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**Caracterização dos compostos derivados de furoxano como inibidores da atividade de bombas de efluxo em *Mycobacterium tuberculosis* e estudo da influência do efluxo e da halotolerância nos perfis de resistência do bacilo**

**Leonardo Biancolino Marino**

**TESE DE DOUTORADO**

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Tese apresentada à Faculdade de Ciências Farmacêuticas – UNESP, Araraquara, como parte dos requisitos para obtenção do título de Doutor em Biociências e Biotecnologia Aplicadas à Farmácia.

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## RESUMO

Tuberculose (TB), com principal agente etiológico o bacilo *Mycobacterium tuberculosis*, é uma doença infecciosa passível de cura. Entretanto, é detentora de uma estatística mundial preocupante, e em 2014 apresentou 9,5 milhões de novos casos e 1,5 milhão de mortes. Além dos altos índices estatísticos, a TB aparece ainda associada a dois fatores agravantes: a co-infecção com HIV (“*Human Immunodeficiency Virus*”) e a crescente emergência de linhagens resistentes aos quimioterápicos como “*multi-drug resistant*” (MDR) e “*extensively drug resistant*” (XDR), que dificultam sobremaneira o tratamento, indicando premência na pesquisa de novos fármacos. A resistência do bacilo manifesta-se a partir de eventos adquiridos (como aquisição de mutações cromossômicas em genes específicos relacionados ao metabolismo de fármacos) ou intrínsecos, como uma parede celular altamente hidrofóbica (devido à grande quantidade de ácidos micólicos). Estes mecanismos de resistência são discutidos amplamente na literatura. Por outro lado, pouco se sabe a respeito da resistência devido as alterações na expressão dos genes das bombas de efluxo, responsáveis pela exclusão de fármacos, bem como dos mecanismos de resistência relacionados a halotolerância bacteriana.

Neste sentido, três assuntos de relevância no combate à tuberculose foram abordados nesta tese. Como primeira frente, avaliou-se inicialmente a expressão de 5 genes codificadores de bombas de efluxo pela técnica de RT-qPCR em 12 isolados clínicos frente ao tratamento com rifampicina, principal fármaco do regime terapêutico da TB. Resultados indicaram que essas proteínas auxiliam na emergência da resistência, sendo indicados pela superexpressão dos genes frente ao tratamento.

Como segunda frente avaliou-se a atividade de 15 compostos inéditos de furoxânicos e benzofuroxânicos quanto à capacidade inibitória frente ao *M. tuberculosis*, citotoxicidade em células VERO, além de ensaios para avaliar seus mecanismos de ação (acumulação de brometo de etídio / inibição de efluxo, liberação de óxido nítrico e *microarray*). Resultados indicaram que a classe de compostos é promissora para o tratamento, com boa atividade inibitória contra o bacilo e considerado grau de seletividade. Ensaios de mecanismo de ação, indicaram atividade independente da inibição do efluxo, da subunidade de isoniazida ou da liberação de óxido nítrico. *Microarray* apontou para alteração em grande quantidade de proteínas ribossômicas, semelhante ao que ocorre com aminoglicosídeos.

Por fim, na terceira frente do estudo avaliou-se os efeitos da halotolerância na emergência de bacilos resistentes a fármacos, tendo como base as diferentes concentrações de cloreto de sódio a que o bacilo é submetido durante o seu ciclo de vida. Os resultados apontaram para aumento da resistência bacteriana com consequente aumento nas concentrações inibitórias de vários fármacos usados para a terapia da TB, quando o bacilo é submetido à concentração de cloreto de sódio encontrada no interior dos macrófagos, indicando uma necessidade de reflexão sobre as novas diretrizes para testes de novos fármacos contra TB.

## ABSTRACT

Tuberculosis (TB) is an infectious disease caused mainly by *Mycobacterium tuberculosis* bacillus, presenting 9.5 million of new cases and 1.5 million of deaths in 2014. Two aggravating factors are associated with these high statistical indexes: a co-infection with HIV ("Human Immunodeficiency Virus") and the increasing emergence of resistant strains represented by MDR ("multi-drug resistant") and XDR ("extreme drug resistant") strains. The bacillus resistance manifests from acquired events (such as acquisition of chromosomal mutations in specific genes related to drug metabolism) or intrinsic, as a highly hydrophobic cell wall (due to the large amount of mycolic acids) and the action of efflux pumps (responsible for drug extrusion). Thus, the thesis project evaluated the expression of five genes encoding efflux pumps by RT-qPCR technique in 12 clinical isolates against rifampicin, the main anti-TB drug used in the standard treatment. Results indicated that these proteins contribute to the emergence of resistance, indicated by overexpression of these genes after treatment.

The second part of the study evaluated the activity of 15 furoxan and benzofuroxan derivative compounds for inhibitory capacity against the *M. tuberculosis*, cytotoxicity in VERO cells, as well as ethidium bromide accumulation and efflux assays. Aiming evaluate their mechanism of action, microarray and assays of nitric oxide release were also done, as they are supposed nitric oxide donors and may present an action by oxidative stress and/or the activity efflux pumps inhibition. Results indicated that the class of compounds is promising for the treatment, with good inhibitory activity against bacillus and substantial degree of selectivity. Mechanism of action assays indicated activity independent of efflux inhibition, of isoniazid subunit' or nitric oxide release. Microarray pointed to changes in expression of ribosomal proteins, similar to what occurs with aminoglycosides.

Finally, the third part of the study demonstrated that the halotolerance developed by *M. tuberculosis* might cause the emergence of drug resistant bacilli, based on the different concentrations of sodium chloride that the bacillus faces during its cycle. The results indicate a need for reflection on the new guidelines for testing new TB drugs. The results pointed to an increase in the minimal inhibitory concentrations of various anti-TB drugs, when the bacillus was subjected to the sodium chloride concentration found in macrophages, indicating a need for reflection on the guidelines for discovery of new anti-TB drugs.

## LISTA DE FIGURAS

<b>Figura 1:</b> Mecanismos de ação de moléculas inibidoras de efluxo.....	24
<b>Figura 2:</b> Estruturas dos anéis heterocíclicos de furazanos e furoxanos. ....	26
<b>Figura 3:</b> Diferentes concentrações de NaCl nos ambientes frequentados pelo <i>M. tuberculosis</i> .....	33
<b>Figura 4:</b> Esquema geral do planejamento para a síntese dos compostos derivados furoxânicos. ....	37
<b>Figura 5:</b> Esquema do ensaio de REMA .....	46
<b>Figura 6:</b> Curvas-padrão demonstrando eficiência de amplificação dos genes pesquisados. ....	49
<b>Figura 7:</b> Expressão de genes de efluxo para isolado clínico CF76.....	56
<b>Figura 8:</b> Expressão de genes de efluxo para isolado clínico CF78.....	57
<b>Figura 9:</b> Expressão de genes de efluxo para isolado clínico CF85.....	58
<b>Figura 10:</b> Expressão de genes de efluxo para isolado clínico CF93.....	59
<b>Figura 11:</b> Expressão de genes de efluxo para isolado clínico CF102.....	60
<b>Figura 12:</b> Expressão de genes de efluxo para isolado clínico CF104.....	61
<b>Figura 13:</b> Expressão de genes de efluxo para isolado clínico CF110.....	62
<b>Figura 14:</b> Expressão de genes de efluxo para isolado clínico CF114.....	63
<b>Figura 15:</b> Expressão de genes de efluxo para isolado clínico CF145.....	64
<b>Figura 16:</b> Expressão de genes de efluxo para isolado clínico CF158.....	65
<b>Figura 17:</b> Expressão de genes de efluxo para isolado clínico CF168.....	66
<b>Figura 18:</b> Expressão de genes de efluxo para isolado clínico CF185.....	67
<b>Figura 19:</b> Expressão de genes de efluxo para <i>M. tuberculosis</i> H <sub>37</sub> Rv .....	68
<b>Figura 20:</b> Ensaio de acúmulo de EtBr.....	73
<b>Figura 21:</b> Ensaio de efluxo de EtBr.....	74
<b>Figura 22:</b> Liberação de NO pelos derivados furoxânicos .....	77
<b>Figura 23:</b> Diagrama representando genes diferencialmente expressos após tratamento com compostos furoxânicos.....	78
<b>Figura 24:</b> Ilustração das proteínas ribossomais alteradas pelo tratamento com PJ10 e PJ21. ....	79
<b>Figura 25:</b> Heatmap mostrando alteração no número de transcritos a partir de genes de proteínas ribossomais. ....	82
<b>Figura 26:</b> CIM <sub>90</sub> dos fármacos testados nas diferentes concentrações de NaCl ....	84

<b>Figura 27:</b> Efeito da concentração de NaCl no meio de cultura para a contagem de UFC após teste de susceptibilidade à canamicina. ....	86
<b>Figura 28:</b> Experimento para avaliação de resistência fenotípica ou seleção de mutantes resistentes ao stress osmótico.....	89
<b>Figura 29:</b> CIM90 de gatifloxacina e canamicina perante às diferentes condições de adaptação ao stress osmótico.....	90

