, *chequerboard* e curva de tempo de morte**

O método *Resazurin Microtiter Assay Plate* (REMA), descrito por Palomino et al. (2002), permitiu a detecção rápida de resistência a múltiplos fármacos, em isolados de *Mtb*, com uma precisão de 97% quando comparado ao método das proporções. O REMA utiliza a resazurina, um corante de oxidação-redução como indicador de viabilidade celular. A

resistência é detectada por uma mudança na cor do indicador, que é diretamente proporcional ao número de micobactérias viáveis no meio. Os métodos colorimétricos representam uma boa alternativa para a rápida detecção de resistência a fármacos em laboratórios com recursos limitados (PALOMINO et al., 2002).

Para avaliar novas opções terapêuticas, estudos com combinações de diferentes fármacos têm sido realizados (REY-JURADO et al., 2013; ZUMLA et al., 2014). O sinergismo entre os fármacos pode ser avaliado utilizando as metodologias *chequerboard* (CALEFFI-FERRACIOLI et al., 2013) e/ou o método de curva de tempo de morte (LIMONCU et al., 2011; PANKUCH, et al., 2008; PILLAI, 2005). É importante enfatizar que o *chequerboard* avalia o efeito bacteriostático dos fármacos (CALEFFI-FERRACIOLI et al., 2013), ao passo que a curva de tempo de morte avalia o efeito bactericida (DE STEENWINKEL et al., 2010).

Recentemente, a técnica de REDCA (*Resazurin Drugs Combination Microtiter Assay*), um *chequerboard* modificado desenvolvido no Laboratório de Bacteriologia Médica da Universidade Estadual de Maringá, que emprega resazurina como indicador de viabilidade celular, reduziu o tempo de incubação do ensaio para *Mtb*, facilitou a leitura e, consequentemente, diminuiu o tempo para obtenção dos resultados (CALEFFI-FERRACIOLI et al., 2013).

Para interpretar o efeito sinérgico entre os fármacos estudados pelo método de REDCA, utiliza-se o Índice da Fração Inibitória (IFI): sendo,  $IFI = (CIM\ A + B / CIM\ A) + (CIM\ B + A / CIM\ B)$ , onde: CIM A + B representa a CIM do fármaco A quando associada ao fármaco B; CIM B + A representa a CIM do fármaco B quando associada ao fármaco A; CIM A representa a CIM do fármaco A quando testado isoladamente; e CIM B representa a CIM do fármaco B quando testado isoladamente. O efeito das combinações dos agentes antimicrobianos é classificado em: sinérgico ( $IFI \leq 0,5$ ); aditivo ou indiferente ou sem interação ( $IFI > 0,5 - 4$ ); e antagônico ( $IFI > 4$ ) (ODDS, 2003; PILLAI, 2005).

A técnica de curva de tempo de morte microbiana se baseia na incubação (35-37°C por 7-8 dias) de culturas acrescidas de antimicrobianos sozinhos e em combinação e, de repiques quantitativos ao longo deste tempo, a fim de determinar o número de bactérias viáveis remanescentes em cada tempo previamente determinado. O sinergismo é definido quando ocorre redução de dois ou mais  $\log_{10}$  UFC/mL para a combinação de fármacos, em comparação com o agente anti-TB mais ativo (LIMONCU et al., 2011).

### **1.6.2 Ensaios de acúmulo de brometo de etídio em presença de IBEs**

Viveiros et al. (2008) propuseram um método fluorimétrico semi-automatizado para estimar a atividade de BEs em bactéria. Por este método, o efluxo de um substrato fluorescente comum, tal como o brometo de etídio (EtBr, sigla do inglês, *ethidium bromide*) é quantificado em toda a célula viva por determinado período de tempo. A cinética do transporte de EtBr é a base para determinar a atividade de efluxo das células. EtBr emite fluorescência fraca em soluções aquosas, e torna-se fortemente fluorescente quando concentrado intracelularmente pela ligação aos ácidos nucléicos o que torna este método importante para monitorar continuamente o transporte do substrato fluorescente através das membranas celulares (PAIXÃO et al., 2009).

Este ensaio permite observar, em tempo real, o acúmulo e o efluxo de EtBr sob diferentes condições fisiológicas (alterações de temperaturas e pH, a presença ou ausência de uma fonte de energia) e na presença ou ausência de IBEs. Dessa forma, este método pode ser utilizado na triagem de IBEs e no monitoramento da superexpressão de BEs (VIVEIROS et al., 2008; PAIXÃO et al., 2009).

### **1.6.3 Microscopia eletrônica**

A microscopia eletrônica difere da microscopia óptica pelo tipo de radiação utilizada e pela maneira como ela é refratada. Na primeira, a radiação empregada é a de feixe de elétrons, sendo ele refratado por meio de lentes eletrônicas, e na segunda as radiações provêm de fontes luminosas e são refratadas em lentes de vidro. Neste sentido, a microscopia eletrônica, seja a tipo varredura (SEM, sigla em inglês, *Scanning Electron Microscopy*) que permite estudar a topografia da superfície de objetos sólidos, ou a tipo transmissão (TEM, sigla em inglês, *Transmission Electron Microscopy*) que permite estudar estruturas de parede e intracelulares, agrega vantagens sobre a microscopia de luz, uma vez que permite maior resolução e consequentemente favorece a melhor observação de detalhes (BOZZOLA; RUSSELL, 1999).

A maioria dos estudos de microscopia eletrônica da ultra-estrutura em *Mtb* foi realizada nas décadas de 1950 e 1960 e carecem de alta resolução para os padrões modernos (DAHL, 2005). Takayama, Wang e Merkal (1973) realizaram um trabalho pioneiro com SEM para avaliar a morfologia das células de *Mtb H<sub>37</sub>Ra* após exposição à INH. No entanto, até o nosso conhecimento, estudos para avaliar da ação dos IBEs utilizando SEM ainda não foram reportados.

#### **1.6.4 Avaliação da expressão gênica relativa por PCR em tempo real**

Grandes avanços em biologia molecular e a disponibilidade de novas informações geradas após o sequenciamento do genoma do *Mtb* aumentaram o conhecimento sobre os mecanismos de resistência aos principais fármacos anti-TB e o papel das BEs (GUPTA et al., 2010a). Existem várias maneiras de investigar alterações induzidas artificialmente ou por agentes naturais, durante um processo biológico na célula. Uma maneira é pesquisar as mudanças ocorridas na transcrição celular, que pode indicar mudanças na proteína correspondente (TEVFIK, 2006). O transcriptoma é dependente do contexto, ou seja, o RNAm, transcrito a partir da informação genômica, varia de níveis de acordo com os aspectos fisiológicos ou patológicos (BUSTIN; NOLAN, 2004).

A PCR quantitativa, em tempo real, a partir do transcrito (RT-qPCR *reverse transcription quantitative real-time PCR*) é um método de quantificação de RNAm, rotineiramente usado para investigar a expressão de um pequeno ou moderado número de genes (TEVFIK, 2006), tais como a expressão de genes de BEs (JIANG et al., 2008; HAO et al., 2011; WANG et al., 2013). Para a avaliação de níveis de expressão de transcritos em larga escala pode-se utilizar a metodologia do microarranjo (*microarray*) como realizado por alguns autores (RAMON-GARCIA et al., 2009; GUPTA et al., 2010a; LIANG et al., 2012). Entretanto, tanto para o RT-qPCR quanto para métodos baseados em *arrays*, os alvos gênicos a serem estudados precisam ser conhecidos.

RT-qPCR tornou-se o ponto de referência para a detecção e quantificação de alvos de RNA e está cada vez mais sendo utilizado em novos ensaios clínicos de diagnóstico (BUSTIN; MUELLER, 2005). O princípio desta técnica baseia-se na coleta contínua de sinal fluorescente a partir de uma ou mais PCRs em um intervalo de ciclos e, posteriormente, realiza a conversão destes sinais a um valor numérico para cada amostra. Quanto mais alto o número de cópias iniciais do ácido nucléico alvo, mais rápido será observado o aumento significativo na fluorescência (BUSTIN; MUELLER, 2005; TEVFIK, 2006).

A detecção química pela RT-qPCR pode estar baseada em sondas (detecção específica) ou não-sondas (inespecífica). O sistema SYBR não utiliza sondas, utiliza o corante SYBR Green que possui ligação altamente específica ao DNA. Neste sistema a dupla-fita de DNA emite fluorescência conforme o acúmulo do produto da PCR durante os ciclos da reação. Como exemplo de sondas específicas, temos o sistema TaqMan, o qual utiliza sondas de hidrólise que são projetadas com especificidade para o DNA alvo complementar (BUSTIN et al., 2005). A diferença mais importante entre o sistema SYBR Green e o sistema TaqMan é que o SYBR Green detectará todo DNA dupla-fita, inclusive produtos de reação não

específicos, assim a especificidade da técnica depende da especificidade do iniciador (*primer*) utilizado. Neste contexto, uma reação bem otimizada é, portanto, essencial para resultados precisos, além da curva de *melting* que deve acompanhar todas as reações de RT-qPCR a fim de monitorar se um único produto da reação está sendo gerado e a ausência da formação de dímeros de *primers* (TEVFIK, 2006).

Os níveis de expressão dos genes podem ser medidos pela quantificação absoluta ou por uma quantificação relativa (ou comparativa), por RT-qPCR (TEVFIK, 2006). A quantificação absoluta correlaciona o sinal de PCR com o número de cópias transcritas usando uma curva de calibração. Já a quantificação relativa mede os níveis de expressão relativos de RNAm comparados a um controle (na ausência de fármacos, por exemplo). Para alcançar melhores resultados de expressão relativa, a normalização apropriada, utilizando genes endógenos, são estratégias necessárias para controlar o erro experimental (BUSTIN et al., 2005).

O gene endógeno, de referência, deve ser expresso de maneira constitutiva em todas as condições, de forma independente do projeto experimental. Alguns dos genes utilizados na normalização do RT-qPCR em *Mtb* são: *16sRNA* (JIANG et al., 2008), gene *polA* (GUPTA et al., 2010b) e gene *Hsp65* (HAO et al., 2011; WANG et al., 2013). Segundo Bustin et al. (2005), o emprego de genes ribossomais (RNAr) ainda é o mais indicado para normalização, já que estes sofrem menos variações que os RNA mensageiros quando expostos as mesmas condições.

O método mais comumente usado para a quantificação relativa é o método  $2^{-\Delta\Delta Ct}$  (LIVAK; SCHMITTGEN, 2001). O cálculo pode ser realizado da seguinte maneira:

Quantificação relativa,  $RQ = 2^{-\Delta\Delta Ct}$ , onde:

$\Delta\Delta Ct = \Delta Ct$  (amostra tratada) –  $\Delta Ct$  (amostra controle); e

$\Delta Ct = (Ct$  gene alvo –  $Ct$  gene de referência);

sendo:  $Ct$  = ciclo *threshold*, número do ciclo da PCR no qual a fluorescência atinge o *threshold*; *Threshold* = Nível arbitrário de fluorescência estabelecido acima do *Baseline* e dentro da região de crescimento exponencial (TEVFIK, 2006).

## 2. JUSTIFICATIVA

O surgimento de *Mtb* multirresistente é uma preocupação mundial, visto que o arsenal de antimicrobianos disponíveis para o tratamento da TB é pequeno. Sendo assim, a descoberta de novos fármacos, ou mesmo associações destes, representam um grande desafio, pois o bacilo apresenta crescimento lento e tem uma parede celular rica em lipídios, o que o torna protegido contra ação de um significativo número de antimicrobianos.

Combinações de diferentes fármacos com atividade anti-TB tem sido relatadas na literatura. Assim, a proposta de ação sinérgica entre estes pode ajudar a encurtar o tempo de tratamento da TB, melhorar a adesão do paciente e encontrar alternativas terapêuticas mais eficazes e seguras para o tratamento da doença principalmente quando causada por *Mtb* MDR e XDR.

Recentemente, a resistência mediada por BEs, as quais podem conferir resistência natural ou adquirida a um ou vários compostos, foi descrita em micobactérias. Estudos envolvendo as BEs mostraram que a atividade destes sistemas pode resultar na redução dos níveis intracelulares de antimicrobianos, o que pode contribuir para a seleção de bactérias resistentes. Portanto, a inativação dessas BEs, surge como grande expectativa para controlar a resistência do bacilo a determinados antimicrobianos.

O uso combinado de substâncias capazes de inibir as BEs com alguns fármacos anti-TB, em estudos *in vitro*, tem demonstrado uma considerável redução nos valores de CIM do antimicrobiano. Nos últimos anos, estudos *in vivo* também têm corroborado com estes achados experimentais mostrando inclusive a redução dos efeitos colaterais dos medicamentos.

Considerando os poucos estudos envolvendo a combinação de fármacos anti-TB com IBEs em *Mtb*; que a maioria dos procedimentos experimentais para a identificação de BEs estão limitados a estudos de expressão em mutantes induzidos em laboratório; e que poucos estudos foram realizados com a cepa de referência *Mtb* H<sub>37</sub>Rv; o presente trabalho visa ampliar os conhecimentos sobre a atividade e o efeito da combinação de antimicrobianos de primeira linha no tratamento da TB com IBEs.

### 3. OBJETIVOS

#### 3.1 Objetivo Geral

Avaliar os efeitos da combinação de antimicrobianos e inibidores de bomba de efluxo na morfologia e expressão gênica em *M. tuberculosis*.

#### 3.2 Objetivos Específicos

- Determinar a concentração inibitória mínima dos inibidores CCCP e VP e, dos antimicrobianos (INH, RIF e EMB) para a cepa de referência *Mtb* H<sub>37</sub>Rv pelo método REMA;
- Propor um ensaio de *chequerboard* modificado com redução no tempo de incubação e maior facilidade de leitura para estudos de sinergismo de antimicrobianos em micobactérias;
- Determinar a ação sinérgica entre IBEs e antimicrobianos clássicos usados na terapia anti-TB pelo *chequerboard* bi-dimensional e o melhor resultado de combinação entre estes fármacos em *Mtb* H<sub>37</sub>Rv;
- Determinar o fármaco com melhor poder de inibição das BEs em *Mtb* H<sub>37</sub>Rv evidenciado pelo acúmulo de EtBr na célula bacteriana por meio da metodologia de fluorimetria;
- Determinar o tempo de exposição dos fármacos que serão utilizados nos ensaios de SEM e RT-qPCR por meio da curva de tempo de morte bacteriana;
- Realizar SEM em *Mtb* H<sub>37</sub>Rv exposto à combinação de fármacos com melhor atividade contra o bacilo pelo método *chequerboard* bi-dimensional;
- Realizar RT-qPCR dos principais genes que codificam para BEs, em *Mtb* H<sub>37</sub>Rv exposto à combinação de fármacos com melhor atividade pelo método *chequerboard* bi-dimensional.

## REFERÊNCIAS

- ADAMS, K. N.; SZUMOWSKI, J. D.; RAMAKRISHNAN, L. Verapamil, and its metabolite Norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. **The Journal of Infectious Diseases**, [S.l.], v. 210, n. 3, p. 456-466, 2014.
- ADAMS, K. N. et al. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. **Cell**, [S.l.], v. 145, n. 1, p. 39-53, 2011.
- BALGANESH, M. al. Efflux pumps of *Mycobacterium tuberculosis* play a significant role in antituberculosis activity of potential drug candidates. **Antimicrobial Agents and Chemotherapy**, Washington, v. 56, n. 5, p. 2643-2651, 2012
- BAMMANN, R. H. et. al. High prevalence of drug-resistant tuberculosis and other mycobacteria among HIV-infected patients in Brazil: a systematic review. **Memórias do Instituto Oswaldo Cruz**, Rio de Janeiro , v. 105, n. 6, p. 838-841, 2010.
- BLOMBERG, B. et. al. The rationale for recommending fixed-dose combination tablets for treatment of tuberculosis. **Bulletin of the World Health Organization**, Geneva, v. 79, n. 1, p. 61-68, 2001.
- BOEHME, C. C. et. al. Rapid molecular detection of tuberculosis and rifampin resistance. **New England Journal of Medicine**, Royston , v. 363, n. 11, p. 1005-1015, 2010.
- BOZZOLA, J. J.; RUSSELL, L. D. **Electron Microscopy**. Boston: Jones and Bartlett Publishers, 1999.
- BRASIL. Ministério da Saúde. Manual Técnico para o Controle da Tuberculose. Secretaria de Políticas de Saúde. Departamento de Atenção Básica. 2002. Disponível em: <[http://bvsms.saude.gov.br/bvs/publicacoes/guia\\_controle\\_tuberculose.pdf](http://bvsms.saude.gov.br/bvs/publicacoes/guia_controle_tuberculose.pdf)> Acesso em 12 fev. 2014
- BRENNAN, P. J.; NIKAIDO, H. The envelope of mycobacteria. **Annual Review of Biochemistry**, Palo Alto, CA, v. 64, p. 29-63, 1995.
- BUSTIN, S. A. et. al. Quantitative real-time RT-PCR-a perspective. **Journal of Molecular Endocrinology**, Bristol, U.K , v. 34, n. 3, p. 597-601, 2005.
- BUSTIN, S. A.; MUELLER, R. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. **Clinical Science Clin Sci**, London, UK, v. 109, n. 4, p. 365-379, 2005.
- BUSTIN, S. A.; NOLAN, T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. **Journal of Biomolecular Techniques**, Santa Fe, NM, v. 15, n. 3, p. 155-166, 2004.

- CALEFFI-FERRACIOLI, K. R. et. al. Fast detection of drug interaction in *Mycobacterium tuberculosis* by a checkerboard resazurin method. **Tuberculosis (Edinb)**, Scotland, v. 93, n. 6, p. 660-663, 2013.
- CALGIN, M. K. et. al. Expression analysis of efflux pump genes among drug-susceptible and multidrug-resistant *Mycobacterium tuberculosis* clinical isolates and reference strains. **Diagnostic Microbiology and Infectious Disease**, United States, v. 76, n. 3, p. 291-297, 2013.
- CARDOSO, R. F. et al. Screening and characterization of mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates obtained in Brazil. **Antimicrobial Agents and Chemotherapy**, Washington, v. 48, p. 3373-3381, 2004.
- CHOUDHURI, B. S. et al. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drrA* and *drrB* of *Mycobacterium tuberculosis*. **The Biochemical Journal**, London, v. 367, p. 279-285, 2002.
- COLL, P. Drugs with activity against *Mycobacterium tuberculosis*. **Enfermedades Infecciosas y Microbiología Clínica**, Spain, v. 21, n. 6, p. 299-307, 2003.
- CONDE, M.; FITERMAN, J.; LIMA, M. Tuberculose. **Sociedade Brasileira de Pneumologia e Tisiologia**. Rio de Janeiro - RJ: Guanabara Koogan Ltda, 2011.
- DA SILVA, P. E. al. Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*. **FEMS Immunology and Medical Microbiology**, England, v. 63, n. 1, p. 1-9, 2011.
- DAHL, J. L. Scanning electron microscopy analysis of aged *Mycobacterium tuberculosis* cells. **Canadian Journal of Microbiology**, [S.l.], v. 51, n. 3, p. 277-281, 2005.
- DALEY, C. L.; CAMINERO, J. A. Management of multidrug resistant tuberculosis. **Seminars in Respiratory and Critical Care Medicine**, United States, v. 34, n. 1, p. 44-59, 2013.
- DANILCHANKA, O.; MAILAENDER, C.; NIEDERWEIS, M. Identification of a novel multidrug efflux pump of *Mycobacterium tuberculosis*. **Antimicrobial Agents and Chemotherapy**, Washington, v. 52, n. 7, p. 2503-2511, 2008.
- DE KNEGT, G. J. et. al. E. Rifampicin-induced transcriptome response in rifampicin-resistant *Mycobacterium tuberculosis*. **Tuberculosis (Edinb)**, Scotland, v. 93, n. 1, p. 96-101, 2013.
- DE ROSSI, E.; AINSA, J. A.; RICCARDI, G. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. **FEMS Microbiology Reviews**, England , v. 30, n. 1, p. 36-52, 2006.
- DE ROSSI, E. et. al. The multidrug transporters belonging to major facilitator superfamily in *Mycobacterium tuberculosis*. **Molecular Medicine**, United States, v. 8, n. 11, p. 714-724, 2002.

DE ROSSI, E. et. al. O. *mmr*, a *Mycobacterium tuberculosis* gene conferring resistance to small cationic dyes and inhibitors. **Journal of Bacteriology**, United States, v. 180, n. 22, p. 6068-6071, 1998.

DE STEENWINKEL, J. E. et. al. Time-kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of *Mycobacterium tuberculosis*. **The Journal of Antimicrobial Chemotherapy**, England , v. 65, n. 12, p. 2582-2589, 2010.

DONALD, P. R.; SCHAAF, H. S. Old and new drugs for the treatment of tuberculosis in children. **Paediatric Respiratory Reviews**, England, v. 8, n. 2, p. 134-141, 2007.

GLAZIOU, P. et. al. Global epidemiology of tuberculosis. **Seminars in Respiratory and Critical Care Medicine**, United States, v. 34, n. 1, p. 3-16, 2013.

GUMBO, T. et. al. Concentration-dependent *Mycobacterium tuberculosis* killing and prevention of resistance by rifampin. **Antimicrobial Agents and Chemotherapy**, Washington, v. 51, n. 11, p. 3781-3788, 2007.

GUPTA, A. K. et. al. Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. **Microbial Drug Resistance**, United States, v. 16, n. 1, p. 21-28, 2010a.

GUPTA, A. K. et. al. *jefA* (Rv2459), a drug efflux gene in *Mycobacterium tuberculosis* confers resistance to isoniazid & ethambutol. **The Indian Journal of Medical Research**, India, v. 132, p. 176-188, 2010b.

GUPTA, S. et. al. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. **American Journal of Respiratory and Critical Care Medicine**, United States, v. 188, n. 5, p. 600-607, 2013.

HAO, P. et. al. The role of ABC efflux pump, Rv1456c-Rv1457c-Rv1458c, from *Mycobacterium tuberculosis* clinical isolates in China. **Folia Microbiologica**, United States, v. 56, n. 6, p. 549-553, 2011.

JIANG, X. et. al. Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR. **Microbial Drug Resistance**, United States, v. 14, n. 1, p. 7-11, 2008.

JIN, J. et. al. Farnesol, a potential efflux pump inhibitor in *Mycobacterium smegmatis*. **Molecules**, Basel, Switzerland, v. 15, n. 11, p. 7750-7762, 2010.

LAWN, S. D. et. al. Screening for HIV-associated tuberculosis and rifampicin resistance before antiretroviral therapy using the Xpert MTB/RIF assay: a prospective study. **PLoS Medicine**, San Francisco, v. 8, n. 7, p. e1001067, 2011a.

LAWN, S. D.; ZUMLA, A. I. Tuberculosis. **Lancet**, London, v. 378, n. 9785, p. 57-72, 2011b.

LEWIS, K. Platforms for antibiotic discovery. **Nature Reviews Drug Discovery**, London, v. 12, n. 5, p. 371-387, 2013.

- LIANG, J. et. al. Genome-wide expression profiling of the response to linezolid in *Mycobacterium tuberculosis*. **Current microbiology**, New York, v. 64, n. 6, p. 530-8, 2012.
- LIBERATO, I. R. et. al. Characteristics of pulmonary tuberculosis in HIV seropositive and seronegative patients in a Northeastern region of Brazil. **Revista da Sociedade Brasileira de Medicina Tropical**, Rio De Janeiro, v. 37, n. 1, p. 46-50, 2004.
- LIMONCU, M. H. et. al. An investigation of the antimicrobial impact of drug combinations against *Mycobacterium tuberculosis* strains. **Turkish Journal of Medical Sciences**, [S.l.], v. 41, n. 4, p. 719-724, 2011.
- LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods**, California, USA, v. 25, n. 4, p. 402-408, 2001.
- LOUW, G. E. et al. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. **American Journal of Respiratory and Critical Care Medicine**, [S.l.], v. 184, n. 2, p. 269-276, 2011.
- LOUW, G. E. et. al. A balancing act: efflux/influx in mycobacterial drug resistance. **Antimicrobial Agents Chemotherapy**, Washington, v. 53, n. 8, p. 3181-3189, 2009.
- MACHADO, D. et. al. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. **PLoS One**, San Francisco, CA v. 7, n. 4, p. e34538, 2012.
- MARTINEZ, J. L.; BAQUERO, F. Mutation frequencies and antibiotic resistance. **Antimicrobial Agents Chemotherapy**, Washington, v. 44, n. 7, p. 1771-1777, 2000.
- MISHRA, M. N.; DANIELS, L. Characterization of the MSMEG\_2631 gene (*mmp*) encoding a multidrug and toxic compound extrusion (MATE) family protein in *Mycobacterium smegmatis* and exploration of its polyspecific nature using biolog phenotype microarray. **Journal of Bacteriology**, Washington, v. 195, n. 7, p. 1610-1621, 2013.
- ODDS, F. C. Synergy, antagonism, and what the chequerboard puts between them. **The Journal of Antimicrobial Chemotherapy**, London, v. 52, n. 1, p. 1, 2003.
- OKANDEJI, B. O. et. al. Synthesis and evaluation of inhibitors of bacterial drug efflux pumps of the major facilitator superfamily. **Bioorganic & Medicinal Chemistry**, Oxford, v. 19, n. 24, p. 7679-7689, 2011.
- PAIXÃO, L. et. al. Fluorometric determination of ethidium bromide efflux kinetics in *Escherichia coli*. **Journal of biological engineering**, London, v. 3, p. 18, 2009.
- PALOMINO, J. C. et. al. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. **Antimicrobial Agents Chemotherapy**, Washington, v. 46, n. 8, p. 2720-2722, 2002.

PALOMINO, J. C.; RAMOS, D. F.; DA SILVA, P. A. New anti-tuberculosis drugs: strategies, sources and new molecules. **Current Medicinal Chemistry**, Schiphol, The Netherlands, v. 16, n. 15, p. 1898-1904, 2009.

PANG, Y. et. al. Study of the rifampin monoresistance mechanism in *Mycobacterium tuberculosis*. **Antimicrobial Agents Chemotherapy**, Washington, v. 57, n. 2, p. 893-900, 2013.

PANKUCH, G. A. et al. Activity of meropenem with and without ciprofloxacin and colistin against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. **Antimicrobial Agents Chemotherapy**, Washington, v. 52, n. 1, p. 333-336, 2008.

PASCA, M. R. et. al. *mmpL7* gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium smegmatis*. **Antimicrobial Agents Chemotherapy**, Washington, v. 49, n. 11, p. 4775-4777, 2005.

PILLAI, S. K.; MOELLERLING, R.C.; ELIOPoulos, G. M. **Antimicrobial Combinations**. 5 ed. New York: Lippincott Williams & Wilkins, 2005.

RAMASWAMY, S. V. et. al. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. **Antimicrobial Agents Chemotherapy**, Washington, v. 44, n. 2, p. 326-336, 2000.

RAMON-GARCIA, S. et. al. Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. **Journal of Antimicrobial Chemotherapy**, London, v. 57, n. 2, p. 252-259, 2006.

RAMON-GARCIA, S. et. al. Role of the *Mycobacterium tuberculosis* P55 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. **Antimicrobial Agents Chemotherapy**, Washington, v. 53, n. 9, p. 3675-3682, 2009.

REY-JURADO, E. et. al. *In vitro* effect of three-drug combinations of antituberculous agents against multidrug-resistant *Mycobacterium tuberculosis* isolates. **International journal of antimicrobial agents**, Amsterdam, v. 41, n. 3, p. 278-280, 2013.

RODRIGUES, L. et. al. Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. **Infection, Genetics and Evolution**, Amsterdam, v. 12, n. 4, p. 695-700, 2012.

RODRIGUES, L. et. al. Role of the *Mmr* efflux pump in drug resistance in *Mycobacterium tuberculosis*. **Antimicrobial Agents Chemotherapy**, Washington, v. 57, n. 2, p. 751-757, 2013.

SCORPIO, A.; ZHANG, Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. **Nature Medicine**, New York, v. 2, n. 6, p. 662-667, 1996.

SHARMA, S. et. al. Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of *Mycobacterium tuberculosis*. **Journal of Antimicrobial Chemotherapy**, London, v. 65, n. 8, p. 1694-1701, 2010.

SHARMA, S. K.; MOHAN, A. Tuberculosis: From an incurable scourge to a curable disease - journey over a millennium. **The Indian Journal of Medical Research**, New Delhi, v. 137, n. 3, p. 455-493, 2013.

SILVA, M. S. et. al. Mutations in *katG*, *inhA*, and *ahpC* genes of Brazilian isoniazid-resistant isolates of *Mycobacterium tuberculosis*. **Journal of Clinical Microbiology**, Washington, v. 41, n. 9, p. 4471-4474, 2003.

SILVA, P. E. et. al. Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. **Antimicrobial Agents Chemotherapy**, Washington, v. 45, n. 3, p. 800-804, 2001.

SMITH, C. V.; SHARMA, V.; SACCHETTINI, J. C. TB drug discovery: addressing issues of persistence and resistance. **Tuberculosis (Edinb)**, Scotland, v. 84, n. 1-2, p. 45-55, 2004.

SOUZA, M. V. N.; VASCONCELOS, T. R. A. Fármacos no combate à tuberculose: passado, presente e futuro. **Química Nova**, São Paulo, v. 28, n. 4, p. 678-682, 2005.

SUN, Z.; ZHANG, Y. Reduced pyrazinamidase activity and the natural resistance of *Mycobacterium kansasii* to the antituberculosis drug pyrazinamide. **Antimicrob Agents Chemotherapy**, USA, v. 43, n. 3, p. 537-42, 1999.

SZUMOWSKI, J. D. et al. Antimicrobial efflux pumps and *Mycobacterium tuberculosis* drug tolerance: evolutionary considerations. **Current Topics in Microbiology and Immunology**, Berlin, v. 374, p. 81-108, 2013.

TAKAYAMA, K.; WANG, L.; MERKAL, R. S. Scanning electron microscopy of the H37Ra strain of *Mycobacterium tuberculosis* exposed to isoniazid. **Antimicrobial Agents Chemotherapy**, Washington, v. 4, n. 1, p. 62-65, 1973.

TELENTI, A. et. al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. **Lancet**, London, v. 341, n. 8846, p. 647-650, 1993.

TEVFIK, D. **Real time-PCR**. Newcastle-upon-Tyne, UK: Taylor & Francis Group, 2006.

VERONESI, R.; FOCACCIA, R. **Tratado de Infectologia**. 4 ed. [S.l.] Atheneu, 2010.

VIVEIROS, M. et. al. New methods for the identification of efflux mediated MDR bacteria, genetic assessment of regulators and efflux pump constituents, characterization of efflux systems and screening for inhibitors of efflux pumps. **Current Drug Targets**, Hilversum, Netherlands, v. 9, n. 9, p. 760-778, 2008.

VIVEIROS, M. et. al. Inhibitors of mycobacterial efflux pumps as potential boosters for anti-tubercular drugs. **Expert Review of Anti-infective Therapy**, England, v. 10, n. 9, p. 983-998, 2012.

WANG, K. et. al. The expression of ABC efflux pump, Rv1217c-Rv1218c, and its association with multidrug resistance of *Mycobacterium tuberculosis* in China. **Current Microbiology**, New York, v. 66, n. 3, p. 222-226, 2013.

- WEBBER, M. A.; PIDDOCK, L. J. The importance of efflux pumps in bacterial antibiotic resistance. **Journal of Antimicrobial Chemotherapy**, London, v. 51, n. 1, p. 9-11, 2003.
- WHO. World Health Organization. **Transforming the fight towards elimination of tuberculosis**. The global plan to stop TB 2011-2015. 2010a. Disponível em: <[http://whqlibdoc.who.int/publications/2010/9789241500340\\_eng.pdf](http://whqlibdoc.who.int/publications/2010/9789241500340_eng.pdf)>. Acesso em: 12 fev. 2014.
- WHO. World Health Organization. **Treatment of Tuberculosis: guidelines for national programmes**. 2010b. Disponível em: <[http://www.who.int/tb/publications/tb\\_treatmentguidelines/en/index.html](http://www.who.int/tb/publications/tb_treatmentguidelines/en/index.html)>. Acesso em: 12 fev. 2014.
- WHO. World Health Organization. **Global tuberculosis report 2013**. 2013. Disponível em: <[http://www.who.int/tb/publications/global\\_report/en/](http://www.who.int/tb/publications/global_report/en/)>. Acesso em: 12 fev. 2014.
- ZECHINI, B.; VERSACE, I. Inhibitors of multidrug resistant efflux systems in bacteria. **Recent Patents on Anti-infective Drug Discovery**, Netherlands, v. 4, n. 1, p. 37-50, 2009.
- ZHANG, Y. et. al. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. **Nature**, London, v. 358, n. 6387, p. 591-593, 1992.
- ZHANG, Y.; YEW, W. W. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. **The International Journal of Tuberculosis and Lung Disease**, Paris, v. 13, n. 11, p. 1320-1330, 2009.
- ZUMLA, A. I. et al. New antituberculosis drugs, regimens, and adjunct therapies: needs, advances, and future prospects. **The Lancet Infectious Disease**, New York, v. 14, n. 4, p. 327-340, 2014.

## CAPÍTULO II

**Artigo 1: “FAST DETECTION OF DRUG INTERACTION IN *Mycobacterium tuberculosis* BY A CHECKERBOARD RESAZURIN METHOD”**

**FAST DETECTION OF DRUG INTERACTION IN *Mycobacterium tuberculosis*  
BY A CHECKERBOARD RESAZURIN METHOD**

Katiany R. Caleffi-Ferracioli<sup>a,b,\*</sup>, Flaviane G. Maltempe<sup>b</sup>, Vera Lúcia D. Siqueira<sup>b</sup> and  
Rosilene F. Cardoso<sup>b</sup>

<sup>a</sup> Postgraduate Program in Health Science, State University of Maringá, Paraná, Brazil

<sup>b</sup> Laboratory of Medical Bacteriology, Department of Clinical Analysis and Biomedicine,  
State University of Maringá, Maringá, Paraná, Brazil.

**\*Corresponding author.** Laboratório de Bacteriologia Médica, Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá. Avenida Colombo, 5790, 87020-900, Maringá, Paraná, Brasil. Telefone: +55 44 3011-5376; Fax: +55 44 3011-4797;  
E-mail: katianyrcf@gmail.com

**Running title:** Synergism detection between drugs in *Mycobacterium tuberculosis*.

## S U M M A R Y

Tuberculosis (TB) is a health public problem and a long combination therapy is necessary to treat patients. In recent years, some drugs, not routinely used in treatment of TB, have appeared as promising new anti-TB therapies in patients with resistance to classical drugs. The aim of this study was: (i) to evaluate a modified checkerboard assay, resazurin drugs combination microtiter assay (REDCA) to detect drugs interaction in *Mycobacterium tuberculosis*; (ii) to evaluate the interaction between isoniazid (INH) or ethambutol (EMB) with levofloxacin (LEVO) in susceptible and resistant *M. tuberculosis* Brazilian clinical isolates. *M. tuberculosis* H<sub>37</sub>Rv ATCC 27294 and 19 clinical isolates (10 resistant and 9 susceptible) were tested. The fractional inhibitory concentration index (FICI) 0.5 was considered synergistic. Synergism in *M. tuberculosis* H<sub>37</sub>Rv and resistant *M. tuberculosis* Brazilian isolates was observed with EMB vs. LEVO. No synergism was observed with INH vs. LEVO by both assays. No statistical difference was observed by the two assays studied. REDCA showed to be a simple assay for detecting synergism between drugs in *M. tuberculosis*. The results with EMB vs. LEVO are promising and it can be a new option in future investigations of drugs interactions against *M. tuberculosis* with the view to reduce EMB adverse effects.

**Keywords:** *M. tuberculosis*; Synergism; REDCA; Drugs

## 1. Introduction

The incidence of tuberculosis (TB) remains high, affecting mostly young adults in their most productive years [1]. According to World Health Organization (WHO), a third of the world's population is infected with *Mycobacterium tuberculosis* (*Mtb*) and 95% of TB deaths are in the developing world [2]. Treatment of TB patients is based on six-month regimen using isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA). A long combination therapy is necessary in order to prevent relapse of the disease and development of drug resistance. In 2011, there were an estimated global prevalence of 630,000 cases of multidrug-resistant TB (MDR-TB) among the world's 12 million prevalent cases of TB [3]. In this case, second-line drugs are used, but most of them are either very toxic and more expensive [4].

In recent years, fluoroquinolones and other drugs, not routinely used in treatment of TB, have emerged as promising new TB therapies in patients with resistance to classical anti-TB drugs [5]. Clinical trials have also suggested that fluoroquinolones such as moxifloxacin or levofloxacin (LEVO) may be useful in shortening the treatment of TB [6]. Yew et al. [7] reported that LEVO was found to be more efficacious than ofloxacin when incorporated into multidrug regimens for treatment of MDR-TB and has been recommended for treatment of MDR-TB in the United States. [8]

As the discovery of new drugs is a challenge, which demands time and money, new combinations of old and known antimicrobials have been studied [9, 10] and synergistic effect against *Mtb* have been demonstrated empirically [10, 11].