## LISTA DE TABELAS

<b>Tabela 1:</b> Exemplo de esquemas terapêuticos utilizados para TB .....	17
<b>Tabela 2:</b> Perfil dos isolados clínicos utilizados no trabalho .....	36
<b>Tabela 3:</b> Características dos compostos sintetizados e avaliados .....	38
<b>Tabela 4:</b> Oligonucleotídeos utilizados na RT-qPCR.....	42
<b>Tabela 5:</b> Eficiências de amplificação e dados de padronização dos <i>primers</i> .....	49
<b>Tabela 6:</b> Expressão de genes de efluxo para isolado clínico CF76 .....	56
<b>Tabela 7:</b> Expressão de genes de efluxo para isolado clínico CF78.....	57
<b>Tabela 8:</b> Expressão de genes de efluxo para isolado clínico CF85 .....	58
<b>Tabela 9:</b> Expressão de genes de efluxo para isolado clínico CF93 .....	59
<b>Tabela 10:</b> Expressão de genes de efluxo para isolado clínico CF102 .....	60
<b>Tabela 11:</b> Expressão de genes de efluxo para isolado clínico CF104 .....	61
<b>Tabela 12:</b> Expressão de genes de efluxo para isolado clínico CF110 .....	62
<b>Tabela 13:</b> Expressão de genes de efluxo para isolado clínico CF114 .....	63
<b>Tabela 14:</b> Expressão de genes de efluxo para isolado clínico CF145 .....	64
<b>Tabela 15:</b> Expressão de genes de efluxo para isolado clínico CF158 .....	65
<b>Tabela 16:</b> Expressão de genes de efluxo para isolado clínico CF168 .....	66
<b>Tabela 17:</b> Expressão de genes de efluxo para isolado clínico CF185 .....	67
<b>Tabela 18:</b> Expressão de genes de efluxo para <i>M. tuberculosis</i> H37Rv.....	68
<b>Tabela 19:</b> Resultados de atividade e citotoxicidade dos derivados furoxânicos utilizando a cepa-padrão <i>M. tuberculosis</i> H <sub>37</sub> Rv ATCC 27294 e células VERO.....	69
<b>Tabela 20:</b> Resultados de atividade dos derivados furoxânicos em 5 isolados clínicos de <i>M. tuberculosis</i> . .....	70
<b>Tabela 21:</b> Resultados de atividade dos derivados furoxânicos em <i>M. smegmatis</i> <i>mc</i> <sup>2</sup> <i>155</i> .....	73
<b>Tabela 22:</b> CIM <sub>90</sub> dos fármacos avaliados em 5 isolados clínicos com diferentes concentrações de NaCl no meio de cultura.....	87

## LISTA DE ABREVIATURAS

ASB - Albumina de Soro Bovino  
BAAR - Bacilos álcool-ácido resistentes  
CIM - Concentração Inibitória Mínima  
CLF - Clofazimina  
CS - Cicloserina  
DMSO - Dimetil-sulfóxido  
EMB - Etambutol  
EST - Estreptomicina  
ETM - Etionamida  
GEN - Gentamicina  
INH – Isoniazida  
KAN - Canamicina  
LORA - *Low Oxygen Recovery Assay*  
MET - Metronidazol  
MOX - Moxifloxacino  
MTB - *Mycobacterium tuberculosis*  
OADC – *Oleic, Albumin, Dextrose, Catalase enrichment*  
PAS - Ácido para-aminossalicílico  
PBS - Solução salina tamponada com fosfato à 1% em pH ≈ 7,0  
PZA - Pirazinamida  
RIF - Rifampicina  
SFB - Soro Fetal Bovino  
TB - Tuberculose  
TB-MDR - TB Multi-Droga-Resistente  
TB-XDR - TB Extensivamente-Droga-Resistente  
REMA- *Resazurin Microtiter Assay*

## SUMÁRIO

<b>RESUMO .....</b>	<b>5</b>
<b>ABSTRACT.....</b>	<b>7</b>
<b>LISTA DE FIGURAS .....</b>	<b>8</b>
<b>LISTA DE TABELAS .....</b>	<b>10</b>
<b>LISTA DE ABREVIATURAS.....</b>	<b>11</b>
<b>CAPÍTULO I.....</b>	<b>14</b>
<b>1.Introdução.....</b>	<b>15</b>
1.1.Contexto Geral e Justificativa.....	15
1.2.Resistência e a participação de bombas de efluxo no processo de resistência.....	19
1.3.Compostos derivados furoxânicos e benzofuroxânicos.....	25
1.4.Halotolerância em Mycobacterium tuberculosis e sua direta implicação na resistência à fármacos.....	30
<b>2. Objetivos.....</b>	<b>34</b>
2.1. Objetivos gerais.....	34
2.2. Objetivos específicos.....	34
<b>3. Material e Métodos .....</b>	<b>35</b>
3.1. Material.....	35
3.1.1. Isolados clínicos e linhagens ATCC.....	35
3.1.2. Compostos sintéticos inéditos .....	37
3.2. Métodos .....	40
3.2.1. Preparo das suspensões de isolados clínicos e da cepa-padrão H <sub>37</sub> Rv de <i>M. tuberculosis</i> .....	40
3.2.2. Tratamento, extração e purificação de RNA.....	40
3.2.3. Transcrição reversa e RT-qPCR.....	41
3.2.4. Ensaio de acumulação e efluxo de brometo de etídio.....	43
3.2.4.1. Determinação da concentração ideal de brometo de etídio.....	43
3.2.4.2. Ensaio de acumulação de brometo de etídio.....	43
3.2.4.3. Ensaio de efluxo de brometo de etídio.....	44
3.2.5. Determinação da Atividade anti- <i>M. tuberculosis in vitro</i> dos Compostos derivados de furoxânicos, dos fármacos padrão e do brometo de etídio frente aos isolados clínicos, ao <i>M. tuberculosis</i> H37Rv e ao <i>M. smegmatis</i> mc <sup>2</sup> 155 (ATCC 700084) .....	45
3.2.6. Ensaio de liberação de NO (óxido nítrico) pelos compostos derivados furoxânicos e benzofuroxânicos .....	46
3.2.7. Ensaio de <i>Microarray</i> .....	47
3.2.7.1. Síntese de cDNA marcado com Cy3 e Cy5.....	47

3.2.7.2. Purificação dos cDNAs marcados com Cy3 e Cy5.....	47
3.2.7.3. Hibridização.....	48
<b>4. Resultados e Discussão .....</b>	<b>48</b>
4.1. Ensaio de RT-qPCR e avaliação da expressão de bombas de efluxo em isolados clínicos frente ao tratamento com RIF .....	48
4.2. Compostos derivados furoxânicos e benzofuroxânicos.....	69
4.2.1. Atividade contra cepa-padrão (H37Rv), isolados clínicos resistentes e citotoxicidade em células VERO.....	69
4.2.2. Ensaio de acumulação e efluxo de brometo de etídio na presença dos derivados furoxânicos em <i>M. smegmatis</i> mc <sup>2</sup> 155 .....	72
4.2.3. Ensaio de liberação de NO pelos compostos derivados furoxânicos.....	75
4.2.4. Ensaio de <i>microarray</i> .....	78
4.3. Halotolerância em <i>Mycobacterium tuberculosis</i> .....	83
<b>5. Conclusões .....</b>	<b>91</b>
<b>6. Referências bibliográficas .....</b>	<b>93</b>
<b>CAPÍTULO II .....</b>	<b>106</b>
<b>Artigos e trabalhos publicados durante o período de Doutorado .</b>	<b>107</b>

# **CAPÍTULO I**

## 1.Introdução

### 1.1.Contexto Geral e Justificativa

Segundo o último relatório da Organização Mundial de Saúde (OMS), no ano de 2014 foram registrados 9,6 milhões de novos casos de Tuberculose (TB), doença infecciosa cujo principal agente é o bacilo *Mycobacterium tuberculosis*, com 1,5 milhão de óbitos (World and Health Organization, 2015). Esses dados apresentados demonstram um cenário de total fragilidade nas políticas de saúde pública ao redor do mundo, considerando que com uma combinação de medidas corretas adotadas para a terapia e ferramentas de diagnóstico mais precisas poderiam praticamente curar todas as pessoas com TB.

Apesar da evidente melhora nas estatísticas de tratamento, com 56 milhões de casos tratados com sucesso entre 1995 e 2011 e uma queda de 45% no índice de mortalidade entre os anos 1990 e 2012 (World and Health Organization, 2014b), alguns fatores contribuem para a elevada prevalência de TB. A co-infecção de *M. tuberculosis* e HIV (“Human Immunodeficiency Virus”) foi responsável pela morte de 400 mil pessoas em 2014, número este considerado inaceitável pela OMS, levando-se em consideração o fato de que muitos desses casos eram passíveis de prevenção (World and Health Organization, 2015). Outro elemento, ainda mais preocupante, é o surgimento de linhagens do bacilo da TB resistentes aos fármacos disponíveis no mercado para a terapia, esgotando ou dificultando as possibilidades de tratamento do paciente infectado, e permitindo assim que a doença se alastre.