To establish a new therapeutic regimen, it is necessary to develop quantitative and reproductive assay to estimate antimicrobial activity when two agents are combined [12]. The synergism of drugs can be studied using checkerboard [13, 15] and/or by the time-kill method [12, 16]. Two of the main difficulties in implementing the classical checkerboard assay for *Mtb* are the long incubation period and difficulty in reading.

There are a couple of methods to detect the viability of actively growing bacteria in a microplate format. Colorimetric methods that use redox indicators or the nitrate reduction assay have received increasing attention because of their simplicity and the absence of any requirement for sophisticated equipment or highly trained personnel [17]. Colorimetric methods are based on the reduction of a colored indicator added to the culture medium after *M. tuberculosis* has been exposed in vitro to different drugs. A change in color of the indicator is directly proportional to the number of viable mycobacteria in the medium [18].

The most common indicators used have been the resazurin (also known as alamar blue, an oxidation-reduction colorimetric indicator) [19, 20] and tetrazolium salts (reduction by metabolically active cells) such as: XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] and MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [21, 22]. No differences in results using resazurin, MTT and XTT indicators have been observed [22, 23]. Sala and Hartkoorn [24] reported that the main method, to date, employed to high and medium-throughput screening of compound libraries is the oxidation-reduction colorimetric method called resazurin-based assay (REMA).

In this sense, the use of resazurin to assess the antimycobacterial activity in drugs interaction assay, has been previously proposed for determination of Minimal Inhibitory Concentration (MIC) by Palomino et al. [25] and Martin et al. [26, 27].

In this context, the aim of this study was: (i) to evaluate a modified checkerboard method, Resazurin drugs combination microtiter assay (REDCA), to determine interaction of drugs *in vitro* against *Mtb* clinical isolates; (ii) to evaluate the interaction between INH or EMB with LEVO in susceptible and resistant *Mtb* Brazilian clinical isolates.

## 2. Materials and methods

### 2.1. Bacterial isolates

Nineteen *Mtb* clinical isolates belonging to the reference center for TB diagnosis denominated LEPAC (Laboratory of teaching and research in clinical analysis), State University of Maringá, Paraná, Brazil were selected based in previously characterized mutations in the *katG* and *inhA* genes [28]: Nine isolates were susceptible to all antimicrobial agents and ten resistant to INH and/or other antituberculous drugs (9 were MDR-TB clinical isolates). *M. tuberculosis* H37Rv (ATCC 27294) wild-type reference strain was used as control. The tested isolates were grown in Middlebrook 7H9 medium (Difco), added with 0.2% glycerol (vol/vol), 0.025% tween 80 (vol/vol) and supplemented with OADC (Oleic acid, albumin, dextrose and catalase) (BBL/Becton-Dickinson, Sparks, MD, USA) for 15 days at 37°C.

### 2.2. Antimicrobials

The antimicrobial agents INH and EMB were provided by Sigma Chemical Co. (USA, Canada) and LEVO by HalexIstar Co. (Brazil, Goiania). The stock solutions and further dilutions were prepared in OADC-supplemented Middlebrook 7H9.

### 2.3. REDCA and classical checkerboard assay

The REDCA was performed in duplicate as described elsewhere with minor changes in time of incubation and reading using as model the interaction between INH vs. LEVO and EMB vs. LEVO, and compared with classical checkerboard assay [12].

Plates were prepared by serially diluting INH (0.25-0.0009 mg/L and 32-0.125 mg/L for susceptible and resistant isolates, respectively) or EMB (64-0.250 mg/L) in the x-axis and LEVO (16-0.0156 mg/L) in the y-axis in a 96-well microtitre plate [14, 29]. One hundred microliter of mycobacterial suspension adjusted to a turbidity of 1.0 McFarland Standard scale and diluted 1:20 was inoculated in each well. The plates were covered with their lids, sealed and incubated at 37 °C in normal atmosphere for 7 days [25] for REDCA and two-three weeks until adequate growth in the control well could be visually read for classical method [12]. After, 30 µl of freshly 0.02% (wt/vol) resazurin solution (Acros, Morris Plains, NJ, USA) were added to each REDCA plate well. Plates were incubated overnight at 37 °C. A blue to pink color change indicated reduction of resazurin and therefore, bacterial growth [25]. The classical checkerboard reading was carried out by visual reading of growth, without resazurin, as previously described [12].

To evaluate the synergistic effect for both assay, the fractional inhibitory concentration index: FICI = (MIC A + B/MIC A) + (MIC B + A/MIC B) was used, where: MIC A + B represents the MIC of drug A when combined with drug B. MIC B + A represents the MIC of drug B when combined with drug A. The MIC A and MIC B represent the MICs of drugs A and B when tested alone, respectively. The results were interpreted by the FICI as: synergism, ≤0.50; indifference, >0.50 and 4; and antagonism, >4 [14].

### 2.4. Statistical analysis

Statistic 7.1 software (StatSoft, 2005) was used for analysis of REDCA compared with Classical Checkerboard Method. The level of significance was considered  $p < 0.05$ .

## 3. Results

The MICs values of INH, EMB and LEVO for *Mtb* H37Rv, susceptible and resistant *Mtb* clinical isolates are listed in Table 1. INH, EMB and LEVO MICs ranged from 0.03 to 32 mg/L, 0.5 to 32 mg/L and 0.06 to 4 mg/L, respectively (Table 1). Altogether, 90% of the clinical isolates were inhibited at MIC 4, 8 and 0.5 mg/L for INH, EMB and LEVO, respectively.

A synergistic effect for the *Mtb* H37Rv reference strain was observed only with EMB vs. LEVO (Table 1).

Discrepant EMB vs. LEVO synergism results were observed by REDCA (two resistant *Mtb* clinical isolates) and by the classical checkerboard (one resistant *Mtb* clinical isolate) (Table 1). However, this result showed no statistical difference between the two assays studied ( $p = 0.959$ ).

Similar for INH vs. LEVO synergism results, no statistical difference ( $p = 0.145$ ) was observed when compared classical checkerboard and REDCA, with FICIs ranging from 0.61 to 2 (Table 1).

#### 4. Discussion

The combination of anti-TB drugs is a potential tool for the treatment of TB as it has been recommended by WHO for decades. Despite the need of emergence of new drugs for TB treatment, there is no short-term prospect of this happening. Drugs that have actions on non *Mtb* complex microorganisms have been used in some situations as the treatment of resistant TB. In this regard, the study of old and known drugs in combination with the classical anti-TB drugs is a valuable alternative.

Comparing classical checkerboard with REDCA assay, some variations in the FICI values were observed. For example, the isolate 57s showed FICI equal to 1 for REDCA and 0.75 for classical checkerboard with EMB vs. LEVO. Although the FICI values are different, both values indicate non-synergism between drugs ( $FICI \geq 0.50$ ). Thus, the changes in the FICI did not show differences in the interpretation (synergic or non synergic) of the observed interaction between the studied drugs with the exception of isolate 69.

The improved performance of REDCA, in detecting EMB vs. LEVO synergism observed in the present study may reside in its bacteriostatic activity against *Mtb* [30]. Using a long incubation time necessary in classical checkerboard, the EMB can lose the bacteriostatic effect thus masking the synergism, because *Mtb* can grow back.

Rastogi et al. [31] reported that combinations of EMB with LEVO were also synergistic in two of five clinical isolates using drug combination classical assays, but not in *Mtb* H37Rv as observed in the present study. Rey-Jurado et al. [32] working with three drugs combinations, LEVO, linezolid (LINE) and EMB, did not observe synergism in *Mtb* H<sub>37</sub>Rv and clinical isolates by classical checkerboard assay.

Although Rastogi et al. [31] found synergistic effect between INH vs. LEVO in *Mtb* clinical isolates, our study did not show this effect by both, classical checkerboard and

REDCA. In this regard, the good correlation between the results, by both assays for the INH vs. LEVO, may have been influenced by the bactericidal activity of INH [33].

Traditionally (and still the gold standard), bacterial viability was determined by counting colony-forming units (CFUs), such as occurs in the Time-Kill method [34]. It is a process that is laborious and time-intensive, mainly for mycobacteria which take longer to grow *in vitro*, but gives an accurate picture of the bactericidal or bacteriostatic activity of a compound [24]. Recently, some promising alternative methods for detecting drug resistance and MICs have been proposed. The colorimetric methods, despite having lack of sensitivity compared with CFU evaluation, with a limit of detection of approximately  $1 \times 10^6$  CFU/mL (for tetrazolium salts) and  $1 \times 10^5$  CFU/mL (for resazurin) [21], are easier to perform and the result can be obtained in shorter incubation time. The possibility of a method for detecting synergism in *M. tuberculosis*, a slow growing mycobacteria, which use a shorter incubation time, is important for accurate results. Nonetheless, Sala and Hartkoorn [24] reported that the main disadvantage of colorimetric methods is that the used dyes measure bacteriostatic rather than bactericidal activity. The REDCA presented in this paper, could detect synergistic effect of EMB and LEVO. An explication for this lies in the fact the REDCA does not need a long incubation time, which could cause the loss of bacteriostatic activity of EMB, thus masking a real result. Additionally, a long incubation time (by the classical checkerboard) generates other disadvantages, such as, evaporation and drying of the plate wells. Beyond a long incubation time can provide water condensation on the lid of the microplate, which can favor fungal contamination.

The main attractiveness of using fluorescent signal by resazurin dye is the read ability. It is known that fluorescence readouts obtained by redox-dye, typically display a background. However, in the use of a fluorescence detecting equipment, the reading should be adjusted for the background level fluorescence originated by the dye and medium [35]. The read ability of REDCA assay, without necessity of using equipment, makes it accessible for its use in laboratories with low financial resources.

The drugs interaction model used in the present study, INH vs. LEVO and EMB vs. LEVO determined by REDCA showed to be efficient comparable to the classical checkerboard ( $p < 0.05$ ), with the advantage of obtaining the result in eight days (seven of incubation plus one for resazurin reading) instead of two-three weeks used in the classical assay. Additional advantage of REDCA is the easiness of reading by visual inspection and the low cost of resazurin.

To our knowledge, there were two other previous works [36, 37] that employed the resazurin to assess the viability and the antimicrobial activity in drugs interaction assay in *Mtb* H<sub>37</sub>Rv and *M. smegmatis*. However, none of them applied a comparison with the classical checkerboard assay in susceptible and resistant *Mtb* clinical isolates including MDR-TB as carried out in the present study. The limitation of this study was the small number of *Mtb* clinical isolates evaluated and the limited classical anti-TB drugs combinations. Nevertheless, the results make us optimistic about the use of REDCA in future investigations of drugs interactions, once it is faster compared to classical checkerboard assay and the use of resazurin, which is inexpensive, makes the visual reading to be more reliable. The relevance of using the drugs combination model proposed in the study, including LEVO, call attention for its potential in treatment of *Mtb*-HIV co-infected patients who are at increased risk for developing MDR-TB disease and also the dose-dependent ocular toxicity of EMB. The finding of two Brazilian clinical MDR-TB isolates that showed EMB vs. LEVO synergism points attention to the benefits of this combination for the treatment of these cases and consequently reduce the adverse effects.

*Funding:* No funding sources.

*Competing interests:* None declared.

*Ethical approval:* Not required.

## References

- [1] Jagielski T, Augustynowicz-Kopec E, Zwolska Z. Epidemiology of tuberculosis: a global, European and Polish perspective. *Wiad Lek* 2010;**63**:230-246.
- [2] WHO. *Global tuberculosis Control 2011*. Geneva: World Health Organization; 2011. <[http://www.who.int/tb/publications/global\\_report/2011/en/](http://www.who.int/tb/publications/global_report/2011/en/)>
- [3] WHO. *Global tuberculosis report 2012*. Geneva: World Health Organization; 2012. <[http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf)>
- [4] Dye C. Tuberculosis 2000-2010: control, but not elimination. *Int J Tuberc Lung Dis* 2000;**4**:146-152.
- [5] Ginsburg AS, Grosset JH, Bishai WR. Fluoroquinolones, tuberculosis, and resistance. *Lancet Infect Dis* 2003;**3**:432-442.
- [6] Lienhardt C, Raviglione M, Spigelman M, Hafner R, Jaramillo E, Hoelscher M, Zumla A, Gheuens J. New drugs for the treatment of tuberculosis: needs, challenges, promise, and prospects for the future. *J Infect Dis* 2012;**205** (2):241-249.
- [7] Yew WW, Chan CK, Leung CC, Chau CH, Tam CM, Wong PC, Lee J. Comparative roles of levofloxacin and ofloxacin in the treatment of multidrug-resistant tuberculosis: preliminary results of a retrospective study from Hong Kong. *Chest* 2003;**124**:1476-1481.
- [8] Small PM, Fujiwara PI. Management of tuberculosis in the United States. *N Engl J Med* 2001;**345**:189-200.
- [9] Rey-Jurado E, Tudo G, Martinez JA, Gonzalez-Martin J. Synergistic effect of two combinations of antituberculous drugs against *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 2012;**92**:260-263.
- [10] Amaral L, Viveiros M. Why thioridazine in combination with antibiotics cures extensively drug-resistant *Mycobacterium tuberculosis* infections. *Int J Antimicrob Agents* 2012;**39**:376-380.
- [11] Dauby N, Muylle I, Mouchet F, Sergysels R, Payen MC. Meropenem/clavulanate and linezolid treatment for extensively drug-resistant tuberculosis. *Pediatr Infect Dis J* 2011;**30**:812-813.
- [12] Bhusal Y, Shiohira CM, Yamane N. Determination of *in vitro* synergy when three antimicrobial agents are combined against *Mycobacterium tuberculosis*. *Int J Antimicrob Agents* 2005;**26**:292-297.

- [13] Bergmann JS, Woods GL. In vitro activity of antimicrobial combinations against clinical isolates of susceptible and resistant *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 1998;2:621-626.
- [14] Pillai SKMJ, R.C.; Eliopoulos, G.M. *Antimicrobial combinations*. New York: Lippincott Williams & Wilkins, 2005.
- [15] Abourashed EA, Galal AM, Shibli AM. Antimycobacterial activity of ferutinin alone and in combination with antitubercular drugs against a rapidly growing surrogate of *Mycobacterium tuberculosis*. *Nat Prod Res* 2011;25:1142-1149.
- [16] Limoncu MH, Ermertcan S, Erac B, Tasli H. An investigation of the antimicrobial impact of drug combinations against *Mycobacterium tuberculosis* strains. *Turkish Journal of Medical Sciences* 2011;41:719-724.
- [17] Palomino JC, Martin A, Portaels F. Rapid drug resistance detection in *Mycobacterium tuberculosis*: a review of colourimetric methods. *Clin Microbiol Infect* 2007;13(8):754-762.
- [18] Yajko DM, Madej JJ, Lancaster MV, Sanders CA, Cawthon VL, Gee B, Babst A, Hadley WK. Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. *J Clin Microbiol* 1995;33:2324-2327.
- [19] Reis RS, Neves I Jr, Lourenco SL, Fonseca LS, Lourenco MC. Comparison of flow cytometric and Alamar Blue tests with the proportional method for testing susceptibility of *Mycobacterium tuberculosis* to rifampin and isoniazid. *J. Clin. Microbiol* 2004;42:2247-2248.
- [20] Sungkanuparph S, Pracharttam R, Thakkinstian A, Buabut B, Kiatatchasai W. Correlation between susceptibility of *Mycobacterium tuberculosis* by microtiter plate alamar blue assay and clinical outcomes. *J. Med. Assoc. Thai* 2002;85:820-824.
- [21] Caviedes L, Delgado J, Gilman RH. Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2002;40:1873-1874.
- [22] De Logu A, Uda P, Pellerano ML, Pusceddu MC, Saddi B, Schivo ML. Comparison of two rapid colorimetric methods for determining resistance of *Mycobacterium tuberculosis* to rifampin, isoniazid, and streptomycin in liquid medium. *Eur J Clin Microbiol Infect Dis* 2001;20:33-39.
- [23] Martin A, Portaels F, Palomino JC. Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *J Antimicrob Chemother* 2007;59,175-183.

- [24] Sala C, Hartkoorn RC. Tuberculosis drugs: new candidates and how to find more. *Future Microbiol* 2011;6(6):617-633.
- [25] Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin Microtiter Assay Plate: Simple and Inexpensive Method for Detection of Drug Resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2002;46:2720-2722.
- [26] Martin A, Paasch F, Docx S, Fissette K, Imperiale B, Ribón W, González LA, Werngren J, Engström A, Skenders G, Juréen P, Hoffner S, Portillo PD, Morcillo N, Palomino JC. Multicentre laboratory validation of the colorimetric redox indicator (CRI) assay for the rapid detection of extensively drug-resistant (XDR) *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2011;66:827-833.
- [27] Martin A, Camacho M, Portaels F, Palomino JC. Resazurin Microtiter Assay Plate Testing of *Mycobacterium tuberculosis* Susceptibilities to Second-Line Drugs: Rapid, Simple, and Inexpensive Method. *Antimicrob Agents Chemother* 2003;47:3616-3619.
- [28] Cardoso RF, Cooksey RC, Morlock GP, Barco P, Cecon L, Forestiero F, Leite CQF, Sato DN, Shikama MD, Mamizuka EM, Hirata RDC, Hirata MH. Screening and characterization of mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates obtained in Brazil. *Antimicrob Agents Chemother* 2004;48:3373-3381.
- [29] Moody JA. Synergism testing: broth microdilution checkerboard and broth macrodilution methods. Washington: DC: American Society for Microbiology, 1992.
- [30] Strupczewska-Januszowa H, Tomaszkiewicz L, Wozniak S. Bacteriostatic effect of ethambutol on tubercle bacilli and other mycobacteria in vitro. *Gruzlica* 1968;36:203-207.
- [31] Rastogi N, Goh KS, Bryskier A, Devallois A. In Vitro Activities of Levofloxacin Used Alone and in Combination with First- and Second-Line Antituberculous Drugs against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1996;40(7):1610-1616.
- [32] Rey-Jurado E, Tudo G, de la Bellacasa JP, Espasa M, Gonzalez-Martin J. In vitro effect of three-drug combinations of antituberculous agents against multidrug-resistant *Mycobacterium tuberculosis* isolates. *Int J Antimicrob Agents* 2013;41:278-280.
- [33] Skinner PS, Furney SK, Kleinert DA, Orme IM. Comparison of activities of fluoroquinolones in murine macrophages infected with *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1995;39:750-753.
- [34] Steenwinkel JEM, Knegt GJ, Kate MT, Belkum AV, Verbrugh HA, Kremer K, Soolingen DV, Bakker-Woudenberg IAJM. Time-kill kinetics of anti-tuberculosis

- drugs, and emergence of resistance, in relation to metabolic activity of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2010;65:2582–2589.
- [35] Hamalainen-Laanaya HK, Orloff MS. Analysis of cell viability using time-dependent increase in fluorescence intensity. *Anal Biochem* 2012;429:32–38.
- [36] Ramon-Garcia S, Martin C, Ainsa JA, De Rossi E. Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. *J Antimicrob Chemother* 2006;57:252-259.
- [37] Ramon-Garcia S, Ng C, Anderson H, Chao JD, Zheng X, Pfeifer T, Av-Gay Y, Roberge M, Thompson CJ. Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen. *Antimicrob Agents Chemother* 2011;55:3861-3869.

Received for publication on 09<sup>th</sup> April 2012

Accepted for publication on 02<sup>nd</sup> November 2012

**Table 1**

MICs values alone and FICI obtained by REDCA and Classical checkerboard assay with isoniazid or ethambutol vs. levofloxacin combinations in *Mycobacterium tuberculosis* H<sub>37</sub>Rv and drug-susceptible and multidrug-resistant (MDR) *Mycobacterium tuberculosis* clinical isolates

Isolate	Susceptibility pattern	REDCA			Classical Checkerboard			REDCA			Classical Checkerboard			REDCA			Classical Checkerboard		
		MIC (mg/L)			MIC (mg/L)			INH / LEVO <sup>a</sup>		INH / LEVO <sup>a</sup>		EMB/LEVO <sup>b</sup>	EMB/LEVO <sup>b</sup>	EMB/LEVO <sup>b</sup>					
		INH	EMB	LEVO	INH	EMB	LEVO	MICs (mg/L)	FICI	MICs (mg/L)	FICI	MICs (mg/L)	FICI	MICs (mg/L)	FICI	MICs (mg/L)	FICI		
H <sub>37</sub> Rv	S	0.03	1	0.50	0.06	2	0.50	0.03/0.50	1.5	0.03/0.125	0.75	0.25/0.125	<b>0.50</b>	0.5/0.125	<b>0.50</b>	0.5/0.125	<b>0.50</b>		
49 S	S	0.25	2	0.25	0.25	2	0.50	0.25/0.25	2	0.25/0.5	2	0.5/0.125	0.75	0.5/0.25	0.75	0.5/0.25	0.75		
57 S	S	0.25	2	0.25	0.25	2	0.50	0.25/0.25	2	0.125/0.125	0.75	1/0.125	1	0.5/0.25	0.75	0.5/0.25	0.75		
80 S	S	0.06	1	0.25	0.06	2	0.25	0.03/0.06	0.74	0.03/0.125	1	0.5/0.125	1	0.5/0.125	0.75	0.5/0.125	0.75		
46 S	S	0.06	2	0.125	0.25	1	0.25	0.06/0.125	2	0.06/0.125	1	0.5/0.07	0.53	0.5/0.125	1	0.5/0.125	1		
9 S	S	0.03	1	0.06	0.06	2	0.125	0.03/0.06	2	0.06/0.125	2	0.5/0.03	1	1/0.007	0.56	1/0.007	0.56		
20 S	S	0.03	1	0.125	0.06	1	0.125	0.03/0.125	2	0.03/0.06	1	0.5/0.06	1	0.5/0.06	1	0.5/0.06	1		
50 S	S	0.06	1	0.125	0.06	NP	0.25	NP	NP	0.03/0.125	1	NP	NP	NP	NP	NP	NP		
58 S	S	0.06	1	0.06	0.06	NP	0.125	0.06/0.06	2	0.06/0.125	2	NP	NP	NP	NP	NP	NP		
65 S	S	0.06	2	0.25	0.06	2	0.5	0.03/0.125	1	0.03/0.125	0.75	1/0.06	0.74	1/0.25	1	1/0.25	1		
18 R	MDR	2	4	0.25	4	4	0.25	0.5/0.125	0.75	2/0.125	1	2/0.06	0.74	2/0.125	1	2/0.125	1		
97 R	MDR	32	8	0.25	4	8	0.50	16/0.125	1	2/0.125	0.75	4/0.125	1	2/0.25	0.75	2/0.25	0.75		
3614 R	MDR	16	32	0.25	4	8	0.25	16/0.25	2	4/0.25	2	32/0.25	2	4/0.125	1	4/0.125	1		
3408 R	MDR	8	32	0.125	16	8	0.125	8/0.125	2	2/0.06	0.61	8/0.06	0.73	4/0.125	1	4/0.125	1		
69 R	MDR	2	4	0.5	4	1	0.125	1/0.08	0.66	1/0.06	0.73	1/0.014	<b>0.28</b>	0.5/0.06	1	0.5/0.06	1		
71 R	MDR	1	1	0.125	4	2	0.50	1/0.125	2	2/0.125	0.75	0.125/0.03	<b>0.36</b>	0.5/0.125	<b>0.50</b>	0.5/0.125	<b>0.50</b>		
73 R	MDR	2	2	2	4	2	4	2/2	2	2/1	0.75	1/1	1	1/2	1	1/2	1		
91 R	R	4	1	0.125	4	1	0.25	2/0.03	0.74	2/0.125	1	0.5/0.06	1	0.5/0.125	1	0.5/0.125	1		

<b>4250 R</b>	MDR	8	1	0.25	4	1	0.25	8/0.25	2	2/0.125	1	0.5/0.125	1	0.5/0.125	2
<b>25252 R</b>	MDR	1	0.50	0.125	4	2	0.25	1/0.25	2	NP	NP	0.5/0.125	2	1/0.008	0.53

\**M. tuberculosis*: (S), Drug-susceptible isolates; (R), Resistance to INH; (MDR), multidrug-resistant; H<sub>37</sub>Rv: wild-type *Mtb* strain; NP, not performed; MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index; REDCA, Resazurin drugs combination microtiter assay.

<sup>a</sup> p = 0.145, t-test; FICI results by REDCA assay vs. Classical checkerboard for INH vs LEVO.

<sup>b</sup> p = 0.959, t-test; FICI results by REDCA assay vs. Classical checkerboard for EMB vs LEVO.

**Artigo 2: “MORPHOLOGICAL CHANGES AND DIFFERENTIALLY EXPRESSED  
EFFLUX PUMP GENES IN *Mycobacterium tuberculosis* EXPOSED TO RIFAMPICIN  
AND VERAPAMIL COMBINATION”**

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

3

4 Katiany R. Caleffi-Ferracioli<sup>a,b</sup>, Renata Claro R. do Amaral<sup>b</sup>, Fernanda Demitto<sup>b</sup>, Flaviane G.  
5 Maltempe<sup>b</sup>, Pedro Henrique Canezin<sup>b</sup>, Regiane B. L. Scodro<sup>b</sup>, Celso V. Nakamura<sup>c</sup>, Clarice  
6 Queico F. Leite<sup>d</sup>, Vera Lúcia D. Siqueira<sup>b</sup> and Rosilene Fressatti Cardoso<sup>b#</sup>

7

8 **Running title:** *M. tuberculosis* changes at drugs combination exposure

9

10 <sup>a</sup>Postgraduate Program in Health Science, State University of Maringa, Parana, Brazil.

11 <sup>b</sup>Laboratory of Medical Bacteriology. Department of Clinical Analysis and Biomedicine, State  
12 University of Maringa, Parana, Brazil.

13 <sup>c</sup>Department of Basic Health Sciences, State University of Maringa, Parana, Brazil.

14 <sup>d</sup>School of Pharmaceutical Sciences, Department of Biological Sciences, Paulista State  
15 University, Araraquara, Sao Paulo, Brazil.

16

17

18 **Address correspondence to:** Rosilene Fressatti Cardoso, rfcardoso@uem.br

19 **#Present Address:** Laboratory of Medical Bacteriology, Department of Clinical Analyses and  
20 Biomedicine, State University of Maringa. Colombo avenue 5790, 87.020-900, Maringa,  
21 Parana, Brazil. *Phone:* +55 44 3011 5375; *Fax:* +55 44 3011 4797

22

Resistance in *Mycobacterium tuberculosis* (*Mtb*) has been attributed to combination of a highly impermeable cell wall, spontaneous mutations, and active efflux pumps (EPs). The aim of the present study was to (i) evaluate *in vitro* combinations of rifampicin (RIF), ethambutol (EMB), or isoniazid (INH) with the inhibitors carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) or verapamil (VP) in *Mtb* H<sub>37</sub>Rv and (ii) evaluate morphological and EP transcriptional changes in *Mtb* H<sub>37</sub>Rv exposed to the drug combination, which showed the best action against the bacillus. The MIC and synergic effects of drug combinations were evaluated by Resazurin Microtiter Plate Assay and Resazurin Drugs Combination Microtiter Assay, respectively. The effects of RIF+VP combination on the morphology and EPs genes expression were evaluated. VP showed the best EP inhibition, and RIF+VP the lower fractional inhibitory concentration index in *Mtb* H<sub>37</sub>Rv. RIF+VP exerted a similar reduction of viable cell counts as RIF by time-kill curve. Wrinkled and rounding cells were the main morphological changes after RIF and VP exposure, respectively. The effects of RIF+VP appeared to be a summation of the observed changes. The overexpression of some EP genes after 72 h to RIF exposure was observed. For RIF+VP exposure at the same time, decrease on EP gene expression was observed. The present study demonstrated the best effect of combination with RIF+VP in *Mtb* H<sub>37</sub>Rv, suggesting that this drug combination may provide advantages over conventional therapies, including a decrease in resistance mediated by EPs, and may help guide further studies with *Mtb* clinical isolates.

42

43 **INTRODUCTION**

44       Tuberculosis (TB) is still one of the most common causes of death worldwide (1),  
45 responsible for 8.6 million new cases and 1.3 million deaths in 2012 (2). The high mortality  
46 and morbidity, especially in poor countries, and emergence of multidrug-resistant (MDR) and  
47 extensively drug-resistant (XDR) *Mycobacterium tuberculosis* (*Mtb*) has become a major  
48 public health concern worldwide (3, 4).

49       Mycobacteria are naturally resistant to most of the commonly used antimicrobial in  
50 the medical clinic because of the slow uptake of drugs across the highly hydrophobic  
51 mycobacterial cell envelop (5, 6). In addition to the unique structure of the bacteria, mutations  
52 of drug target genes are known to be an important mechanism of resistance (1). However,  
53 explaining the observed MDR to first-line drugs with known mutations has not yet been  
54 possible (7).

55       Recently, the resistance of *Mtb* has been attributed to an active drug efflux  
56 mechanism. Bacterial efflux pumps (EPs) are membrane proteins that are capable of actively  
57 transporting a broad range of substrates, including antimicrobial agents (5, 8). The  
58 constitutive or inducible expression of efflux systems in response to treatment contributes to a  
59 decrease in the intracellular concentration of drugs and thus natural or acquired resistance (9-  
60 12).

61       *Mtb* has some of the largest numbers of putative drug EP genes in its genome (4).  
62 Some EPs have been described and well characterized in *Mtb* as belonging to the adenosine  
63 triphosphate binding cassette (ABC), major facilitator superfamily (MFS), resistance  
64 nodulation division (RND), and small multidrug resistance (SMR) families (3-5, 10, 12, 13).  
65 Some EPs have been reported to play a role in resistance to anti-TB drugs, such as rifampicin  
66 (RIF), ethambutol (EMB) and isoniazid (INH) (3, 9, 14-17).

67       Efflux pump inhibitors (EPIs) have been described and tested in drugs combinations  
68       to increase their intracellular concentration and restore the activity of standard antimicrobial  
69       (18). Some EPIs, such as carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), valinomycin,  
70       and dinitrophenol, affect transmembrane electrochemical potential. Other EPIs include the  
71       vesicular monoamine inhibitor reserpine, proton pump inhibitor omeprazol, and calcium  
72       channel antagonist verapamil (VP) (19). However, the mechanisms involved in the induction  
73       and regulation of EPs are not yet fully understood (4). Not all currently available anti-TB  
74       drugs are considered substrates for EPs (20).

75       Efflux pump-mediated resistance has become relevant because these systems help  
76       bacteria extrude antimicrobial until relevant mutations emerge in the *Mycobacterium* genome  
77       (21). Promising *in vivo* results have been reported with VP as an adjunctive therapy in an  
78       attempt to accelerate the treatment of TB (22).

79       The aim of the present study was to (*i*) evaluate *in vitro* combinations of RIF, EMB,  
80       and INH with EPIs (CCCP and VP) in *Mtb* H<sub>37</sub>Rv and (*ii*) evaluate morphological and EP  
81       transcriptional changes in *Mtb* H<sub>37</sub>Rv exposed to the drug combination that showed the best  
82       action against the bacillus.

83

84

## 85       **MATERIALS AND METHODS**

86       **Bacterial culture.** *M. tuberculosis* H<sub>37</sub>Rv (ATCC 27294), a wildtype strain, was used  
87       throughout the study. Bacterial cells were grown at 35-37°C for 15 days in Middlebrook 7H9  
88       medium (Difco Laboratories, Detroit, MI, USA) supplemented with 10% (v/v) oleic acid-  
89       bovine serum albumin-dextrose catalase enrichment (OADC, BBL/Becton-Dickinson, Sparks,  
90       MD, USA), with the addition of 0.2% glycerol (v/v) and 0.025% Tween 80 (v/v).

91

92 **Antimicrobial agents and efflux pump inhibitors.** The antimicrobial agents RIF, INH,  
93 EMB, CCCP, and VP were provided by Sigma (St. Louis, MO, USA). Stock solutions were  
94 prepared and stored at -80°C. INH, EMB, and VP were prepared in distillate water. CCCP  
95 was prepared in dimethyl sulfoxide (DMSO; Synth, Diadema/SP, Brazil). Rifampicin was  
96 prepared in methanol:water (1:10, v/v). Further dilutions for the study were prepared in  
97 OADC-supplemented Middlebrook 7H9 with the following concentration ranges: RIF  
98 (0.0005-0.25 µg/mL), INH (0.0009-0.25 µg/mL), EMB (0.125-32 µg/mL), CCCP (0.39-100  
99 µg/mL), and VP (3.90-1,000 µg/mL). The final DMSO and methanol concentrations had no  
100 effect on *Mtb* growth.

101

102 **Determination of MIC.** The MIC for *Mtb* H<sub>37</sub>Rv was determined in triplicate for each drug  
103 (antimicrobial agents and EPIs) using the Resazurin Microtiter Assay Plate (REMA) as  
104 described by Palomino et al. (23). The MIC was assessed by a color change from blue to pink,  
105 which indicates a reduction of resazurin by bacterial growth.

106

107 **Checkerboard assay.** The interactions between anti-TB drugs (INH, RIF and EMB) and EPIs  
108 (CCCP and VP) were evaluated using the Resazurin Drugs Combination Microtiter Assay  
109 (REDCA) as described previously by Caleffi-Ferracioli et al. (24). The fractional inhibitory  
110 concentration index (FICI) was used to evaluate the interaction effect, and the results were  
111 interpreted as synergism (FICI ≤ 0.50), indifference/additive (FICI > 0.50-4), and antagonism  
112 (FICI > 4) (25).

113

114 **Accumulation of ethidium bromide.** The ethidium bromide (EtBr) MIC for *Mtb* was first  
115 determined by the REMA method (23) and then EtBr accumulation in *Mtb* cells was assessed  
116 by fluorometry (11, 26). *Mtb* H<sub>37</sub>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 was reached. The culture was centrifuged at 2,880 x g for 10 min, and 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  $\mu$ l aliquots of bacterial suspension were transferred to microplate wells that contained 0.25  $\mu$ g/mL EtBr (0.5 $\times$ MIC) (26). Ten microliters of CCCP and VP at a final concentration of 0.5 $\times$ MIC was added to the corresponding well in the microplate and incubated at 25°C for 15 min. Fluorescence was determined for the bacterial suspension in the absence of CCCP and VP as a reference assay. Fluorescence relative to EtBr-loaded cells was acquired every 51 s for 60 min at 37°C in a VICTOR<sup>2</sup> D fluorometer (PerkinElmer, Santa Clara, CA, USA) using 530/25 nm as the excitation wavelengths and 590/20 nm as the detection wavelengths, respectively (11). The relative fluorescence values were obtained by normalizing the data against the background fluorescence of EtBr. The relative final fluorescence (RFF) for each assay 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 under reference conditions (without EPIs) (11).

133

134 **Time-kill curve assay.** The time-kill curve assay was performed with the drug combination  
135 that showed the lowest FICI by REDCA and using the 0.5 $\times$ MIC of each drug. A culture of  
136 5mL of *Mtb* H<sub>37</sub>Rv ( $7.5 \times 10^6$  CFU (colony-forming unit)/mL) in OADC-supplemented  
137 Middlebrook 7H9 medium was exposed to RIF and VP alone and the RIF+VP combination  
138 and continuously shaken at 96 rpm at 35-37°C. A growth control without drugs was included.  
139 Aliquots (0.1 ml) were removed at 0, 1, 2, 3, 5, 7 days and serially diluted ( $10^{-1}$ ,  $10^{-3}$  and  $10^{-5}$ )  
140 in sterile saline to avoid RIF and VP carry-over. Afterward, 20  $\mu$ l of each dilution was  
141 immediately seeded on OADC-supplemented Middlebrook 7H11 (Difco Laboratories,

142 Detroit, MI, USA). The plates were incubated at 35-37°C for 21 days, and the colonies were  
143 counted. The time-kill curve assays were performed in duplicate. Log CFU values were  
144 plotted against time (in days) to obtain the time-kill curve. Synergy was defined as a decrease  
145 of  $\geq 2 \log_{10}$  CFU/mL compared with the most active single drug (27, 28).

146

147 **Scanning electron microscopy.** The RIF and VP combination that showed the lowest FICI  
148 by REDCA was selected for scanning electron microscopy (SEM). *Mtb* H<sub>37</sub>Rv was exposed to  
149 the 0.5×MIC of VP, RIF, and RIF+VP for 16 and 72 h at 35-37°C. Subcultures on OADC-  
150 supplemented Middlebrook 7H11 were performed to ensure the absence of contamination.  
151 After drug exposure, the cells were fixed with 2.5% glutaraldehyde (Sigma) in 0.1 M  
152 cacodylate buffer (Electron Microscopy Science, Hatfield, PA, USA) for at least 2 h at 4°C.  
153 The treated cells were placed on a glass support with poly-L-lysine (Sigma), dehydrated in  
154 graded ethanol, critical-point-dried in CO<sub>2</sub>, coated with gold, and observed in a Shimadzu SS-  
155 550 (Kyoto, Japan) scanning electron microscope. An average of 20-30 microscopic fields in  
156 each sample were selected by random scanning and photographed. SEM was performed in  
157 duplicate with different cultures to ensure reproducibility of the obtained data.