A transmissão de TB ocorre pessoa-a-pessoa através de gotículas (aerossóis) contendo o bacilo, expelidas por indivíduos com doença na forma ativa ao tossir, falar ou espirrar. Essas gotículas são então inaladas por indivíduos saudáveis, podendo ou não se estabelecer a infecção (Jereb *et al.*, 2003). Aproximadamente 10 a 30% dos expostos adoecem (desenvolvendo a chamada TB primária), e 60% a 90% apresentam resposta imune celular capaz de conter a infecção; sendo que nos últimos a TB pode existir na sua forma latente (Ward, Hoye e Talaat, 2008) (Manabe e Bishai, 2000). O processo de TB primária ocorre quando macrófagos alveolares fagocitam os bacilos, mas são incapazes de eliminá-los, os quais permanecem viáveis dentro das pequenas lesões granulomatosas pulmonares, mesmo após a ativação de células T específicas, permitindo sua disseminação (Kaufmann, 2001). Já o estado latente é definido como a presença do patógeno no interior dos macrófagos (devido à

fagocitose), o que induz uma resposta pró-inflamatória e o início do recrutamento de outros macrófagos (para a formação de células gigantes multinucleadas), linfócitos T, plasmócitos e até mesmo fibroblastos, para a formação do chamado “granuloma”. Esse complexo é o responsável por conter a bactéria que permanece em um estado não-replicativo (“dormente”), considerado não infeccioso e que pode permanecer assim por anos. No entanto, razões como imunodeficiência (geralmente atrelada ao HIV), deficiência nutricional, idade, consumo de exagerado de álcool, entre outros, podem levar esses bacilos à sofrerem uma reativação, tornando-se infecciosos, o que resultaria então no desenvolvimento da forma ativa da TB no indivíduo (Russell, 2007).

A complexidade da doença em aspectos como ciclo e interação com o hospedeiro podem gerar fronteiras de difícil transposição à terapia. O tratamento padrão para TB, sem considerar as particularidades “caso-a-caso” (tais como infecção por cepas resistentes, imunodeficiência, idade do paciente, etc), é feito através de duas etapas: uma fase intensiva e uma fase de continuação (manutenção). Durante a fase intensiva, com a duração de dois meses, os quatro fármacos de primeira-linha são utilizados: isoniazida (INH), rifampicina (RIF), etambutol (EMB) e pirazinamida (PZA). Ao término desse período deve-se iniciar a fase de continuação, com a duração de 4 meses, e a administração de INH e RIF, com o objetivo de reduzir a chance de falhas no tratamento e a possibilidade de recorrência da doença (World Health Organization, 2010) (Balganesh, Alzari e Cole, 2008). Quando casos de resistência ou não responsividade ao tratamento são encontrados, a complexidade do mesmo é alterada consideravelmente, com um aumento na duração da terapia e a introdução de outros fármacos. Nesses casos, cabe ao responsável pelo tratamento a escolha entre adotar um regime padrão (com base nos perfis de resistência já encontrados na região do paciente) ou um regime individualizado (considerando as particularidades do paciente e principalmente baseando-se em testes de sensibilidade à fármacos do bacilo isolado do paciente). Independentemente da decisão tomada, o tratamento deve-se prolongar por uma média de 20 meses, com um aumento da etapa intensiva para até 8 meses e da etapa de manutenção para até 12 meses. A exigência por fármacos capazes de eliminar efetivamente as bactérias resistentes com diferentes mecanismos de ação, leva à adição quase obrigatória nesses casos de uma fluoroquinolona, um fármaco injetável (aminoglicosídeos), etionamida e D-cicloserina ao regime terapêutico (World and Health Organization, 2014a). A **tabela 1** a seguir evidencia a diferença em

complexidade de esquemas terapêuticos propostos para o tratamento de TB causada por bacilo sensível e TB causada por bacilo resistente, de acordo com as nomenclaturas propostas pela OMS.

**Tabela 1:** Exemplo de esquemas terapêuticos utilizados para TB

Tuberculose causada por:	Esquema terapêutico
Bacilo sensível aos principais fármacos do tratamento.	2RHEZ / 4RH
Bacilo resistente aos principais fármacos do tratamento (descoberta por Teste de Susceptibilidade à Fármacos ou não responsividade ao tratamento).	8Km <sup>6</sup> -Lfx <sup>7</sup> -Eto <sup>7</sup> -Cs <sup>7</sup> -Z <sup>7</sup> / 12Lfx <sup>7</sup> -Eto <sup>7</sup> -Cs <sup>7</sup> -Z <sup>7</sup>

**Tabela 1.** Exemplo do esquema terapêutico adotado para pacientes que desenvolveram a doença infectados por bacilo sensível ou bacilo resistente aos principais fármacos do tratamento. Números indicados antes da sigla indicam por quantos meses o esquema de tratamento deverá ser adotado. Números sobrescritos indicam por quantos dias da semana o fármaco deverá ser utilizado. O símbolo de barra (/) indica a separação entre as fases intensiva e de manutenção do tratamento. Legenda: R (rifampicina), H (isoniazida), E (etambutol), Z (pirazinamida), Km (canamicina), Lfx (levofloxacina), Eto (etionamida), Cs (D-cicloserina).

Resistência em TB é diferenciada de acordo com a capacidade de cada cepa de evadir-se do tratamento, possibilitando assim uma subclassificação em 4 grupos: cepas monorresistentes (que apresentam resistência à apenas um dos fármacos da terapia), cepas MDR-TB (“*multidrug-resistant*”, que são resistentes a pelo menos INH e RIF), XDR-TB (“*extensively drug-resistant*”, que além de resistentes à INH e RIF apresentam resistência às fluoroquinolonas e pelo menos a um dos fármacos injetáveis que são: amicacina, capreomicina e canamicina) e TDR-TB (“*totally drug-resistant*”, que como o próprio nome diz seriam resistentes a todos os fármacos já testados para TB, porém o termo ainda é novo e necessita de maiores estudos para ser aceito) (World and Health Organization, 2014b). De qualquer forma, e mesmo com algumas dúvidas pairando sobre a denominação, a presença de bacilos TDR-TB (pela primeira vez reportados por (Velayati *et al.*, 2009) no Irã, mas também já encontrados na Itália (Migliori *et al.*), Índia (Udwadia *et al.*, 2012) e África do Sul (Klopper *et al.*,

2013)) alertam para uma situação de extrema gravidade, onde é possível observar que a capacidade de adaptação da bactéria superou os esforços de indústrias e profissionais em desenvolver novos fármacos que pudessem efetivamente erradicar o *M. tuberculosis*.

Vários podem ser os mecanismos responsáveis pela resistência demonstrada pelo bacilo, tais como: (1) parede celular altamente hidrofóbica, com maciça presença de ácidos micólicos; (2) ação de bombas de efluxo (Da Silva *et al.*, 2011; Banerjee *et al.*, 1996; Singh *et al.*, 2011); (3) ocorrência de mutações cromossômicas em genes que codificam para proteínas que são alvos dos principais fármacos de tratamento ou para proteínas responsáveis pela ativação de pró-fármacos (Green e Garneau-Tsodikova, 2013). Para inibir a dispersão da doença, a resistência a fármacos deve ser rapidamente superada de uma maneira segura. Para isso, torna-se necessário minar esses mecanismos usados pelo bacilo, através da descoberta de novos fármacos capazes de agir em novos alvos ou em alvos já conhecidos, porém em outros sítios. Tais descobertas convergem para a necessidade de se elaborar novas terapias que possam atingir o maior número possível de vias celulares, de modo a inibir completamente o metabolismo bacteriano (Green e Garneau-Tsodikova, 2013).

Apesar da existência no momento de 19 moléculas em etapas pré-clínicas e clínicas como candidatas ao tratamento de TB (Working Group on New TB Drugs, 2014), desde o descobrimento da RIF em 1963, pelo *Dow-Lepetit Research Laboratories*, em Milão, pouco avanço foi realizado nesse sentido (Sensi, 1983). Somente no final de 2012 que, o FDA (*Food and Drug Administration*), pressionado pela necessidade de melhorias no tratamento da doença (principalmente com relação à resistência), acelerou a aprovação e garantiu licença provisória a um novo fármaco, a bedaquilina (TMC207, Sirturo™). Tal fato marca potencialmente o início de uma nova era na descoberta de novas moléculas com atividade antimicobacteriana, apontando para uma maior eficiência de compostos capazes de agir em novos alvos presentes na bactéria. Bedaquilina é uma representante da classe das diarilquinolinas, assim como o SQ109, que inibe a subunidade F0 da cadeia de transferência de prótons da ATP sintase, colocando em colapso todo o sistema de energia do bacilo (Andries *et al.*, 2005; Rivers e Mancera, 2008). Além dos novos compostos, outros fármacos inicialmente desenvolvidos para outras finalidades estão sendo repropostos para TB, incluindo oxazolidinonas, clofazimina e  $\beta$ -lactâmicos (Wong, Cohen e Bishai, 2013).

A pesquisa das atividades biológicas de novos fármacos contra TB é uma das principais linhas de pesquisa do nosso Laboratório que, em parceria com laboratórios de química orgânica e inorgânica, nacionais e internacionais, vem obtendo compostos com atividade promissoras anti-TB (Oliveira *et al.*, 2013; Pavan *et al.*, 2011; Aguiar *et al.*, 2015). Em parceria com Laboratório de Pesquisa e Desenvolvimento de Fármacos (Lapdesf, FCFar – UNESP Araraquara) foram sintetizados 15 compostos inéditos derivados de furoxano e benzofuroxano para serem avaliados neste trabalho de tese, tendo como referência inúmeras citações bibliográficas indicando a capacidade destes compostos de interferir na atividade de transportadores e de bombas de efluxo, através da liberação de NO e nitração dos mesmos.

Considerando o panorama geral da doença, a necessidade de novos alvos terapêuticos contra o *Mycobacterium tuberculosis* e uma melhor compreensão dos mecanismos pelos quais o bacilo consegue se esquivar do tratamento, bem como de novos fármacos contra *M. tuberculosis* a presente tese foi desenvolvida em 3 frentes: (1) avaliar a importância e comportamento de bombas de efluxo em isolados clínicos tratados com RIF (fármaco considerado o mais importante da terapia); (2) avaliar derivados furoxânicos e benzofuroxânicos como compostos anti-TB e como possíveis inibidores de bombas de efluxos; e (3) compreensão do fenômeno de halotolerância em *Mycobacterium tuberculosis* e aumento dos níveis de resistência aos fármacos utilizados em condições de alta concentração de cloreto de sódio.

## **1.2. Resistência e a participação de bombas de efluxo no processo de resistência**

De uma maneira geral, resistência em *M. tuberculosis* é um processo extremamente favorecido, podendo ser dividida em dois grupos: adquirida e intrínseca. Uma possível explicação para o desenvolvimento desse fator de maneira tão natural, consiste no fato de que, tendo que se multiplicar em ambientes extremamente hostis durante a infecção, o bacilo evoluiu adquirindo uma alta capacidade de adaptação (Karunakaran e Davies, 2000; Gillespie, 2002).

Como discutido acima, a resistência pode ser adquirida por pressão seletiva, que ocorre por meio de mutações cromossômicas que afetam proteínas relacionadas à ativação de fármacos ou que são diretamente o alvo dos mesmos. Dessa forma, essas

alterações podem levar à perda de função da proteína (por exemplo, proteína ativadora de pró-fármaco), alteração física do alvo (especificamente do sítio de ligação do fármaco), além de inativação enzimática do fármaco utilizado na terapia (Green e Garneau-Tsodikova, 2013).

Com relação à diminuição da susceptibilidade do bacilo de maneira intrínseca, o processo pode ocorrer influenciado por dois fatores: (1) uma parede celular extremamente lipofílica, majoritariamente composta por ácidos micólicos e que diminui a permeabilidade de compostos, funcionando como uma barreira natural e (2) atividade aumentada de bombas de efluxo (Jarlier e Nikaido, 1994; De Rossi, Ainsa e Riccardi, 2006; Da Silva *et al.*, 2011).

Em média, 30% dos isolados resistentes à INH (INH<sup>r</sup>) e 5% dos isolados resistentes à RIF (RIF<sup>r</sup>) não apresentam mutações em genes relacionados a fármaco-resistência (Louw *et al.*, 2009), o que sugere que uma atividade aumentada de bombas de efluxo podem estar mediando este fenótipo (Spies *et al.*, 2008), como já foi reportado em muitas bactérias Gram-positivas e Gram-negativas (Kohler, Pechere e Plesiat, 1999).

Bombas de efluxo bacterianas estão distribuídas em 5 famílias, sendo que duas delas são as principais, por serem maiores e mais ancestrais: “*ATP-binding cassette superfamily*” (ABC) e “*Major Facilitator Superfamily*” (MFS). As outras três famílias são menores e de desenvolvimento mais recente: “*Small Multidrug Resistance*” (SMR), “*Resistance-Nodulation-cell Division family*” (RND) e “*Multidrug and Toxic Compounds Extrusion Family*” (MATE) (De Rossi, Ainsa e Riccardi, 2006).

“*ABC transporters*” são encontrados tanto em eucariotos quanto em procariotos, sendo responsáveis pelo transporte de várias moléculas em geral (íons, aminoácidos, peptídeos, antibióticos, polissacarídeos e proteínas), sendo dependentes da hidrólise de ATP (trifosfato de adenosina) para o seu funcionamento (Ames, 1993) (Braibant, Gilot e Content, 2000; Silva *et al.*, 2009). São compostos em geral, por dois domínios de membrana hidrofóbicos (MSDs – “*Membrane Spanning Domains*”) associados com dois domínios citoplasmáticos de ligação à nucleotídeos (NBDs – “*Nucleotide Binding Domains*”) (Braibant, Gilot e Content, 2000). Em procariotos, essa família possui além da função de exportadora, a função de importadora de moléculas, sendo que para esta última atividade é indispensável a presença da proteína ligante de substrato (SBP – “*Substrate Binding Protein*”), que se encontra no *Mycobacterium tuberculosis* associada à membrana citoplasmática por meio de uma cauda lipídica, de maneira

semelhante ao que ocorre nas bactérias Gram-positivas (Braibant, Gilot e Content, 2000). Algumas das bombas pertencentes à essa família já foram descritas como responsáveis pela diminuição da susceptibilidade de cepas MDR a determinados fármacos, como por exemplo DrrABC (Choudhuri *et al.*, 2002), Rv2686c-Rv2687c-Rv2688c (Pasca *et al.*, 2004), Rv0194 (Danilchanka, Mailaender e Niederweis, 2008), Rv1217c-Rv1218c (Pan *et al.*, 2012), estando relacionadas a resistência frente a EMB, fluoroquinolonas, estreptomicina (STR), INH e RIF.

“*MFS transporters*” realizam o fluxo, entre a célula micobacteriana e o meio, de açúcares simples, oligossacarídeos, inositóis, fármacos, aminoácidos, nucleosídeos, ésteres organofosfatados, metabólitos do ciclo de Krebs, além de uma variedade de ânions e cátions inorgânicos, utilizando a força motriz de prótons para o funcionamento (Silva *et al.*, 2009; De Rossi *et al.*, 2002). É um grupo de transportadores dividido em subfamílias de acordo com o substrato transportado, e possuem regiões conservadas, sugerindo que desempenham o papel estrutural vital ou funcional, além da interação com substrato. De acordo com dados provenientes de análises bioinformáticas, essas proteínas apresentam em sua maioria 12 segmentos transmembrana, podendo existir até 15, sendo que as porções C e N terminal encontram-se no citoplasma celular. Apesar das pequenas variações estruturais de acordo com as subfamílias, esses transportadores apresentam um padrão conformacional típico, conhecido como “*MFS fold*” (Yan, 2013; De Rossi *et al.*, 2002). A participação dessa família de bombas de efluxo na resistência de linhagens micobacterianas aos fármacos RIF, INH, STR, ofloxacina (OFL), fluoroquinolonas, EMB e etionamida (ETH) já foi reportada, sendo identificadas principalmente as bombas: Rv1258c (Ainsa *et al.*, 1998; Jiang, Xin *et al.*, 2008), Rv1410c (Silva, P. E. A. *et al.*, 2001; Jiang, Xin *et al.*, 2008), Rv1634 (De Rossi *et al.*, 2002), Rv2459 (Gupta, Anuj Kumar *et al.*, 2010) e Rv2846 (Wilson *et al.*, 1999).

O papel do efluxo na resistência micobacteriana foi primeiramente estudado em *Mycobacterium smegmatis*, quando Liu e colaboradores (Liu, Takiff e Nikaido, 1996) descobriram o primeiro sistema responsável por essa atividade no bacilo, o sistema LfrA. Nesse estudo, o gene *lfrA* de bactérias resistentes à quinolonas (*M. smegmatis* mc<sup>2</sup>-552) foi clonado e o plasmídeo resultante transformado em bactérias sensíveis, as quais passaram a apresentar fenótipo de resistência à quinolona após a superexpressão do gene. Esse estudo levou às investigações de outros fármacos,

resultando na descoberta do sistema TetV (De Rossi *et al.*, 1998), que conferia resistência à tetraciclina e do transportado Tap (Ainsa *et al.*, 1998), cuja superexpressão resulta em resistência à tetraciclina e aminoglicosídeos.

A importância das bombas de efluxo em mediar fenótipos de resistência aos antimicrobianos fica mais evidente quando comparamos o perfil de susceptibilidade/resistência de *M. smegmatis* com o de *M. tuberculosis* frente à PZA. A primeira bactéria é naturalmente resistente à PZA, embora apresente uma atividade de pirazinamidase normal. Tal discrepância foi completamente compreendida após o estudo de Zhang e colaboradores (Zhang *et al.*, 1999), no qual foi constatado que o ácido pirazinóico (metabólito ativo da PZA) era eficientemente expelido do microrganismo, e que tal expulsão poderia ser evitada com a adição de um composto inibidor de bombas de efluxo (EPI – “*Efflux Pump Inhibitor*”), no caso a reserpina.