158

159 **Efflux pump gene expression study**

160 **RNA extraction.** *Mtb* H<sub>37</sub>Rv was exposed to VP, RIF, and RIF+VP (0.5×MIC) for 16 and 72 h  
161 at 35-37°C. Total RNA from *Mtb* H<sub>37</sub>Rv growth was extracted in two time-independent  
162 experiments and purified using RNeasy Mini Kit Plus (Qiagen Biotechnology, Valencia, CA,  
163 USA) according to the manufacturer's instructions. Quantification/purity and quality  
164 assessments were performed with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA).  
165 RNA quality (RIN > 9.0) was assessed using an Agilent BioAnalyzer. Contaminating DNA

166 was removed by prior treatment with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA)  
167 according to the manufacturer's instructions.

168

169 **Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).** First-  
170 strand cDNA was synthesized with RT Superscript III (Invitrogen, Carlsbad, CA, USA) using  
171 a random primer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's  
172 instructions. qPCR was performed using SYBR green PCR master mix (Applied Biosystems,  
173 Foster City, CA, USA). Specific primers (Promega, Madison, WI, USA) for the EPs are listed  
174 in the Table 1. Melting curves for each reaction were assessed, and each sample was run in  
175 triplicate. A negative control was performed. The 16S RNA (*rrs*) gene was used to normalize  
176 all of the reactions. A reference assay was conducted with *Mtb* H<sub>37</sub>Rv grown in the absence of  
177 drugs. The relative quantification of target gene expression was calculated by the 2<sup>-ΔΔCT</sup>  
178 method (29). The data analysis was performed using a one-way test with SAS 9.0 software  
179 (SAS OnlineDoc 9, SAS Institute, Cary, NC, USA), followed by the Tukey *post hoc* test.  
180 Values of *p* < 0.01 were considered statistically significant.

181

182

## 183 RESULTS

184 **MICs and checkerboard.** The MICs for RIF, EMB, INH, CCCP, and VP were 0.004, 2.0,  
185 0.03, 3.125, and 125 µg/mL, respectively. The FICIs observed by REDCA were 0.75 and 1.5  
186 for RIF+VP and RIF+CCCP, respectively. For the other drug combinations, the FICI was 2  
187 (Table 2).

188

189 **EtBr accumulation.** The  $0.5\times$ MIC of EtBr did not affect cell viability (influx-efflux in  
190 equilibrium). EtBr efflux was inhibited by VP and CCCP (Fig. 1). The RFF was 0.544 for VP  
191 and 0.154 for CCCP.

192

193 **Time-kill curve assay.** The assay was performed for VP alone (EPI that showed the largest  
194 intracellular EtBr accumulation), RIF+VP (combination that showed the lowest FICI by  
195 REDCA), and RIF alone (antimicrobial of choice) (Fig. 2). No decrease  $\geq 2 \log_{10}$ CFU/mL  
196 was observed with *Mtb* H<sub>37</sub>Rv exposed to RIF+VP compared with RIF alone.

197

198 **Morphological changes.** Some morphological changes occurred over the time of bacillus  
199 exposure to the drugs. Figures 3.1a and b show the appearance of drug-unexposed cells at 16  
200 and 72 h, respectively, with dimorphic rod-shaped cells, a smooth surface, and typical cord  
201 formation at the two exposure times. Figures 3.2, 3.3, and 3.4 show the aspects of treated  
202 cells. Beyond the bacterial clumps, wrinkled and rounding cells were observed in *Mtb* H<sub>37</sub>Rv  
203 exposed to RIF and VP, respectively, mainly at 72 h of drug exposure. The effect of RIF on  
204 cell morphology was less evident (Fig. 3.2a and b) compared with cells exposed to VP (Fig.  
205 3.3a and b). A summation of effects caused by RIF and VP exposure alone was observed with  
206 the RIF+VP combination, with a larger intensity at 72 h of exposure (Fig. 3.4a and b).

207

208 **Differential efflux pump expression.** Figure 4 shows the relative quantification of the  
209 transcript levels of 12 selected EP genes in *Mtb* H<sub>37</sub>Rv exposed to the  $0.5\times$ MIC of RIF, VP,  
210 and RIF+VP for 16 h (Fig. 4a) and 72 h (Fig. 4b). After 16 h of drug exposure, compared with  
211 the control (absence of drugs), a significant difference in relative expression ( $p \leq 0.01$ ) was  
212 detected in six EP genes (*Rv1458*, *Rv1218*, *Rv1819*, *Rv2846*, *Rv1258* and *Rv1410*) for RIF,  
213 nine EP genes (*Rv1456*, *Rv1458*, *Rv1218*, *Rv1457*, *Rv1819*, *Rv2846*, *Rv1258*, *Rv2942* and

214 *Rv1410*) for VP, and seven EP genes (*Rv1458*, *Rv1218*, *Rv1457*, *Rv1819*, *Rv1258*, *Rv1217*  
215 and *Rv2459*) for RIF+VP. At 72 h, a significant difference in the relative expression ( $p \leq$   
216 0.01) for the EP genes was also observed in ten genes (*Rv1456*, *Rv3065*, *Rv1458*, *Rv1457*,  
217 *Rv2846*, *Rv1258*, *Rv2942*, *Rv1217*, *Rv2459* and *Rv1410*) for RIF, three genes (*Rv3065*,  
218 *Rv1457*, and *Rv1410*) for VP, and four genes (*Rv1457*, *Rv2846*, *Rv1258* and *Rv2942*) for  
219 RIF+VP.

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

224

225

## 226 DISCUSSION

227 Recent evidence suggests that EPIs can prevent anti-TB drug resistance or even restore  
228 drug susceptibility in *Mtb* (15, 21, 30). However, the effect of anti-TB drugs plus EPI  
229 combinations on the morphology and expression of particular genes is unclear and has not  
230 been investigated in depth. In the present study, the activity of RIF, INH or EMB (first-line  
231 anti-TB drugs) combined with CCCP or VP was assessed in *Mtb* H<sub>37</sub>Rv. Additionally,  
232 changes in bacillus morphology and EP gene transcription induced by RIF, VP, and the  
233 RIF+VP combination were analyzed.

234 Although no synergistic effects were revealed by the REDCA assay between the  
235 classical anti-TB drugs and EPIs in *Mtb* H<sub>37</sub>Rv, the RIF+VP combination showed the best  
236 effect (FICI = 0.75). This finding corroborates the EtBr accumulation assay, in which VP  
237 showed a greater capacity of EtBr accumulation (RFF = 0.544), indicating better EP  
238 inhibition compared with CCCP (RFF = 0.154) in *Mtb* H<sub>37</sub>Rv.

239       The present results encouraged us to perform a time-kill curve assay for the RIF+VP  
240      combination. The activity of RIF+VP combination in reducing viable cell counts was  
241      comparable to RIF alone. The observed additive effect on MIC decreases in the bacillus  
242      exposure to RIF+VP combination, by REDCA, was confirmed by time kill curve in *Mtb*  
243      H<sub>37</sub>Rv. The activity of RIF in reducing viable cell counts was time-dependent, which is  
244      consistent with Steenwinkel et al. (31). A bacteriostatic effect was observed with exposure of  
245      the bacillus to VP alone at a sub-inhibitory concentration.

246       Few studies have investigated morphological changes after exposure to drugs in *Mtb*.  
247      Most electron microscopy studies with *Mtb* were performed in the 1950s and 1960s, with a  
248      lack of high resolution compared to modern standards (32). To our knowledge, no  
249      morphological study, by SEM, of *Mtb* after RIF or VP exposure has been published to date.

250       The *Mtb* H<sub>37</sub>Rv times of exposure to RIF+ VP in the present study were 16 and 72 h  
251      for the evaluation of morphological and transcriptional changes. The exposure times were  
252      based on the *Mtb* generation time and activity in reducing viable cell counts reflected by the  
253      time-kill curve. Sub-inhibitory drug concentrations were used to evaluate the effect of the  
254      combination and reduce the effect of stress on the bacteria according to previous studies (33,  
255      34). A long exposure time and high drug concentration may cause toxic side effects and not  
256      reflect a primary morphological change or true transcriptional profile related to exposure to  
257      the RIF+VP combination.

258       The *Mtb* H<sub>37</sub>Rv control cells had a rod-shaped appearance, with a smooth surface and  
259      typical cord formation. The ability of cell division to form transient branching structures (35)  
260      was observed in the present study (Fig. 3.1a).

261       The main morphological changes were wrinkled and rounding *Mtb* H<sub>37</sub>Rv cells,  
262      which were intensified with 72 h of exposure to RIF and VP, respectively (Fig. 3.2b and

263 3.3b). The effect of the RIF+VP combination was a summation of the observed changes by  
264 RIF and VP exposure alone (Fig. 3.4a and b).

265 We can infer consistency of the results obtained with the time-kill curve and  
266 morphological changes. The effect of RIF on the morphology of the bacillus was more  
267 evident at 72 h, which is consistent with the time-kill curve results, in which lower viable cell  
268 counts were found at this time compared with 16 h. Interestingly, with VP exposure, some  
269 morphological changes in the bacillus were observed, including the induction of cell  
270 rounding, but viable cell counts were not appreciably affected by 72 h exposure, demonstrated  
271 by the time-kill curve.

272 The morphological changes observed by SEM are consistent with the presently  
273 known mechanism of action of the drugs studied. Cell wrinkling was observed with RIF  
274 exposure, which may be attributable to the intracellular action of this drug. The main biotarget  
275 of RIF is the  $\beta$  subunit of DNA-dependent RNA polymerase, encoded by the *rpoB* gene,  
276 which suppresses the transcription process (36, 37). The morphological changes (i.e., cell  
277 rounding) observed by SEM with VP exposure agree with its action on the cell wall. VP is a  
278 well-known calcium channel antagonist and inhibitor of human membrane protein *P*-  
279 glycoprotein (*P*-gp) (19). *P*-gp expression is responsible for the efflux of a wide range of  
280 drugs, including antimicrobials and anticancer agents (38).

281 Recent evidence suggests that bacterial EPs are involved in physiological processes,  
282 such as cell wall division, homeostasis, metabolite secretion, and the transport of substrates,  
283 including drugs (8, 39). Some EPs have been used in humans for the treatment of some  
284 pathologies because of their antiarrhythmic, antihypertensive, antiulcer, and antiemetic effects  
285 (19). *In vivo* (15, 22) and *in vitro* (12, 18) studies of *Mtb* have shown the applicability of  
286 some EPs in restoring the susceptibility of MDR clinical isolates to anti-TB drugs.

287 Based on a thorough literature review of EPs in mycobacteria, 12 EP genes were  
288 chosen for the present study, in which the overexpression of EPs from the ABC (19, 33, 40),  
289 MFS (8, 12, 41), RND (13), and SMR (3, 11) families has been associated with resistance in  
290 *Mtb*.

291 The best inhibitory effect on *Mtb* H<sub>37</sub>Rv EPs in the present study was observed with  
292 16 h exposure to VP alone. This is consistent with the time-kill curve results, in which initial  
293 bacillus growth inhibition was observed. Growth was then restored after this time when VP  
294 exhibited a bacteriostatic effect.

295 For RIF, overexpression of most of the studied EP genes was observed at 72 h of  
296 exposure. A parallel may be observed with the time-kill curve assay results. Fig. 2 shows  
297 adaptation of the bacillus to RIF beginning at this time in an attempt to restore their  
298 subsequent growth. Notably, RIF is a hydrophobic antimicrobial and may enter the cell by  
299 diffusion through the hydrophobic bilayer (42); thus, the active EPs extrude antimicrobial out  
300 of the cell, allowing for the typical drug tolerance phenomenon.

301 The observed expression changes over RIF exposure time are consistent with Calgin et  
302 al. (5), who postulated that an increase in EP expression in *Mtb* clinical isolates during  
303 treatment can drive constitutive or inducible EP expression and lead to an increase in the  
304 MICs of anti-TB drugs and consequently render the bacillus more resistant.

305 Resistance in *Mtb* has long been assumed to arise mainly from spontaneous mutations  
306 in specific genes that are related to the target drugs and then, the selection of a resistant  
307 bacillus by a neglected treatment. Additionally to not explained resistance by known  
308 mutation, an increased efflux activity sustained by a pressure of a sub-inhibitory  
309 concentrations of anti-TB drug could favor the development of the spontaneous mutants (21,  
310 43).

311           Although eight of the studied genes, *Rv1410c* (8), *Rv1456c* (44), *Rv1457c* (44),  
312   *Rv1458c* (44), *Rv1258c* (41), *Rv1217c* (9, 40), *Rv1819c* (3), and *Rv1218c* (9, 40), have  
313   already been reported to be overexpressed in mycobacteria exposed to RIF, the  
314   overexpression of the *Rv3065*, *Rv2846*, *Rv2942*, and *Rv2459* EP genes was demonstrated in  
315   the present study at 72 h. Overexpression of the *Rv3065 (mmr)* (3, 11, 45, 46), *Rv1258c (tap-*  
316   *like gene)* (41, 45, 46), *Rv1410c (p55)* (41, 45, 46), *Rv1819* (3), *Rv2459 (jefA)* (3, 16, 46),  
317   *Rv2942 (mmpL7)* (45, 46), and *Rv2846c (efpA)* (45) genes has also been reported previously  
318   in mycobacteria exposed to INH and EMB.

319           Gupta et al. (3) and Pang et al. (14) did not find significant expression of the *Rv3065*  
320   gene in resistant *Mtb* clinical isolates exposed to RIF, as observed in the present study in *Mtb*  
321   H<sub>37</sub>Rv at 72 h of exposure to the 0.5×MIC of RIF. One possible explanation for this  
322   difference may be related to the exposure time and RIF concentration, which were not  
323   specified by Gupta et al. (3) or Pang et al. (14), respectively. According to Rodrigues et al.  
324   (11), *Rv3065* overexpression was induced by drug exposure for a long period of time, and a  
325   similar effect may have occurred with *Rv2846*, *Rv2942*, and *Rv2459* in the present study.

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

331           The time-kill curve assay showed a decrease in viable cell counts with the RIF+VP  
332   combination similar to RIF exposure alone. The expression study showed an EP inhibitory  
333   effect with combination exposure, which was more pronounced throughout the period of  
334   exposure than RIF exposure alone. The use of a RIF+VP combination may decrease the

335 resistance mediated by EPs. This theory is shared by Ramon-Garcia et al. (47) who studied *M.*  
336 *fortuitum* and other EPIs.

337 Some studies have shown that EPIs may also block antimicrobial efflux in *Mtb*-  
338 infected macrophages, leading to an increase in intracellular drug levels and an increase in  
339 drug actions on the bacillus (48-50). According to Adams et al. (49), VP is perhaps the most  
340 promising inhibitor for further evaluation as an adjunctive TB agent, given its ability to  
341 reverse macrophage-induced tolerance to RIF. Thus, the role of EPs in promoting drug  
342 tolerance opens up a potentially powerful approach to shortening TB treatment.

343 This combination therapy could be a promising alternative for the treatment of patients  
344 with TB, including TB/HIV co-infection. This co-infection, mainly infection with *Mtb* MDR  
345 strains, is associated with difficult therapeutic management. Despite promising results, the  
346 RIF+VP combination should be investigated more thoroughly to determine proper VP  
347 dosages for combined therapy by considering its metabolism by liver enzymes (22).

348 Finally, the present study demonstrated the combined effect between RIF, EMB or  
349 INH with EPIs (CCCP or VP) in *Mtb* H<sub>37</sub>Rv. The use of VP in combination with anti-TB  
350 drugs, mainly RIF, may provide advantages over conventional therapy, including a decrease  
351 in resistance mediated by EPs. This suggestion is based on the morphological changes  
352 observed, including wrinkled and rounding cells, after RIF+VP exposure. These effects are  
353 consistent with the known mechanism of action of each drug. We also observed differential  
354 expression of some EP genes, including some changes that have not been previously reported,  
355 associated with this drug combination. The results obtained with *Mtb* H<sub>37</sub>Rv help guide  
356 further studies with *Mtb* clinical isolates.

357

358

359

360 **Acknowledgements**

361           We would like to thank Vânia Cristina Desoti and Kátia Aparecida Kern Cardoso for  
362           their assistant with fluorometry and the electron microscopes, Complexo de Centrais de  
363           Apoio a Pesquisa (COMCAP), Laboratório de Ensino and Pesquisa em Análises Clínicas  
364           (LEPAC). This work was supported by Programa de Apoio à Pesquisa Básica e  
365           Aplicada/Fundação Araucária; Coordenação de Aperfeiçoamento de Pessoal de Nível  
366           Superior (CAPES) and Programa de Pós-Graduação em Ciências da Saúde da Universidade  
367           Estadual de Maringá.

368

369 **REFERENCES**

- 370     1. **Louw GE, Warren RM, Gey van Pittius NC, McEvoy CR, Van Helden PD, Victor TC.** 2009. A balancing act: efflux/influx in mycobacterial drug resistance. *Antimicrob Agents Chemother* **53**:3181-3189.
- 371     2. **WHO.** 2013. Global tuberculosis report 2013, World Health Organization, Geneva,  
372           Switzerland.
- 373     3. **Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, Sharma VD.** 2010. Microarray analysis of efflux pump genes in multidrug-resistant  
374           *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs.  
375           *Microb Drug Resist* **16**:21-28.
- 376     4. **da Silva PE, Von Groll A, Martin A, Palomino JC.** 2011. Efflux as a mechanism  
377           for drug resistance in *Mycobacterium tuberculosis*. *FEMS Immunol Med Microbiol*  
378           **63**:1-9.
- 379     5. **Calgin MK, Sahin F, Turegun B, Gerceker D, Atasever M, Koksal D, Karasartova D, Kiyan M.** 2013. Expression analysis of efflux pump genes among

- 384 drug-susceptible and multidrug-resistant *Mycobacterium tuberculosis* clinical isolates  
385 and reference strains. Diagn Microbiol Infect Dis **76**:291-297.
- 386 6. **Brennan PJ, Nikaido H.** 1995. The envelope of mycobacteria. Annu Rev Biochem  
387 **64**:29-63.
- 388 7. **Silva PE, Bigi F, Santangelo MP, Romano MI, Martin C, Cataldi A, Ainsa JA.**  
389 2001. Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and  
390 *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **45**:800-804.
- 391 8. **Ramon-Garcia S, Martin C, Thompson CJ, Ainsa JA.** 2009. Role of the  
392 *Mycobacterium tuberculosis* P55 efflux pump in intrinsic drug resistance, oxidative  
393 stress responses, and growth. Antimicrob Agents Chemother **53**:3675-3682.
- 394 9. **de Knecht GJ, Bruning O, ten Kate MT, de Jong M, van Belkum A, Endtz HP,  
395 Breit TM, Bakker-Woudenberg IA, de Steenwinkel JE.** 2013. Rifampicin-induced  
396 transcriptome response in rifampicin-resistant *Mycobacterium tuberculosis*.  
397 Tuberculosis (Edinb) **93**:96-101.
- 398 10. **Danilchanka O, Mailaender C, Niederweis M.** 2008. Identification of a novel  
399 multidrug efflux pump of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother  
400 **52**:2503-2511.
- 401 11. **Rodrigues L, Villegas C, Bailo R, Viveiros M, Ainsa JA.** 2013. Role of the Mmr  
402 efflux pump in drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents  
403 Chemother **57**:751-757.
- 404 12. **Balganesh M, Dinesh N, Sharma S, Kuruppath S, Nair AV, Sharma U.** 2012.  
405 Efflux pumps of *Mycobacterium tuberculosis* play a significant role in antituberculosis  
406 activity of potential drug candidates. Antimicrob Agents Chemother **56**:2643-2651.