A alteração/indução da expressão de bombas de efluxo foi recentemente proposta como um evento inicial no surgimento da resistência (Srivastava *et al.*, 2010; Schmalstieg *et al.*, 2012). Num experimento utilizando doses dinâmicas de EMB (ou seja, através do sistema de “*hollow fiber*”, o ambiente era realimentado no decorrer do tempo, ao contrário de experimentos padrões que utilizam apenas doses estáticas do fármaco), Srivastava e colaboradores (Srivastava *et al.*, 2010) verificaram o aparecimento de bacilos resistentes em 14 dias de experimento. Este tempo é considerado curto para a aquisição de uma mutação cromossomal, além da possibilidade de bacilos pré-resistentes terem sido incluídos no ensaio ser muito pequena, uma vez que partiram de um inóculo inicial muito pouco concentrado e com quantidade diminuída de bactérias. Demonstraram também a existência de uma co-tolerância à INH, reforçando a hipótese de indução da expressão de carreadores comuns entre os dois antimicrobianos. No mesmo estudo, a adição de reserpina ao ensaio diminuiu em 64 vezes a frequência de mutação quando comparado ao controle que continha apenas EMB, fato este que se apresenta como mais uma evidência da ação do efluxo como fator inicial no processo de tolerância bacteriana frente a antimicrobianos. O mesmo resultado foi obtido por Schmalstieg e colaboradores trabalhando com o modelo de *Mycobacterium avium* subsp. *hominissius* (Schmalstieg *et al.*, 2012), no qual foi demonstrado a emergência de uma subpopulação resistente após o décimo dia de experimento com o tratamento de azitromicina, população essa que foi incapaz de multiplicar-se na presença de um inibidor de bomba de efluxo

(tioridazina). Além disso, resultados de qPCR indicaram um aumento na expressão de proteínas de efluxo pertencentes às famílias ABC e MFS (proteínas MAV\_3306 e MAV\_1406), chegando à uma variação de 56 vezes na expressão para uma delas. Tal modelo permitiu uma extrapolação da realidade para outras espécies de micobactérias e indicou a resposta imediata do efluxo mediando resistência.

Diante do exposto, dois aspectos contrastantes surgem no contexto e devem ser considerados: embora bombas de efluxo representem verdadeiras barreiras na descoberta de novos fármacos anti-TB, elas acabam surgindo também como potenciais alvos terapêuticos, reforçando a idéia da necessidade de busca por moléculas que possam inibi-las, permitindo a retenção de outros fármacos no interior do bacilo, o que aumentaria o tempo de vida intracelular, bem como a eficácia terapêutica dos mesmos.

Inibição de bomba de efluxo pode ocorrer seguindo diversos mecanismos, sendo os principais: (1) interferência em etapas regulatórias da expressão de bombas de efluxo, (2) mudanças na estrutura química do antimicrobiano, impedindo sua fixação na bomba como substrato específico, (3) interferência na formação da estrutura física dos componentes da bomba de efluxo, (4) inibição da ligação do substrato (antimicrobiano) por mecanismos competitivos ou não competitivos através do uso de outros compostos, (5) bloqueio dos poros da bomba pelos quais ocorrem o efluxo e (6) interferência na disponibilidade da energia requerida para o correto funcionamento da bomba (Askoura *et al.*, 2011). Um esquema desses principais mecanismos pode ser visto na **Figura 1**.

**Figura 1:** Mecanismos de ação de moléculas inibidoras de efluxo

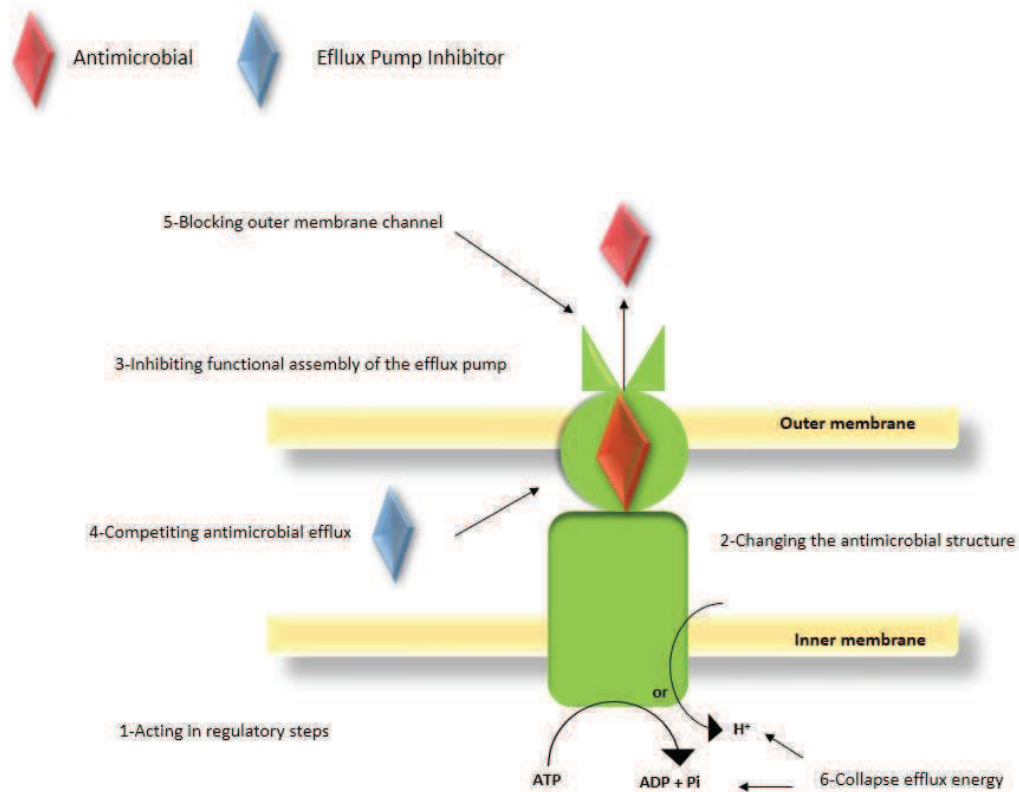


Figura 1: Possíveis mecanismos de ação de moléculas EPIs. Adaptada de Askoura et al. (Askoura et al., 2011).

Ao longo dos anos, muitos EPIs têm sido identificados: por exemplo, reserpina, que aumenta a sensibilidade de *Bacillus subtilis* a compostos tóxicos (Neyfakh, Bidnenko e Chen, 1991), e em *M. tuberculosis* (como citado acima) (Zhang *et al.*, 1999), esse composto aumenta a acumulação de ácido pirazinóico, aumentando consequentemente a sensibilidade à PZA. Verapamil funciona também de uma maneira similar, bloqueando *ABC transporters* e aumentando a susceptibilidade à OFL de bacilos resistentes à quinolonas (Singh *et al.*, 2011). O efluxo pode ser inibido de outras maneiras por moléculas como a clorpromazina e a tioridazina, que diminuem a resistência de *Staphylococcus aureus* à meticilina (Kristiansen *et al.*, 2006) e aumentam a susceptibilidade de *M. avium* ATCC25291 e *M. smegmatis* mc<sup>2</sup>155 à eritromicina (Rodrigues *et al.*, 2008). CCCP (Carbonilcianeto m-clorofenil-hidrazona), fármaco que desestabiliza o gradiente de prótons, interferindo com a produção de ATP (essencial ao efluxo), aumenta a sensibilidade de *M. avium* ATCC25291 à amicacina, EMB e tioridazina (Rodrigues *et al.*, 2008). Há também outros registros de verapamil,

CCCP e reserpina diminuindo a concentração inibitória mínima de tetraciclina e STR em *M. smegmatis* mc<sup>2</sup>155 (Silva, P. E. *et al.*, 2001).

Estudos mais recentes demonstraram também a eficácia da introdução de verapamil em um possível regime de tratamento de cepas clínicas com bedaquilina (sendo que estas apresentavam diferentes padrões de resistência a fármacos de primeira-linha). Em um desses trabalhos, o EPI foi capaz de diminuir de 8 a 16 vezes a CIM da bedaquilina após sua introdução no ensaio (Gupta *et al.*, 2013). A efetividade do verapamil foi reportada pelo mesmo grupo *in vivo*, demonstrando uma diferença de até 1,06 log na contagem de UFC (Unidades formadoras de colônias) entre os grupo tratado com bedaquilina e o grupo tratado com bedaquilina na presença de verapamil (Gupta *et al.*, 2014).

Utilizando uma gama maior de conhecidos EPIs (verapamil, tioridazina e clorpromazina), Coelho e seus colaboradores (Coelho *et al.*, 2015) foram capazes de demonstrar, de maneira geral, uma redução nas concentrações inibitórias mínimas de rifampicina, isoniazida, amicacina e ofloxacina em isolados clínicos MDR-TB e na cepa padrão H<sub>37</sub>Rv (sensível), quando na presença dos compostos EPIs. Além dessas mudanças realmente significativas (redução de até 64 vezes da concentração do fármaco quando na presença do EPI), foram apresentadas evidências de que bacilos MDR-TB podem apresentar uma maior quantidade de bombas de efluxo que bacilos pan suscetíveis.