- 407 13. **Pasca MR, Guglierame P, De Rossi E, Zara F, Riccardi G.** 2005. mmpL7 gene of  
408 *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium*  
409 *smegmatis*. *Antimicrob Agents Chemother* **49**:4775-4777.
- 410 14. **Pang Y, Lu J, Wang Y, Song Y, Wang S, Zhao Y.** 2013. Study of the rifampin  
411 monoresistance mechanism in *Mycobacterium tuberculosis*. *Antimicrob Agents*  
412 *Chemother* **57**:893-900.
- 413 15. **Louw GE, Warren RM, Gey van Pittius NC, Leon R, Jimenez A, Hernandez-**  
414 **Pando R, McEvoy CR, Grobbelaar M, Murray M, van Helden PD, Victor TC.**  
415 2011. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant  
416 *Mycobacterium tuberculosis* through efflux. *Am J Respir Crit Care Med* **184**:269-276.
- 417 16. **Gupta AK, Reddy VP, Lavania M, Chauhan DS, Venkatesan K, Sharma VD,**  
418 **Tyagi AK, Katoch VM.** 2010. jefA (Rv2459), a drug efflux gene in *Mycobacterium*  
419 *tuberculosis* confers resistance to isoniazid & ethambutol. *Indian J Med Res* **132**:176-  
420 188.
- 421 17. **Sarathy JP, Dartois V, Lee EJ.** 2012. The role of transport mechanisms in  
422 *Mycobacterium tuberculosis* drug resistance and tolerance. *Pharmaceuticals (Basel)*  
423 **5**:1210-1235.
- 424 18. **Viveiros M, Leandro C, Amaral L.** 2003. Mycobacterial efflux pumps and  
425 chemotherapeutic implications. *Int J Antimicrob Agents* **22**:274-278.
- 426 19. **Zechini B, Versace I.** 2009. Inhibitors of multidrug resistant efflux systems in  
427 bacteria. *Recent Pat Antiinfect Drug Discov* **4**:37-50.
- 428 20. **Palomino JC, Ramos DF, da Silva PA.** 2009. New anti-tuberculosis drugs:  
429 strategies, sources and new molecules. *Curr Med Chem* **16**:1898-1904.

- 430 21. **Viveiros M, Martins M, Rodrigues L, Machado D, Couto I, Ainsa J, Amaral L.**  
431 2012. Inhibitors of mycobacterial efflux pumps as potential boosters for anti-  
432 tubercular drugs. *Expert Rev Anti Infect Ther* **10**:983-998.
- 433 22. **Gupta S, Tyagi S, Almeida DV, Maiga MC, Ammerman NC, Bishai WR.** 2013.  
434 Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an  
435 efflux inhibitor. *Am J Respir Crit Care Med* **188**:600-607.
- 436 23. **Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F.** 2002.  
437 Resazurin microtiter assay plate: simple and inexpensive method for detection of drug  
438 resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **46**:2720-  
439 2722.
- 440 24. **Caleffi-Ferracioli KR, Maltempe FG, Siqueira VL, Cardoso RF.** 2013. Fast  
441 detection of drug interaction in *Mycobacterium tuberculosis* by a checkerboard  
442 resazurin method. *Tuberculosis (Edinb)* **93**:660-663.
- 443 25. **Pillai S, Moellering R, Eliopoulos GM.** 2005. Antimicrobial combinations, 5th ed  
444 ed. Lippincott Williams & Wilkins, New York.
- 445 26. **Rodrigues L, Sampaio D, Couto I, Machado D, Kern WV, Amaral L, Viveiros M.**  
446 2009. The role of efflux pumps in macrolide resistance in *Mycobacterium avium*  
447 complex. *Int J Antimicrob Agents* **34**:529-533.
- 448 27. **Bhusal Y, Shiohira CM, Yamane N.** 2005. Determination of in vitro synergy when  
449 three antimicrobial agents are combined against *Mycobacterium tuberculosis*. *Int J*  
450 *Antimicrob Agents* **26**:292-297.
- 451 28. **Limoncu MH, Ermertcan S, Erac B, Tasli H.** 2011. An investigation of the  
452 antimicrobial impact of drug combinations against *Mycobacterium tuberculosis*  
453 strains. *Turk J Med Sci* **41**:719-724.

- 454 29. **Livak KJ, Wills QF, Tipping AJ, Datta K, Mittal R, Goldson AJ, Sexton DW,**  
455 **Holmes CC.** 2013. Methods for qPCR gene expression profiling applied to 1440  
456 lymphoblastoid single cells. *Methods* **59**:71-79.
- 457 30. **Okandeji BO, Greenwald DM, Wroten J, Sello JK.** 2011. Synthesis and evaluation  
458 of inhibitors of bacterial drug efflux pumps of the major facilitator superfamily.  
459 *Bioorg Med Chem* **19**:7679-7689.
- 460 31. **de Steenwinkel JE, de Knegt GJ, ten Kate MT, van Belkum A, Verbrugh HA,**  
461 **Kremer K, van Soolingen D, Bakker-Woudenberg IA.** 2010. Time-kill kinetics of  
462 anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity  
463 of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* **65**:2582-2589.
- 464 32. **Dahl JL.** 2005. Scanning electron microscopy analysis of aged *Mycobacterium*  
465 *tuberculosis* cells. *Can J Microbiol* **51**:277-281.
- 466 33. **Gupta AK, Chauhan DS, Srivastava K, Das R, Batra S, Mittal M, Goswami P,**  
467 **Singhal N, Sharma VD, Venkatesan K, Hasnain SE, Katoch VM.** 2006. Estimation  
468 of efflux mediated multi-drug resistance and its correlation with expression levels of  
469 two major efflux pumps in mycobacteria. *J Commun Dis* **38**:246-254.
- 470 34. **Liang J, Tang X, Guo N, Zhang K, Guo A, Wu X, Wang X, Guan Z, Liu L, Shen**  
471 **F, Xing M, Li L, Yu L.** 2012. Genome-wide expression profiling of the response to  
472 linezolid in *Mycobacterium tuberculosis*. *Curr Microbiol* **64**:530-538.
- 473 35. **Dahl JL.** 2004. Electron microscopy analysis of *Mycobacterium tuberculosis* cell  
474 division. *FEMS Microbiol Lett* **240**:15-20.
- 475 36. **Lewis K.** 2013. Platforms for antibiotic discovery. *Nat Rev Drug Discov* **12**:371-387.
- 476 37. **Silva PEA, Palomino JC.** 2011. Molecular basis and mechanisms of drug resistance  
477 in *Mycobacterium tuberculosis*: classical and new drugs. *J Antimicrob Chemother*  
478 **66**:1417-1430.

- 479 38. **van Veen HW, Konings WN.** 1997. Multidrug transporters from bacteria to man:  
480 similarities in structure and function. *Semin Cancer Biol* **8**:183-191.
- 481 39. **Piddock LJ.** 2006. Multidrug-resistance efflux pumps - not just for resistance. *Nat  
482 Rev Microbiol* **4**:629-636.
- 483 40. **Wang K, Pei H, Huang B, Zhu X, Zhang J, Zhou B, Zhu L, Zhang Y, Zhou FF.**  
484 2013. The expression of ABC efflux pump, Rv1217c-Rv1218c, and its association  
485 with multidrug resistance of *Mycobacterium tuberculosis* in China. *Curr Microbiol*  
486 **66**:222-226.
- 487 41. **Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, Wang H.** 2008. Assessment  
488 of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by  
489 real-time reverse transcription PCR. *Microb Drug Resist* **14**:7-11.
- 490 42. **Nguyen L, Thompson CJ.** 2006. Foundations of antibiotic resistance in bacterial  
491 physiology: the mycobacterial paradigm. *Trends Microbiol* **14**:304-312.
- 492 43. **Schmalstieg AM, Srivastava S, Belkaya S, Deshpande D, Meek C, Leff R, van  
493 Oers NS, Gumbo T.** 2012. The antibiotic resistance arrow of time: efflux pump  
494 induction is a general first step in the evolution of mycobacterial drug resistance.  
495 *Antimicrob Agents Chemother* **56**:4806-4815.
- 496 44. **Hao P, Shi-Liang Z, Ju L, Ya-Xin D, Biao H, Xu W, Min-Tao H, Shou-Gang K,  
497 Ke W.** 2011. The role of ABC efflux pump, Rv1456c-Rv1457c-Rv1458c, from  
498 *Mycobacterium tuberculosis* clinical isolates in China. *Folia Microbiol (Praha)*  
499 **56**:549-553.
- 500 45. **Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M.** 2012. Contribution of  
501 efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex.  
502 *Infect Genet Evol* **12**:695-700.

- 503 46. **Machado D, Couto I, Perdigao J, Rodrigues L, Portugal I, Baptista P, Veigas B,**  
504 **Amaral L, Viveiros M.** 2012. Contribution of efflux to the emergence of isoniazid  
505 and multidrug resistance in *Mycobacterium tuberculosis*. PLoS One **7**:e34538.
- 506 47. **Ramon-Garcia S, Martin C, Ainsa JA, De Rossi E.** 2006. Characterization of  
507 tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium*  
508 *fortuitum*. J Antimicrob Chemother **57**:252-259.
- 509 48. **Adams KN, Takaki K, Connolly LE, Wiedenhoff H, Winglee K, Humbert O,**  
510 **Edelstein PH, Cosma CL, Ramakrishnan L.** 2011. Drug tolerance in replicating  
511 mycobacteria mediated by a macrophage-induced efflux mechanism. Cell **145**:39-53.
- 512 49. **Adams KN, Szumowski JD, Ramakrishnan L.** 2014. Verapamil, and Its Metabolite  
513 Norverapamil, Inhibit Macrophage-induced, Bacterial Efflux Pump-mediated  
514 Tolerance to Multiple Anti-tubercular Drugs. J Infect Dis **210**(3): 456-466.
- 515 50. **Szumowski JD, Adams KN, Edelstein PH, Ramakrishnan L.** 2013. Antimicrobial  
516 efflux pumps and *Mycobacterium tuberculosis* drug tolerance: evolutionary  
517 considerations. Curr Top Microbiol Immunol **374**:81-108.
- 518

519 **TABLE 1** Primers used to assess relative efflux pump gene expression by qPCR.

<b>Efflux pump gene</b>	<b>Transporter family</b>	<b>Sequences (5'- 3')</b>	<b>Amplicon size (bp)</b>	<b>Reference</b>
Rv 2942	RND	Fw- TACCCAAGCTGGAAACAA Rv- CCGTCAGAATAGAGGAACAG	214	(45)
Rv 3065	SMR	Fw- AACCAGCCTGCTCAAAAG Rv- CAACCACCTTCATCACAGA	221	(45)
Rv 2846	MFS	F- ATGGTAATGCCTGACATCC Rv- CTACGGGAAACCAACAAAG	131	(45)
Rv 1410c	MFS	Fw- AGTGGAAATAAGCCAGTAA R- TGGTTGATGTCGAGCTGT	198	(45)
Rv1258c	MFS	Fw- AGTTATAGATCG GCTGGATG Rv- GTGCTGTTCCCGAAATAC	268	(45)
Rv 2459	MFS	Fw- CATCTCATGGTGTT CGT G Rv- CGGTAGCACACAGACAATAG	232	(46)
Rv 1456c	ABC	Fw- GAGTCGCACCAAGAACATGC Rv- TCGCTGTTGGTTGCCTAC	90	(44)
Rv 1457c	ABC	Fw- GTAGCACCGAGTCGTTG Rv- ATCTCCACCGCATTCAACC	80	(44)
Rv 1458c	ABC	Fw- CAGTCCAAGTACCTCAATG Rv- GCGATACGGGTCAATAAC	163	(44)
Rv 1218c	ABC	Fw- CCGCAAGGCGTCTAGTGAA Rv- TGGACCCGTTGATGGAAAA	173	(40)
Rv 1217c	ABC	Fw- CGGTGAGGTTGGCGTAG Rv- CGGTCGGAATCTGGAAA	150	(40)
Rv 1819c	ABC	Fw- CGGTGATTCTTCACAGC Rv- CCGACAGATTCCATCCATT	351	(41)
16s RNA		Fw- CAAGGCTAAAACCAAAGGA Rv- GGACTTAACCCAACATCTCA	197	(45)

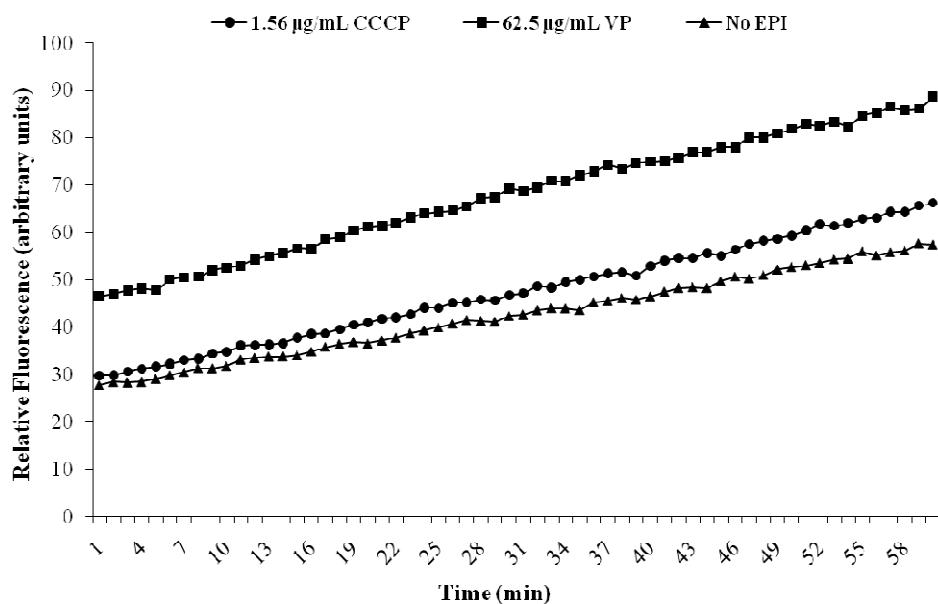
520 F, forward. R, reverse.  
521

522 **TABLE 2** MIC and FICI values for the classical drugs, efflux pump inhibitors, and drug  
 523 combinations in *Mycobacterium tuberculosis* H<sub>37</sub>Rv.

Drugs combination	MIC ( $\mu\text{g/mL}$ )					REDCA	
	INH	EMB	RIF	CCCP	VP	MICs ( $\mu\text{g/mL}$ ) 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

524 RIF, Rifampicin; EMB, Ethambutol; INH, Isoniazid; VP, Verapamil and CCCP, *m*-  
 525 chlorophenyl-hydrazone. FICI, Fractional Inhibitory Concentration Index; REDCA,  
 526 Resazurin Drugs Combination Microtiter Assay; -, not performed.

527

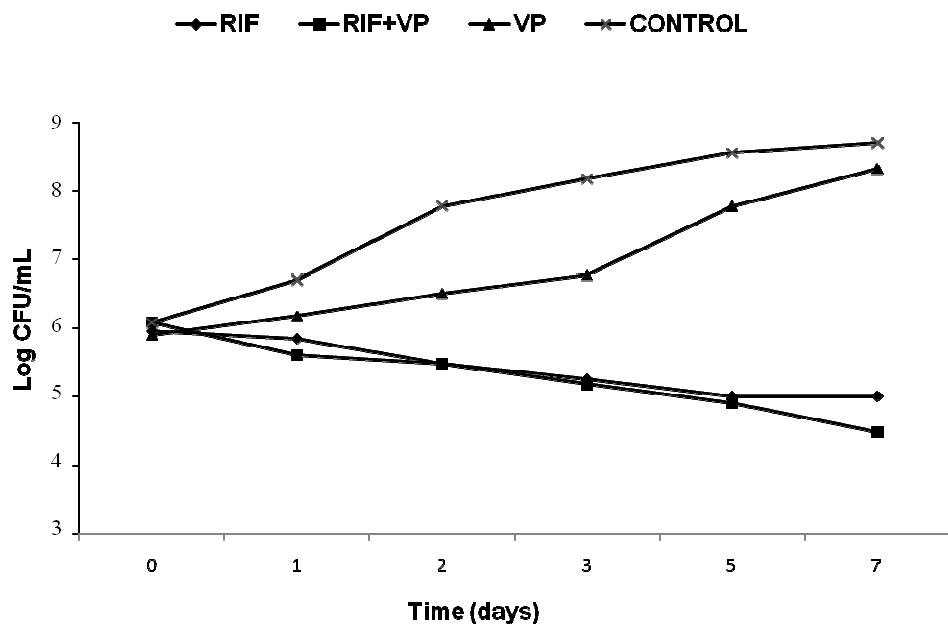


528 **FIG 1** Efflux of ethidium bromide (EtBr) by *Mycobacterium tuberculosis* H<sub>37</sub>Rv by  
 529 fluorometry. The assays were conducted at 35-37°C, with or without an efflux pump inhibitor  
 530 (EPI). Relative fluorescence was obtained by normalizing the data to the background  
 531 fluorescence of EtBr. The efflux of EtBr was inhibited by verapamil (VP) and *m*-

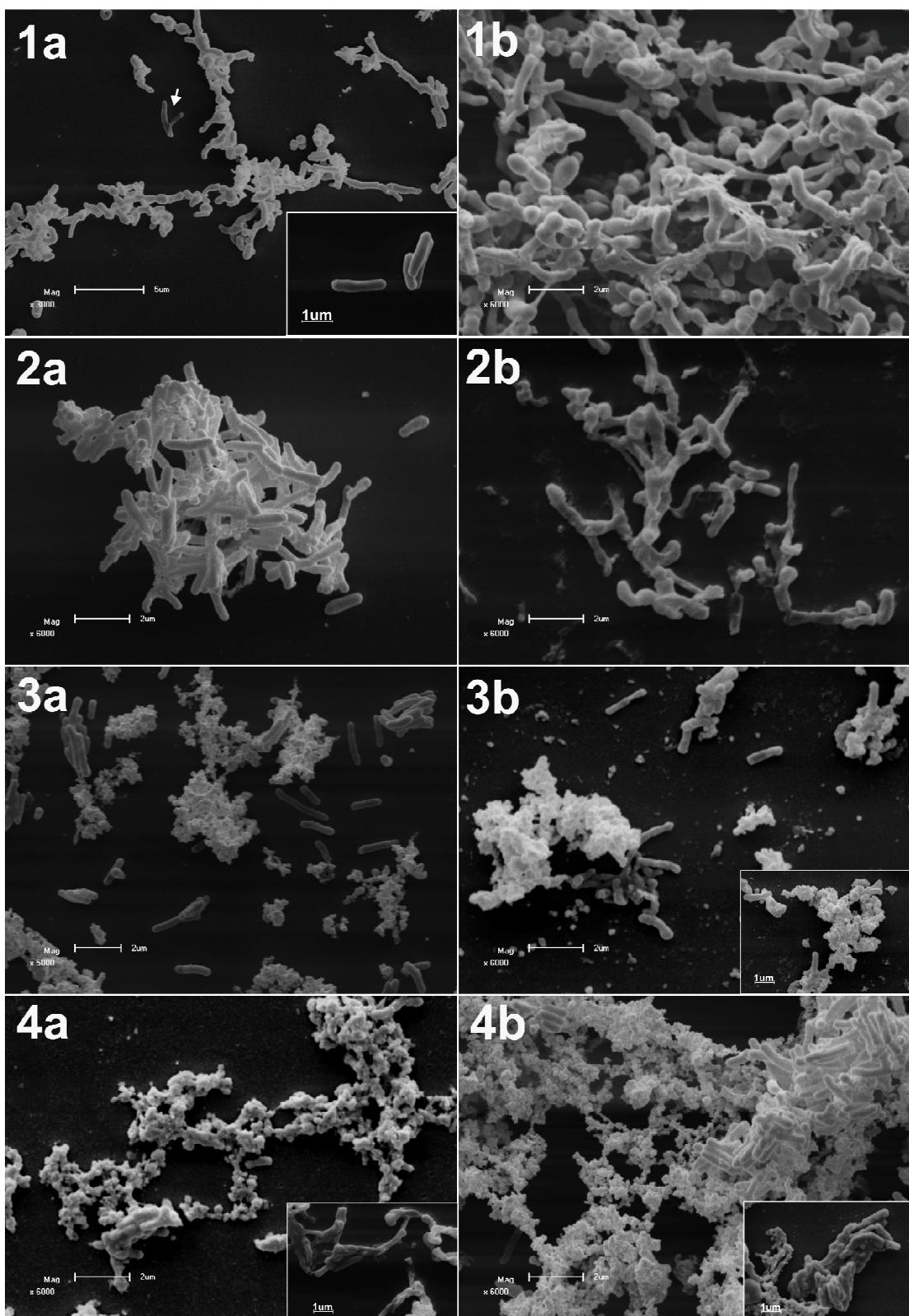
532 chlorophenyl-hydrazone (CCCP) at  $0.5\times\text{MIC}$ . Relative final fluorescence (RFF) was  
533 calculated for each EPI.

534

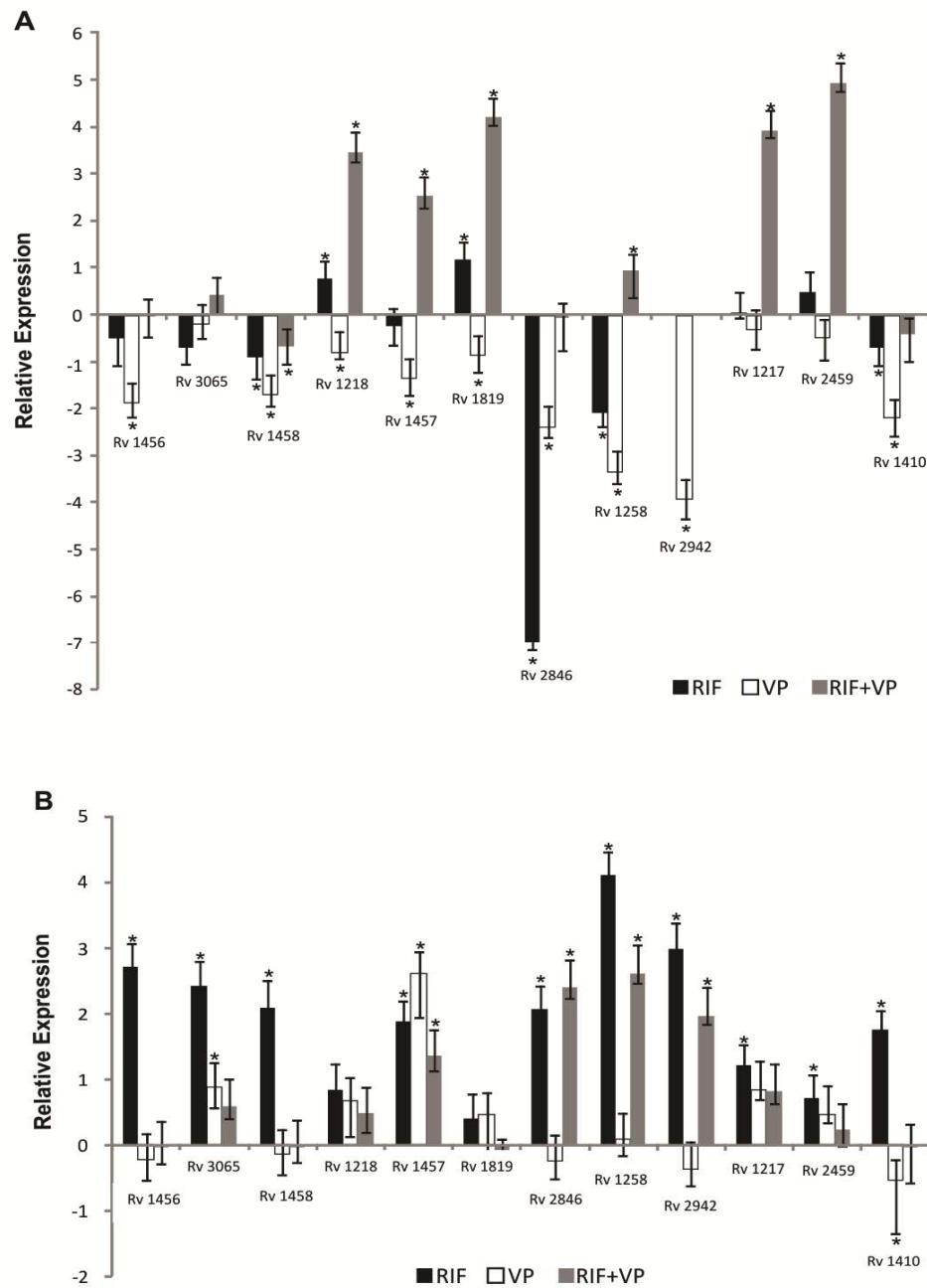
535



536 **FIG 2** Time-kill curve results of *Mycobacterium tuberculosis* using  $0.5\times\text{MIC}$  of rifampicin  
537 (RIF) and verapamil (VP) alone and in combination (RIF+VP) for 7 days. Each data point  
538 (days 0, 1, 2, 3, 5, and 7) represents the mean number of viable bacterial cell counts in  
539 duplicate experiments.



540 **FIG 3** Scanning electron micrograph of *Mycobacterium tuberculosis* (*Mtb*) after 16 h (a) and  
 541 72 h (b) of exposure to a sub-inhibitory concentration (0.5×MIC) of rifampicin (2) or  
 542 verapamil (3) alone and in combination (4). A control of *Mtb* cells without drugs is also  
 543 shown (1). The arrow (1a) shows *Mtb* cell division. Inset magnification: 1a = 18,000×; 3b and  
 544 4a-b = 10,000×.



545

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

## CAPÍTULO III

### CONCLUSÕES

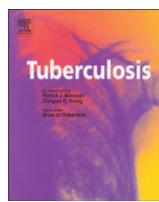
- A metodologia de REDCA foi bem aceita pela área científica e facilitou o estudo de combinação de fármacos em *Mtb*. Devido aos bons resultados obtidos com o modelo da associação entre INH ou EMB com LEVO, o REDCA foi utilizado no estudo da combinação de antimicrobianos anti-TB e IBEs;
- A melhor atividade, entre as combinações de fármacos testadas em *Mtb H<sub>37</sub>Rv*, foi obtida com RIF+VP;
- Os resultados dos estudos de crescimento, morfologia e de expressão de alguns genes que codificam para BEs em *Mtb H<sub>37</sub>Rv*, após exposição à RIF+VP, mostram a ação combinada destes fármacos e a diminuição da resistência do bacilo mediada por BEs a RIF, o que torna esta associação uma terapia alternativa e promissora para o tratamento de casos de tuberculose causada por bacilo resistente a RIF.

### PERSPECTIVAS FUTURAS

Tendo em vista a necessidade do surgimento de novas opções terapêuticas para o tratamento da TB e, que resultados promissores foram obtidos com a combinação de antimicobacterianos e IBEs no modelo *Mtb H<sub>37</sub>Rv*, estudos adicionais com isolados clínicos de *Mtb* resistentes serão conduzidos para um melhor entendimento do funcionamento dos sistemas de efluxo de fármacos, bem como, como ocorre a inibição dos mesmos no bacilo. Adicionalmente, outros genes que codificam para outras BEs e a realização de microscopia eletrônica de transmissão serão também alvos de pesquisas futuras pelo nosso grupo de estudos de mecanismos de resistência em *Mtb* no Laboratório de Bacteriologia Médica, a fim de melhor compreender o efeito da combinação de fármacos anti-TB com IBEs.

## ANEXOS

**Artigo Publicado na revista *Tuberculosis***



## DRUG DISCOVERY AND RESISTANCE

Fast detection of drug interaction in *Mycobacterium tuberculosis* by a checkerboard resazurin method

Katiany R. Caleffi-Ferracioli <sup>a, b,\*</sup>, Flaviane G. Maltempe <sup>b</sup>, Vera Lúcia D. Siqueira <sup>b</sup>, Rosilene F. Cardoso <sup>b</sup>

<sup>a</sup> Postgraduate Program in Health Science, State University of Maringá, Paraná, Brazil

<sup>b</sup> Laboratory of Medical Bacteriology, Department of Clinical Analysis and Biomedicine, State University of Maringá, Maringá, Paraná, Brazil

## ARTICLE INFO

## Article history:

Received 9 May 2013

Received in revised form

1 September 2013

Accepted 4 September 2013

## Keywords:

*Mycobacterium tuberculosis*

Synergism

REDCA

Drugs

## SUMMARY

Tuberculosis (TB) is a health public problem and a long combination therapy is necessary to treat patients. In recent years, some drugs, not routinely used in treatment of TB, have appeared as promising new anti-TB therapies in patients with resistance to classical drugs. The aim of this study was: (i) to evaluate a modified checkerboard assay, Resazurin drugs combination microtiter assay (REDCA) to detect drugs interaction in *Mycobacterium tuberculosis*; (ii) to evaluate the interaction between isoniazid (INH) or ethambutol (EMB) with levofloxacin (LEVO) in susceptible and resistant *M. tuberculosis* Brazilian clinical isolates. *M. tuberculosis* H<sub>37</sub>R<sub>V</sub> ATCC 27294 and 19 clinical isolates (10 resistant and 9 susceptible) were tested. The fractional inhibitory concentration index (FICI)  $\leq 0.5$  was considered synergistic. Synergism in *M. tuberculosis* H<sub>37</sub>R<sub>V</sub> and resistant *M. tuberculosis* Brazilian isolates was observed with EMB vs. LEVO. No synergism was observed with INH vs. LEVO by both assays. No statistical difference was observed by the two assays studied. REDCA showed to be a simple assay for detecting synergism between drugs in *M. tuberculosis*. The results with EMB vs. LEVO are promising and it can be a new option in future investigations of drugs interactions against *M. tuberculosis* with the view to reduce EMB adverse effects.

© 2013 Elsevier Ltd. All rights reserved.

## 1. Introduction

The incidence of tuberculosis (TB) remains high, affecting mostly young adults in their most productive years [1]. According to World Health Organization (WHO), a third of the world's population is infected with *Mycobacterium tuberculosis* (*Mtb*) and 95% of TB deaths are in the developing world [2]. Treatment of TB patients is based on six-month regimen using isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA). A long combination therapy is necessary in order to prevent relapse of the disease and development of drug resistance. In 2011, there were an estimated global prevalence of 630,000 cases of multidrug-resistant TB (MDR-TB) among the world's 12 million prevalent cases of TB [3]. In this case, second-line drugs are used, but most of them are either very toxic and more expensive [4].

\* Corresponding author. Laboratório de Bacteriologia Médica, Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Avenida Colombo, 5790, 87020-900 Maringá, Paraná, Brazil. Tel.: +55 44 3011 5376; fax: +55 44 3011 4797.

E-mail addresses: [katianyrcf@gmail.com](mailto:katianyrcf@gmail.com), [krcaleffi@yahoo.com.br](mailto:krcaleffi@yahoo.com.br) (K.R. Caleffi-Ferracioli).

In recent years, fluoroquinolones and other drugs, not routinely used in treatment of TB, have emerged as promising new TB therapies in patients with resistance to classical anti-TB drugs [5]. Clinical trials have also suggested that fluoroquinolones such as moxifloxacin or levofloxacin (LEVO) may be useful in shortening the treatment of TB [6]. Yew et al. [7] reported that LEVO was found to be more efficacious than ofloxacin when incorporated into multidrug regimens for treatment of MDR-TB and has been recommended for treatment of MDR-TB in the United States. [8]

As the discovery of new drugs is a challenge, which demands time and money, new combinations of old and known antimicrobials have been studied [9,10] and synergistic effect against *Mtb* have been demonstrated empirically [10,11].

To establish a new therapeutic regimen, it is necessary to develop quantitative and reproductive assay to estimate antimicrobial activity when two agents are combined [12]. The synergism of drugs can be studied using checkerboard [13–15] and/or by the time-kill method [12,16]. Two of the main difficulties in implementing the classical checkerboard assay for *Mtb* are the long incubation period and difficulty in reading.

There are a couple of methods to detect the viability of actively growing bacteria in a microplate format. Colorimetric methods that

**Table 1**

MICs values alone and FICI obtained by REDCA and Classical checkerboard assay with isoniazid or ethambutol vs. levofloxacin combinations in *Mycobacterium tuberculosis* H<sub>37</sub>R<sub>V</sub> and drug-susceptible and multidrug-resistant (MDR) *Mycobacterium tuberculosis* clinical isolates.

Isolate	Susceptibility pattern	REDCA			Classical checkerboard			REDCA		Classical checkerboard		
		MIC (mg/L)			MIC (mg/L)			INH/LEVO*		INH/LEVO*		EMB/LEVO†
		INH	EMB	LEVO	INH	EMB	LEVO	MICs (mg/L)	FICI	MICs (mg/L)	FICI	MICs (mg/L)
H <sub>37</sub> R <sub>V</sub>	S	0.03	1	0.50	0.06	2	0.50	0.03/0.50	1.5	0.03/0.125	0.75	0.25/0.125
49 S	S	0.25	2	0.25	0.25	2	0.50	0.25/0.25	2	0.25/0.5	2	0.5/0.125
57 S	S	0.25	2	0.25	0.25	2	0.50	0.25/0.25	2	0.125/0.125	0.75	1/0.125
80 S	S	0.06	1	0.25	0.06	2	0.25	0.03/0.06	0.74	0.03/0.125	1	0.5/0.125
46 S	S	0.06	2	0.125	0.25	1	0.25	0.06/0.125	2	0.06/0.125	1	0.5/0.07
9 S	S	0.03	1	0.06	0.06	2	0.125	0.03/0.06	2	0.06/0.125	2	0.5/0.03
20 S	S	0.03	1	0.125	0.06	1	0.125	0.03/0.125	2	0.03/0.06	1	0.5/0.06
50 S	S	0.06	1	0.125	0.06	NP	0.25	NP	NP	0.03/0.125	1	NP
58 S	S	0.06	1	0.06	0.06	NP	0.125	0.06/0.06	2	0.06/0.125	2	NP
65 S	S	0.06	2	0.25	0.06	2	0.5	0.03/0.125	1	0.03/0.125	0.75	1/0.06
18 R	MDR	2	4	0.25	4	4	0.25	0.5/0.125	0.75	2/0.125	1	2/0.06
97 R	MDR	32	8	0.25	4	8	0.50	16/0.125	1	2/0.125	0.75	4/0.125
3614 R	MDR	16	32	0.25	4	8	0.25	16/0.25	2	4/0.25	2	32/0.25
3408 R	MDR	8	32	0.125	16	8	0.125	8/0.125	2	2/0.06	0.61	8/0.06
69 R	MDR	2	4	0.5	4	1	0.125	1/0.08	0.66	1/0.06	0.73	1/0.014
71 R	MDR	1	1	0.125	4	2	0.50	1/0.125	2	2/0.125	0.75	0.125/0.03
73 R	MDR	2	2	2	4	2	4	2/2	2	2/1	0.75	1/1
91 R	R	4	1	0.125	4	1	0.25	2/0.03	0.74	2/0.125	1	0.5/0.06
4250 R	MDR	8	1	0.25	4	1	0.25	8/0.25	2	2/0.125	1	0.5/0.125
25252 R	MDR	1	0.50	0.125	4	2	0.25	1/0.25	2	NP	NP	0.5/0.125

*M. tuberculosis*: S: Drug-susceptible isolates; R: Resistance to INH; MDR: multidrug-resistant; H<sub>37</sub>R<sub>V</sub>: wild-type *Mtb* strain; NP, not performed; MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index; In bold, Synergistic effect (FICI ≤ 0.50); REDCA, Resazurin drugs combination microtiter assay.

\* p = 0.145, t-test; FICI results by REDCA assay vs. classical checkerboard for INH vs. LEVO.