Esses resultados corroboram cada vez mais com a participação de bombas de efluxo mediando a resistência do bacilo, mas por outro lado acabam por sugerir-las como um excelente alvo para o tratamento, fornecendo esperança na problemática mundial da TB.

### **1.3. Compostos derivados furoxânicos e benzofuroxânicos**

Compostos heterocíclicos aromáticos constituídos de um anel de cinco membros contendo um átomo de oxigênio e dois de nitrogênio são chamados de oxadiazóis (**Figura 2**). Derivados oxadiazóis, com um grupamento *N*-óxido, o 1,2,5-oxadiazol-2-*N*-óxido, são conhecidos como furoxanos.

**Figura 2:** Estruturas dos anéis heterocíclicos de furazanos e furoxanos.

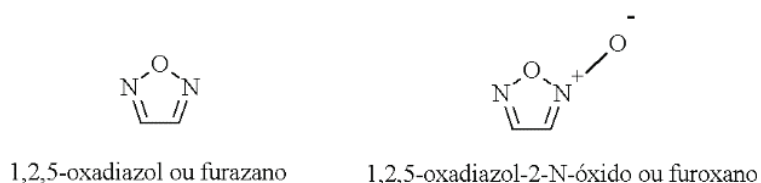


Figura 2: Estruturas dos anéis heterocíclicos de furazanos e furoxanos.

Ações biológicas dos mais variados tipos vêm sendo reportadas para essas moléculas, grande parte devido à hipótese de doação de óxido nítrico (NO). A importância desses compostos e a estratégia de uso dos mesmos não representa nada mais do que uma tentativa de mimetizar as reações normais do corpo humano frente à infecções e presença de microrganismos patogênicos. O NO endógeno funciona como potente agente antimicrobiano, e juntamente com seus derivados reativos são liberados pelos macrófagos como arma de defesa contra invasores (Boiani *et al.*, 2008; Fang, 1997). Inúmeros são os trabalhos com evidências do aumento de NO no sítio de infecção em humanos e modelos experimentais de animais (Ochoa *et al.*, 1991; Nicholson *et al.*, 1996; Hassan *et al.*, 2002), explicado pela indução da isoforma de NO sintase (NOS2) por citocinas pró-inflamatórias, como IFN- $\gamma$ , TNF- $\alpha$ , IL-1, IL-2 e fatores de virulência bacterianos como por exemplo LPS (lipopolissacarídeos). Em situações críticas de infecção, a ausência ou insuficiência do funcionamento da NO-sintase podem gerar consequências drásticas para o indivíduo com um aumento desenfreado na replicação bacteriana ou parasitária (Stenger *et al.*, 1996; Macmicking *et al.*, 1997).

Uma demonstração clara das possibilidades de uso desses compostos foi vista no trabalho de Fruttero e colaboradores (Fruttero *et al.*, 2010), no qual a atividade de bombas de efluxo foi diretamente ligada à resistência de células cancerosas aos tratamentos com fármacos antineoplásicos (Fruttero *et al.*, 2010). Observou-se que o baixo suprimento de oxigênio às células cancerosas e a baixa produção de NO endógeno poderiam levar ao surgimento de linhagens MDR em células de câncer de cólon humano, e que a restauração da produção de NO seria capaz de reverter o processo (Riganti *et al.*, 2005). Nesse contexto surgiram os compostos furoxânicos, capazes de liberar NO sob a ação de grupamentos tióis presentes nas proteínas

celulares (Fruttero *et al.*, 2010). O mecanismo mais provável para tal liberação envolveria a interação da região eletrofílica da posição 3 do anel furoxânico com o grupamento –SH nucleofílico dos tióis, permitindo assim a abertura do anel e a liberação de NO (Fruttero *et al.*, 2010). Dessa forma, foi comprovada a hipótese de que compostos derivados de furoxano eram capazes de interferir na atividade de transportadores e de bombas de efluxo, através da liberação de NO e nitração dos mesmos (Fruttero *et al.*, 2010). A interação das moléculas de fenil-sulfonil-furoxânicos propostas no trabalho ocorreu com transportadores P-gp (“*P-glycoprotein*” ou ABC-B1) e MRP1-6 (“*multidrug resistance associated proteins*” ou ABC-C1-6) de células MDCK, restaurando grande parte da sensibilidade das mesmas à doxorrubicina (Fruttero *et al.*, 2010). Assim, os derivados furoxânicos têm sido sugeridos como inibidores de bombas de efluxo, apresentando resultados promissores como uma ferramenta terapêutica.

Resultados semelhantes foram encontrados por Zou e colaboradores (Zou *et al.*, 2011) após a síntese e avaliação da atividade anticâncer de tetrahidroisoquinolinas que traziam em suas estruturas um anel furoxânico. O trabalho utilizou células de leucemia mielóide humana K562, além de sua variante K562/A02 resistente à doxorrubicina. Os resultados apontaram uma atividade sinérgica entre doxorrubicina e os compostos, sendo que a associação com um dos derivados permitiu um aumento de potência de até 32 vezes da doxorrubicina na linhagem K562/A02. A inibição de transportadores nesse trabalho foi demonstrada através de um ensaio fluorimétrico com rodamina, demonstrando a acumulação da mesma no interior das células K562/A02 após o tratamento com os compostos derivados de furoxano e atingindo valores muito próximos do grupo controle positivo, o qual utilizou verapamil como composto inibidor de efluxo.

A utilização desses anéis aromáticos heterocíclicos também vem sendo estudada e explorada para o tratamento de parasitoses como a leishmaniose e tripanossomíase. Vários trabalhos apontam para a eficácia desses derivados frente às diferentes espécies de *Leishmania* e *Trypanosoma* responsáveis pelo aparecimento das doenças, tais como: *L. amazonensis* (Hernandez *et al.*, 2013; Dutra *et al.*, 2014), *L. braziliensis* (Boiani *et al.*, 2008) e *L. pifanoi* (Boiani *et al.*, 2008), além do *T. cruzi* (Hernandez *et al.*, 2013; Boiani *et al.*, 2008) para o caso da Doença de Chagas. No trabalho de Hernández e colaboradores (Hernandez *et al.*, 2013), a

característica determinante mostrada pelos compostos sintetizados e testados (híbridos de furoxano com N-acilhidrazonas) foi a seletividade, uma vez que apesar de apresentarem perfis de atividade um pouco inferiores à anfotericina B contra *L. amazonensis*, apresentaram uma seletividade superior ao fármaco controle, chegando a índices 50 vezes maiores, quando considerados os testes de citotoxicidade com macrófagos murinos. A atividade dos diferentes furoxânicos desse trabalho contra *Trypanosoma cruzi* foi avaliada frente às formas amastigotas e epimastigotas do parasita, apresentando em geral um perfil semelhante ao que foi observado para *Leishmania*, com atividade menor que o fármaco controle (nifurtimox) e seletividade aumentada quando testadas células de mamíferos como macrófagos murinos e células VERO (células epiteliais de rim do macaco *Cercopithecus aethiops*).

No estudo de Boiani e colaboradores (Boiani *et al.*, 2008), mais uma vez a atividade dos derivados furoxânicos contra os parasitas causadores de leishmaniose e tripanossomíase foi reforçada. Contando com uma biblioteca de compostos derivados de furoxano e nitratos de alquila, a estratégia dos autores foi uma tentativa de correlacionar a atividade antiparasitária com a capacidade de doação de óxido nítrico. De uma maneira geral, os compostos foram ainda mais ou equipotentes que os fármacos comumente utilizados na terapia contra o *T. cruzi*, nifurtimox e benzonidazol. A toxicidade demonstrada para *L. braziliensis* e *L. pifanoi* também atingiu bons índices, lembrando os resultados obtidos pelo estudo anteriormente citado. O trabalho foi um pouco além na tentativa de elucidação do mecanismo de ação, utilizando composto de furazano como controles. Foi identificado que a ausência ou substituição do grupo N-óxido levava à uma perda significativa de atividade, de pelo menos 10 vezes em alguns casos. Suportando o possível papel da liberação de óxido nítrico como principal mecanismo de ação dos compostos, aparecem os dados comparativos entre os derivados com grupamentos 3-sulfonil (mais ativos) e 4-sulfonil. A maior atividade do primeiro grupo pode ser diretamente relacionada com uma facilidade maior dos compostos sofrerem ação dos grupos tióis das proteínas, liberando NO mais facilmente devido a uma maior proximidade do grupamento substituinte com o grupo N-óxido.