† p = 0.959, t-test; FICI results by REDCA assay vs. classical checkerboard for EMB vs. LEVO.

use redox indicators or the nitrate reduction assay have received increasing attention because of their simplicity and the absence of any requirement for sophisticated equipment or highly trained personnel [17]. Colorimetric methods are based on the reduction of a colored indicator added to the culture medium after *M. tuberculosis* has been exposed *in vitro* to different drugs. A change in color of the indicator is directly proportional to the number of viable mycobacteria in the medium [18]. The most common indicators used have been the resazurin (also known as alamar blue, an oxidation–reduction colorimetric indicator) [19,20] and tetrazolium salts (reduction by metabolically active cells) such as: XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] and MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [21,22]. No differences in results using resazurin, MTT and XTT indicators have been observed [22,23]. Sala and Hartkoorn [24] reported that the main method, to date, employed to high and medium-throughput screening of compound libraries is the oxidation–reduction colorimetric method called resazurin-based assay (REMA).

In this sense, the use of resazurin to assess the antimycobacterial activity in drugs interaction assay, has been previously proposed for determination of Minimal Inhibitory Concentration (MIC) by Palomino et al. [25] and Martin et al. [26,27].

In this context, the aim of this study was: (i) to evaluate a modified checkerboard method, Resazurin drugs combination microtiter assay (REDCA), to determine interaction of drugs *in vitro* against *Mtb* clinical isolates; (ii) to evaluate the interaction between INH or EMB with LEVO in susceptible and resistant *Mtb* Brazilian clinical isolates.

## 2. Materials and methods

### 2.1. Bacterial isolates

Nineteen *Mtb* clinical isolates belonging to the reference center for TB diagnosis denominated LEPAC (Laboratory of teaching and research in clinical analysis), State University of Maringá, Paraná,

Brazil were selected based in previously characterized mutations in the *katG* and *inhA* genes [28]: Nine isolates were susceptible to all antimicrobial agents and ten resistant to INH and/or other antituberculous drugs (9 were MDR-TB clinical isolates). *M. tuberculosis* H<sub>37</sub>R<sub>V</sub> (ATCC 27294) wild-type reference strain was used as control. The tested isolates were grown in Middlebrook 7H9 medium (Difco), added with 0.2% glycerol (vol/vol), 0.025% tween 80 (vol/vol) and supplemented with OADC (Oleic acid, albumin, dextrose and catalase) (BBL/Becton-Dickinson, Sparks, MD, USA) for 15 days at 37 °C.

### 2.2. Antimicrobials

The antimicrobial agents INH and EMB were provided by Sigma Chemical Co. (USA, Canada) and LEVO by Halexisstar Co. (Brazil, Goiania). The stock solutions and further dilutions were prepared in OADC-supplemented Middlebrook 7H9.

### 2.3. REDCA and classical checkerboard assay

The REDCA was performed in duplicate as described elsewhere with minor changes in time of incubation and reading using as model the interaction between INH vs. LEVO and EMB vs. LEVO, and compared with classical checkerboard assay [12].

Plates were prepared by serially diluting INH (0.25–0.0009 mg/L and 32–0.125 mg/L for susceptible and resistant isolates, respectively) or EMB (64–0.250 g/mL) in the x-axis and LEVO (16–0.0156 mg/L) in the y-axis in a 96-well microtitre plate [14,29]. One hundred microliter of mycobacterial suspension adjusted to a turbidity of 1.0 McFarland Standard scale and diluted 1:20 was inoculated in each well. The plates were covered with their lids, sealed and incubated at 37 °C in normal atmosphere for 7 days [25] for REDCA and two-three weeks until adequate growth in the control well could be visually read for classical method [12]. After, 30 µL of freshly 0.02% (wt/vol) resazurin solution (Acros, Morris Plains, NJ, USA) were added to each REDCA plate well. Plates were incubated overnight at 37 °C. A blue to pink color change indicated

reduction of resazurin and therefore, bacterial growth [25]. The classical checkerboard reading was carried out by visual reading of growth, without resazurin, as previously described [12].

To evaluate the synergistic effect for both assay, the fractional inhibitory concentration index:  $FICI = (\text{MIC A} + \text{B}/\text{MIC A}) + (\text{MIC B} + \text{A}/\text{MIC B})$  was used, where: MIC A + B represents the MIC of drug A when combined with drug B. MIC B + A represents the MIC of drug B when combined with drug A. The MIC A and MIC B represent the MICs of drugs A and B when tested alone, respectively. The results were interpreted by the FICI as: synergism,  $\leq 0.50$ ; indifference,  $>0.50$ –4; and antagonism,  $>4$  [14].

#### 2.4. Statistical analysis

Statistic 7.1 software (StatSoft, 2005) was used for analysis of REDCA compared with Classical Checkerboard Method. The level of significance was considered  $p < 0.05$ .

### 3. Results

The MICs values of INH, EMB and LEVO for *Mtb* H<sub>37</sub>R<sub>V</sub>, susceptible and resistant *Mtb* clinical isolates are listed in Table 1. INH, EMB and LEVO MICs ranged from 0.03 to 32 mg/L, 0.5–32 mg/L and 0.06–4 mg/L, respectively (Table 1). Altogether, 90% of the clinical isolates were inhibited at MIC 4, 8 and 0.5 mg/L for INH, EMB and LEVO, respectively.

A synergistic effect for the *Mtb* H<sub>37</sub>R<sub>V</sub> reference strain was observed only with EMB vs. LEVO (Table 1).

Discrepant EMB vs. LEVO synergism results were observed by REDCA (two resistant *Mtb* clinical isolates) and by the classical checkerboard (one resistant *Mtb* clinical isolate) (Table 1). However, this result showed no statistical difference between the two assays studied ( $p = 0.959$ ).

Similar for INH vs. LEVO synergism results, no statistical difference ( $p = 0.145$ ) was observed when compared classical checkerboard and REDCA, with FICIs ranging from 0.61 to 2 (Table 1).

### 4. Discussion

The combination of anti-TB drugs is a potential tool for the treatment of TB as it has been recommended by WHO for decades. Despite the need of emergence of new drugs for TB treatment, there is no short-term prospect of this happening. Drugs that have actions on non *Mtb* complex microorganisms have been used in some situations as the treatment of resistant TB. In this regard, the study of old and known drugs in combination with the classical anti-TB drugs is a valuable alternative.

Comparing classical checkerboard with REDCA assay, some variations in the FICI values were observed. For example, the isolate 57s showed FICI equal to 1 for REDCA and 0.75 for classical checkerboard with EMB vs. LEVO. Although the FICI values are different, both values indicate non-synergism between drugs ( $FICI \geq 0.50$ ). Thus, the changes in the FICI did not show differences in the interpretation (synergic or non synergic) of the observed interaction between the studied drugs with the exception of isolate 69.

The improved performance of REDCA, in detecting EMB vs. LEVO synergism observed in the present study may reside in its bacteriostatic activity against *Mtb* [30]. Using a long incubation time necessary in classical checkerboard, the EMB can lose the bacteriostatic effect thus masking the synergism, because *Mtb* can grow back.

Rastogi et al. [31] reported that combinations of EMB with LEVO were also synergistic in two of five clinical isolates using drug combination classical assays, but not in *Mtb* H<sub>37</sub>R<sub>V</sub> as observed in

the present study. Rey-Jurado et al. [32] working with three drugs combinations, LEVO, linezolid (LINE) and EMB, did not observe synergism in *Mtb* H<sub>37</sub>R<sub>V</sub> and clinical isolates by classical checkerboard assay.

Although Rastogi et al. [31] found synergistic effect between INH vs. LEVO in *Mtb* clinical isolates, our study did not show this effect by both, classical checkerboard and REDCA. In this regard, the good correlation between the results, by both assays for the INH vs. LEVO, may have been influenced by the bactericidal activity of INH [33].

Traditionally (and still the gold standard), bacterial viability was determined by counting colony-forming units (CFUs), such as occurs in the Time-Kill method [34]. It is a process that is laborious and time-intensive, mainly for mycobacteria which take longer to grow *in vitro*, but gives an accurate picture of the bactericidal or bacteriostatic activity of a compound [24]. Recently, some promising alternative methods for detecting drug resistance and MICs have been proposed. The colorimetric methods, despite having lack of sensitivity compared with CFU evaluation, with a limit of detection of approximately  $1 \times 10^6$  CFU/mL (for tetrazolium salts) and  $1 \times 10^5$  CFU/mL (for resazurin) [21], are easier to perform and the result can be obtained in shorter incubation time. The possibility of a method for detecting synergism in *M. tuberculosis*, a slow growing mycobacteria, which use a shorter incubation time, is important for accurate results. Nonetheless, Sala and Hartkoorn [24] reported that the main disadvantage of colorimetric methods is that the used dyes measure bacteriostatic rather than bactericidal activity. The REDCA presented in this paper, could detect synergistic effect of EMB and LEVO. An explication for this lies in the fact the REDCA does not need a long incubation time, which could cause the loss of bacteriostatic activity of EMB, thus masking a real result. Additionally, a long incubation time (by the classical checkerboard) generates other disadvantages, such as, evaporation and drying of the plate wells. Beyond a long incubation time can provide water condensation on the lid of the microplate, which can favor fungal contamination.

The main attractiveness of using fluorescent signal by resazurin dye is the read ability. It is known that fluorescence readouts obtained by redox-dye, typically display a background. However, in the use of a fluorescence detecting equipment, the reading should be adjusted for the background level fluorescence originated by the dye and medium [35]. The read ability of REDCA assay, without necessity of using equipment, makes it accessible for its use in laboratories with low financial resources.

The drugs interaction model used in the present study, INH vs. LEVO and EMB vs. LEVO determined by REDCA showed to be efficient comparable to the classical checkerboard ( $p < 0.05$ ), with the advantage of obtaining the result in eight days (seven of incubation plus one for resazurin reading) instead of two-three weeks used in the classical assay. Additional advantage of REDCA is the easiness of reading by visual inspection and the low cost of resazurin.

To our knowledge, there was two other previous works [36,37] that employed the resazurin to assess the viability and the antimicrobial activity in drugs interaction assay in *Mtb* H<sub>37</sub>R<sub>V</sub> and *Mycobacterium smegmatis*. However, none of them applied a comparison with the classical checkerboard assay in susceptible and resistant *Mtb* clinical isolates including MDR-TB as carried out in the present study. The limitation of this study was the small number of *Mtb* clinical isolates evaluated and the limited classical anti-TB drugs combinations. Nevertheless, the results make us optimistic about the use of REDCA in future investigations of drugs interactions, once it is faster compared to classical checkerboard assay and the use of resazurin, which is inexpensive, makes the visual reading to be more reliable. The relevance of using the drugs combination model proposed in the study, including LEVO, call

attention for its potential in treatment of *Mtb*-HIV co-infected patients who are at increased risk for developing MDR-TB disease and also the dose-dependent ocular toxicity of EMB. The finding of two Brazilian clinical MDR-TB isolates that showed EMB vs. LEVO synergism points attention to the benefits of this combination for the treatment of these cases and consequently reduce the adverse effects.

**Ethical approval:** Not required.

**Funding:** No funding sources.

**Competing interests:** None declared.

## References

- [1] Jagielski T, Augustynowicz-Kopeć E, Zwolska Z. Epidemiology of tuberculosis: a global, European and polish perspective. *Wiad Lek* 2010;63:230–46.
- [2] WHO. Global tuberculosis control 2011. Geneva: World Health Organization; 2011. <[http://www.who.int/tb/publications/global\\_report/2011/en/](http://www.who.int/tb/publications/global_report/2011/en/)>.
- [3] WHO. Global tuberculosis report 2012. Geneva: World Health Organization; 2012. <[http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf)>.
- [4] Dye C. Tuberculosis 2000–2010: control, but not elimination. *Int J Tuberc Lung Dis* 2000;4:146–52.
- [5] Ginsburg AS, Grosset JH, Bishai WR. Fluoroquinolones, tuberculosis, and resistance. *Lancet Infect Dis* 2003;3:432–42.
- [6] Lienhardt C, Ravaglione M, Spigelman M, Hafner R, Jaramillo E, Hoelscher M, Zumla A, Gheuens J. New drugs for the treatment of tuberculosis: needs, challenges, promise, and prospects for the future. *J Infect Dis* 2012;205(2):241–9.
- [7] Yew WW, Chan CK, Leung CC, Chau CH, Tam CM, Wong PC, Lee J. Comparative roles of levofloxacin and ofloxacin in the treatment of multidrug-resistant tuberculosis: preliminary results of a retrospective study from Hong Kong. *Chest* 2003;124:1476–81.
- [8] Small PM, Fujiwara PI. Management of tuberculosis in the United States. *N Engl J Med* 2001;345:189–200.
- [9] Rey-Jurado E, Tudo G, Martinez JA, Gonzalez-Martin J. Synergistic effect of two combinations of antituberculous drugs against *Mycobacterium Tuberculosis*. *Tuberculosis (Edinb)* 2012;92:260–3.
- [10] Amaral L, Viveiros M. Why thioridazine in combination with antibiotics cures extensively drug-resistant *Mycobacterium tuberculosis* infections. *Int J Antimicrob Agents* 2012;39:376–80.
- [11] Dauby N, Muylle I, Mouche F, Sergysels R, Payen MC. Meropenem/clavulanate and linezolid treatment for extensively drug-resistant tuberculosis. *Pediatr Infect Dis J* 2011;30:812–3.
- [12] Bhusal Y, Shiohira CM, Yamane N. Determination of *in vitro* synergy when three antimicrobial agents are combined against *Mycobacterium tuberculosis*. *Int J Antimicrob Agents* 2005;26:292–7.
- [13] Bergmann JS, Woods GL. *In vitro* activity of antimicrobial combinations against clinical isolates of susceptible and resistant *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 1998;2:621–6.
- [14] Pillai SK, Moellering Jr RC, Eliopoulos GM. Antimicrobial combinations. In: Lorian V, editor. *Antibiotics in laboratory medicine*. 5th ed. New York: Lippincott Williams & Wilkins; 2005. p. 365–440.
- [15] Abourashed EA, Galal AM, Shibli AM. Antimycobacterial activity of ferutinin alone and in combination with antitubercular drugs against a rapidly growing surrogate of *Mycobacterium tuberculosis*. *Nat Prod Res* 2011;25:1142–9.
- [16] Limoncu MH, Ermertcan S, Erac B, Tasli H. An investigation of the antimicrobial impact of drug combinations against *Mycobacterium tuberculosis* strains. *Turk J Med Sci* 2011;41:719–24.
- [17] Palomino JC, Martin A, Portaels F. Rapid drug resistance detection in *Mycobacterium tuberculosis*: a review of colourimetric methods. *Clin Microbiol Infect* 2007;13(8):754–62.
- [18] Yajko DM, Madej JJ, Lancaster MV, Sanders CA, Cawthon VL, Gee B, Babst A, Hadley WK. Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. *J Clin Microbiol* 1995;33:2324–7.
- [19] Reis RS, Neves Jr I, Lourenco SL, Fonseca LS, Lourenco MC. Comparison of flow cytometric and alamar blue tests with the proportional method for testing susceptibility of *Mycobacterium tuberculosis* to rifampin and isoniazid. *J Clin Microbiol* 2004;42:2247–8.
- [20] Sungkanuparph S, Pracharttam R, Thakkinstian A, Buabut B, Kiatatchasai W. Correlation between susceptibility of *Mycobacterium tuberculosis* by microtitre plate alamar blue assay and clinical outcomes. *J Med Assoc Thai* 2002;85:820–4.
- [21] Caviedes L, Delgado J, Gilman RH. Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2002;40:1873–4.
- [22] De Logu A, Uda P, Pellerano ML, Pusceddu MC, Saddi B, Schivo ML. Comparison of two rapid colorimetric methods for determining resistance of *Mycobacterium tuberculosis* to rifampin, isoniazid, and streptomycin in liquid medium. *Eur J Clin Microbiol Infect Dis* 2001;20:33–9.
- [23] Martin A, Portaels F, Palomino JC. Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *J Antimicrob Chemother* 2007;59:175–83.
- [24] Sala C, Hartkoorn RC. Tuberculosis drugs: new candidates and how to find more. *Future Microbiol* 2011;6(6):617–33.
- [25] Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2002;46:2720–2.
- [26] Martin A, Paasch F, Docx S, Fissette K, Imperiale B, Ribón W, González LA, Werengren J, Engström A, Skenders G, Juréen P, Hoffner S, Portillo PD, Morcillo N, Palomino JC. Multicentre laboratory validation of the colorimetric redox indicator (CRI) assay for the rapid detection of extensively drug-resistant (XDR) *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2011;66:827–33.
- [27] Martin A, Camacho M, Portaels F, Palomino JC. Resazurin microtiter assay plate testing of *Mycobacterium tuberculosis* susceptibilities to second-line drugs: rapid, simple, and inexpensive method. *Antimicrob Agents Chemother* 2003;47:3616–9.
- [28] Cardoso RF, Cooksey RC, Morlock GP, Barco P, Cecon L, Forestiero F, Leite CQF, Sato DN, Shikama MD, Mamizuka EM, Hirata RDC, Hirata MH. Screening and characterization of mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates obtained in Brazil. *Antimicrob Agents Chemother* 2004;48:3373–81.
- [29] Moody JA. Synergism testing: broth microdilution checkerboard and broth macrodilution methods. Washington: DC: American Society for Microbiology; 1992.
- [30] Strupczewska-Januszowa H, Tomaszkiewicz L, Wozniak S. Bacteriostatic effect of ethambutol on tubercle bacilli and other mycobacteria in vitro. *Gruzica* 1968;36:203–7.
- [31] Rastogi N, Goh KS, Bryskier A, Devallois A. *In vitro* activities of levofloxacin used alone and in combination with first- and second-line antituberculous drugs against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1996;40(7):1610–6.
- [32] Rey-Jurado E, Tudo G, de la Bellacasa JP, Espasa M, Gonzalez-Martin J. *In vitro* effect of three-drug combinations of antituberculous agents against multidrug-resistant *Mycobacterium tuberculosis* isolates. *Int J Antimicrob Agents* 2013;41:278–80.
- [33] Skinner PS, Furney SK, Kleinert DA, Orme IM. Comparison of activities of fluoroquinolones in murine macrophages infected with *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1995;39:750–3.
- [34] Steenwinkel JEM, Knegt GJ, Kate MT, Belkum AV, Verbrugh HA, Kremer K, Soelingen DV, Bakker-Woudenberg IAMJ. Time-kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2010;65:2582–9.
- [35] Hamalainen-Laanaya HK, Orloff MS. Analysis of cell viability using time-dependent increase in fluorescence intensity. *Anal Biochem* 2012;429:32–8.
- [36] Ramon-Garcia S, Martin C, Ainsa JA, De Rossi E. Characterization of tetracycline resistance mediated by the efflux pump tap from *Mycobacterium fortuitum*. *J Antimicrob Chemother* 2006;57:252–9.
- [37] Ramon-Garcia S, Ng C, Anderson H, Chao JD, Zheng X, Pfeifer T, Av-Gay Y, Roberge M, Thompson CJ. Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen. *Antimicrob Agents Chemother* 2011;55:3861–9.