Tratando-se de uma doença infecciosa causada por bactéria, a relação da TB com NO e seus derivados reativos não é diferente do que ocorre com os parasitas. Após a infecção por *M. tuberculosis*, as células de defesa estimulam a atividade da enzima

óxido nítrico sintase induzida (iNOS), sinalizadas e ativadas inicialmente pela presença de IFN- $\gamma$  (Jagannath, Actor e Hunter, 1998). Todo o curso da infecção, assim como o resultado final da mesma e o sucesso ou não da bactéria no ambiente, dependerá das relações bactéria-hospedeiro e capacidade do bacilo de se adaptar frente às investidas do sistema imunológico hospedeiro. O fato é que a resposta imunológica envolvida cria um ambiente de total *stress* óxido-redutivo para o *M. tuberculosis*, com a presença de espécies reativas de oxigênio (ROS), espécies reativas de nitrogênio (RNS), pH ácido e hipóxia. Voskuil e colaboradores (Voskuil *et al.*, 2003) demonstraram que o NO, como parte do processo de *stress* óxido-redutivo gerado pelo hospedeiro, seria um dos importantes fatores para determinar o estado replicativo ou não-replicativo (estado de latência) do bacilo, sendo de extrema importância para conter o avanço da doença. Além disso, a produção massiva de NO leva a danos em proteínas e lipídeos bacterianos, além de afetar diretamente os ácidos nucleicos por causar deaminação, gerar sítios abásicos e romper de fitas de DNA (ácido desoxirribonucleico) ou RNA (ácido ribonucleico) (Chan *et al.*, 1995). Outras interações do NO também podem ser maléficas à bactéria, tais como interações com grupos heme, grupamentos tióis, resíduos aromáticos e radicais tirosil, promovendo como resultado final uma inativação ou mau funcionamento proteico (Yang, Yuk e Jo, 2009; Gow, Thom e Ischiropoulos, 1998).

Nesse contexto, o trabalho acima já citado de Hernández e colaboradores (Hernandez *et al.*, 2013) também propôs a utilização de moléculas derivadas de furoxano para o combate ao *M. tuberculosis*, testando aproximadamente 20 compostos inéditos contra a cepa padrão H<sub>37</sub>Rv e um isolado clínico resistente à INH. Os resultados obtidos apontaram valores muito baixos para a Concentração Inibitória Mínima (CIM) para 3 compostos, indicando excelente atividade, além da capacidade de agir em isolados resistentes.

A compilação dos dados presentes na literatura indica um caminho promissor para os compostos derivados de furoxano e benzofuroxano para o tratamento da TB, com boas referências quanto a seus perfis de atividade e citotoxicidade, além da possibilidade de atuarem por um mecanismo duplo: inibição de proteínas responsáveis pela extrusão de fármacos (bombas de efluxo) e *stress* oxidativo.

#### **1.4. Halotolerância em *Mycobacterium tuberculosis* e sua direta implicação na resistência à fármacos**

Halotolerância é o nome dado à capacidade que um organismo tem de sobreviver e constantemente adaptar-se ao *stress* iônico, lançando mão de alterações fisiológicas que minimizem o impacto das flutuações na concentração salina do ambiente, ou sendo dependente de um mecanismo passivo que tenderá a sempre igualar o conteúdo iônico dos ambientes extra e intracelulares (Vreeland, 1987).

Evidências de osmoadação (fenômeno principal que leva à halotolerância) já foram estudadas em bactérias como *Escherichia coli* (Smirnova, Krasnykh e Oktyabrsky, 2001; Mclaggan *et al.*, 1990) e *Lactococcus lactis* (Zhang *et al.*, 2010). Para adaptar-se à condição de elevada osmolaridade, *E. coli* inicialmente acumula grandes quantidades de íons potássio (K<sup>+</sup>) e contra-íons glutamato, como uma forma de recuperação de seu turgor (Mclaggan *et al.*, 1990; Masip, Veeravalli e Georgiou, 2006). Tais alterações em uma primeira fase, podem ser desastrosas à bactéria, devido ao desbalanço eletrolítico criado, necessitando assim de uma segunda fase de adaptação, na qual é necessária a acumulação de componentes osmoprotetores, tais como: carnitina, betaína, prolina e glicina (Smirnova, Krasnykh e Oktyabrsky, 2001; Mclaggan *et al.*, 1990; Masip, Veeravalli e Georgiou, 2006). Outra alteração encontrada, e com certeza a mais importante do estudo, foi que após o choque osmótico, as quantidades de glutathione (GSH) foram aumentadas no interior da bactéria, e que mutantes incapazes de sintetizá-la (inserção de uma mutação no gene *gshA*, capaz de abolir a síntese de GSH) eram também prejudicados em sua taxa de multiplicação em meios de média osmolaridade. O papel exato da glutathione no mecanismo de adaptação à pressão osmótica continua por ser elucidado, porém acredita-se que está diretamente relacionado ao seu papel antioxidante, hipótese essa reforçada por outro trabalho (Smirnova, Muzyka e Oktyabrsky, 2000), no qual foi observada uma superexpressão dos genes *soxS* e *katE*, ambos com atividade antioxidante, após um aumento da osmolaridade. Uma indução de uma superóxido dismutase (SodA), no mesmo trabalho, aponta que os mecanismos de defesa e adaptação da bactéria frente a um *stress* osmótico podem ser muito semelhantes aos que ocorrem frente ao *stress* oxidativo (Masip, Veeravalli e Georgiou, 2006).

O trabalho com *L. lactis* (Zhang *et al.*, 2010) reforçou o aspecto osmoprotetor de glutathione, uma vez que o grupo cultivado na presença de GSH (o microrganismo

nesse caso não é capaz de sintetizá-la) apresentou melhores resultados de multiplicação em comparação ao grupo controle (sem a suplementação com GSH), quando desafiados com uma concentração de cloreto de sódio de 5M (NaCl). Todas essas evidências apontam para a capacidade dos microrganismos em regular sua complexa rede metabólica de uma forma a se beneficiar frente às adversidades que o meio pode impor.

Algumas particularidades experimentadas pelo *M. tuberculosis* ao longo de seu “ciclo”, desde o início da infecção até o estabelecimento da doença o tornam um microrganismo muito peculiar. Como citado anteriormente, uma vez no interior do corpo humano, o bacilo tem que sobreviver enfrentando as mais variadas adversidades, tais como: carência nutricional, acidez, hipóxia, ambientes com ROS e RNS (Ehrt e Schnappinger, 2009). Além desses desafios, o bacilo acaba por enfrentar também um *stress* osmótico, com a pressão exercida pelos eletrólitos do organismo hospedeiro e que irão com absoluta certeza influenciar no resultado final da infecção. Provavelmente, o principal responsável pelas alterações que ocorrem na bactéria devido ao *stress* osmótico é o cloreto de sódio (NaCl), cuja concentração varia drasticamente entre os ambientes que a bactéria frequenta, desde o ar até o interior do corpo humano (**Figura 3**) (Tan *et al.*, 2013; Zabner *et al.*, 1998; Bourque, 2008).

A importância de íons no microambiente do *M. tuberculosis* durante a infecção vem sendo discutida extensivamente (Tan *et al.*, 2013; Payne, 1993; Rengarajan, Bloom e Rubin, 2005; Sasseti e Rubin, 2003). Em um dos últimos trabalhos na área, foi descoberto que íons  $\text{Cl}^-$  não apenas definiam funções importantes relacionadas à osmolaridade no bacilo, como também representavam um fator sensorial importante, cujo aumento da concentração no interior do macrófago indica mudanças mediadas pelo sistema imunológico, como a acidificação do ambiente do fagossomo (Tan *et al.*, 2013). Dessa forma, o aumento da concentração de íons  $\text{H}^+$  no fagossomo requisitaria a entrada também de um contra-íon para atingir o equilíbrio, no caso o  $\text{Cl}^-$ . A partir de um aumento na concentração de  $\text{Cl}^-$ , o bacilo torna-se hábil para reagir bioquimicamente ao ambiente ao seu redor e desencadear mudanças necessárias à sua sobrevivência em um ambiente hostil.

Recentemente, Hatzios e colaboradores (Hatzios *et al.*, 2013) reportaram a descoberta de um sistema serina/treonina dependente de quinase em *M. tuberculosis*, que atua como um mensageiro, sendo responsável por sentir as condições osmóticas

do meio em que o bacilo se encontra, e a partir disso desencadear todas as respostas adequadas para a adaptação bacteriana. Na verdade, o modelo proposto no trabalho é a primeira descrição de um complexo híbrido de um sistema de dois componentes (TCS – *Two Component System*), muito encontrado em eucariotos, com um básico sistema regulatório transcricional típico de procaritos, que é mediado por fatores  $\sigma$ . De uma maneira básica, TCS são sistemas compostos por uma proteína associada à membrana da bactéria (que funciona como uma espécie de sensor com função quinase, ou seja, com capacidade para fosforilar posteriormente outra proteína) e uma proteína efetora, que regulará expressão gênica a partir de sua fosforilação pela proteína sensora. O proposto é que, após submetido a um *stress* osmótico, o *M. tuberculosis* percebe o ambiente hostil através de sua proteína de membrana PknD (sensor). A função quinase da proteína sensora é ativada, fosforilando o seu substrato Rv0516c (nomeada após o trabalho como OprA – *Osmosensory protein A*), que inicialmente atua como um fator anti- $\sigma$ , ou seja, inibindo o fator transcricional SigF. A fosforilação de OprA permite a liberação do fator SigF do complexo inibitório, permitindo sua ligação à RNA polimerase, recrutando-a para a região promotora e ativando a transcrição de genes relacionados com a remodelagem de peptidoglicanos, resposta a *stress* oxidativo e fatores de virulência. De uma maneira resumida, o *stress* osmótico leva à alteração de uma complexa rede metabólica do bacilo, resultando em mudanças que podem inclusive afetar a sensibilidade da bactéria aos fármacos do tratamento. No mesmo trabalho, os autores verificaram que um mutante com transposon integrado no gene *Rv0516c* (mimetizando a situação de *stress* osmótico, onde OprA é fosforilada e perde sua função de inibição de SigF) tornou-se mais resistente ao tratamento com alguns antimicrobianos como ampicilina, penicilina e vancomicina.

**Figura 3:** Diferentes concentrações de NaCl nos ambientes frequentados pelo *M. tuberculosis*

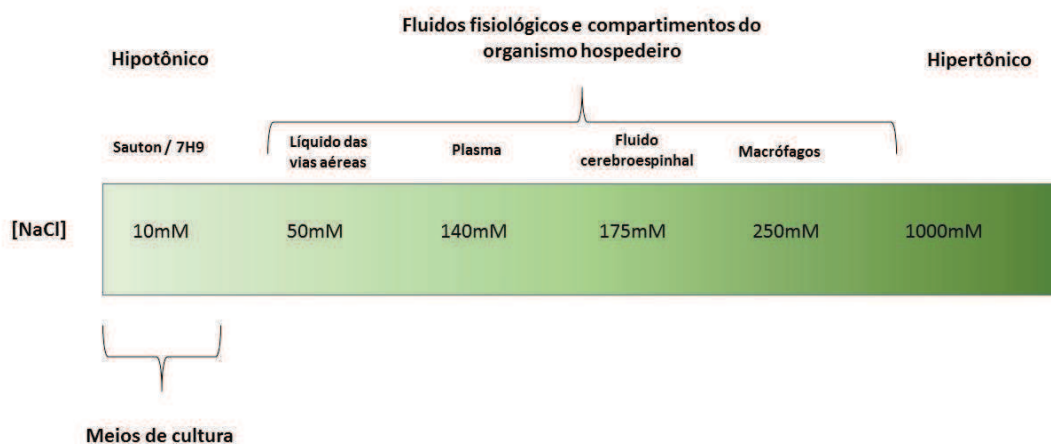


Figura 3: Diferentes concentrações de NaCl nos ambientes experimentados pelo *M. tuberculosis*. desde o ambiente externo (ar) até o interior do corpo humano. A figura também retrata a concentração de NaCl nos principais meios de cultura utilizados para o bacilo em laboratório. Figura adaptada do trabalho de Larrouy-Maumus e colaboradores, 2015 (em revisão para publicação).

Compilando os dados acima fica nítida a importância de se compreender quais as alterações sofridas pela bactéria após o *stress*, e as consequências dessas alterações, principalmente no que tange à sensibilidade do bacilo aos fármacos de tratamento. A preocupação e a necessidade de reflexão tornam-se ainda mais evidentes quando comparadas as concentrações de NaCl no interior do corpo humano (como por exemplo 250mM nos macrófagos) e nos meios de cultura utilizados para pesquisa científica (aproximadamente 10mM), inclusive para os testes de susceptibilidade à fármacos. O questionamento, a partir desses dados, seria se os pesquisadores estão testando seus fármacos *in vitro* na real bactéria que causa a TB, ou em uma bactéria muito mais fraca, que ainda não sofreu as alterações requisitadas pela alta salinidade do ambiente. Um engano como esse, na etapa do teste de susceptibilidade à fármacos, poderia levar à etapas subsequentes moléculas sem potencial terapêutico (“falso-positivas”), acarretando em desperdício de tempo no processo já tão burocrático de lançamento de novos fármacos no mercado.

Diante disso, o projeto da presente tese visou também avaliar a ação *in vitro* de fármacos já utilizados para a terapia da TB em condições mais próximas da realidade,

respeitando as condições de alta salinidade de alguns dos ambientes enfrentados pela bactéria no corpo humano. Os resultados ajudaram a compreender o comportamento fenotípico da bactéria, a influência do NaCl nos perfis de resistência/sensibilidade à fármacos, além de auxiliar na compreensão inicial das modificações do bacilo para adaptar-se ao *stress* osmótico.

## **2. Objetivos**

### **2.1. Objetivos gerais**

Determinação do papel de bombas de efluxo mediando resistência à RIF em isolados clínicos de *M. tuberculosis* selecionados da coleção de isolados clínicos do Laboratório de Micobacteriologia Prof. Dr. Hugo David, caracterização da atividade e mecanismo de ação dos compostos derivados de furoxano e benzofuroxano e influência da halotolerância nos perfis de resistência / susceptibilidade do *M. tuberculosis* aos fármacos utilizados na terapia anti-TB.

### **2.2. Objetivos específicos**

-Caracterização de isolados clínicos de *M. tuberculosis* quanto à expressão de bombas de efluxo relacionada aos seus perfis de resistência após o tratamento com RIF;

-Determinação da Concentração Inibitória Mínima (CIM) dos compostos derivados de furoxano e benzofuroxano frente aos isolados clínicos resistentes e sensíveis de nossa coleção;

-Avaliação do potencial de inibição de bombas de efluxo por compostos derivados de furoxano e benzofuroxano através de ensaio de acumulação e efluxo de brometo de etídio;

-Avaliação da liberação de NO no interior da bactéria pelos compostos derivados de furoxano e benzofuroxano, utilizando DAF-FM DA (diacetato de diaminofluoresceína) como repórter;

-Estudo do possível mecanismo de ação dos compostos furoxânicos e benzofuroxânicos baseado na resposta da bactéria por ensaio de *microarray*;

-Estudo inicial da influência da halotolerância (com base nas condições experimentadas pelo bacilo durante uma provável infecção) nos perfis de resistência / susceptibilidade do *M. tuberculosis* frente aos principais fármacos da terapia anti-TB.

### **3. Material e Métodos**

#### **3.1. Material**

##### **3.1.1. Isolados clínicos e linhagens ATCC**

Isolados clínicos de *M. tuberculosis* provenientes do Instituto Clemente Ferreira (centro nacional de referência no tratamento de TB localizado na cidade de São Paulo-SP) previamente caracterizados por nosso laboratório quanto à resistência fenotípica (determinado pela técnica do BACTEC™ MGIT™ 960) e genotípica (sequenciamento) frente a INH e RIF. Foram utilizados em todo o estudo (incluindo as 3 frentes abordadas na tese) 17 isolados clínicos, cujos perfis são apresentados na **tabela 2**.

As linhagens controle utilizadas nos ensaios foram *Mycobacterium tuberculosis* H<sub>37</sub>Rv (ATCC 27294) (ensaios envolvendo RT-qPCR e determinação de CIM) e *Mycobacterium smegmatis* mc<sup>2</sup>155 (ATCC 700084) (ensaios de acumulação de brometo de etídio).

**Tabela 2:** Perfil dos isolados clínicos utilizados no trabalho

Isolado Clínico	Perfil Fenotípico			Perfil Genotípico											
	CIM da RIF por REMA ( $\mu\text{g.mL}^{-1}$ )	BACTEC™ MGIT™ 960		INH									RIF		
		INH	RIF	<i>inhA</i>			<i>katG</i>			<i>ahpC</i>			<i>rpoB</i> *		
				Nucleotídeo	Códon	Aminoácido	Nucleotídeo	Códon	Aminoácido	Nucleotídeo	Códon	Aminoácido	Nucleotídeo	Códon	Aminoácido
CF46	1,00	R	R		n.e.		G > C	315	Ser > Thr		n.e.		CA > TG	526	His > Cys
CF76	>25,00	R	R		n.e.		G > C	315	Ser > Thr		n.e.		C > T	531	Ser > Leu
CF78	2,00	R	R		n.e.			n.e.			n.e.			n.e.	
CF81	6,25	R	R		n.e.		G > C	315	Ser > Thr		n.e.		A > T	516	Asp > Val
CF85	2,00	R	R		n.e.		G > C	315	Ser > Thr		n.e.			n.e.	
CF93	125,00	R	R		n.e.			n.e.			n.e.		C > T	531	Ser > Leu
CF97	25,00	R	R		n.e.			n.e.			n.e.		C > G	531	Ser > Trp
CF102	0,10	S	S		n.e.			n.e.			n.e.			n.e.	
CF104	25,00	R	R		n.e.		G > C	315	Ser > Thr		n.e.		C > T	531	Ser > Leu
CF110	25,00	R	R		n.e.			n.e.			n.e.			n.e.	
CF114	0,12	R	R	C > T	-15			n.e.			n.e.			n.e.	
CF145	25,00	R	R		n.e.		G > C	315	Ser > Thr		n.e.			n.e.	
CF152	25,00	R	R	C > T	-15			n.e.			n.e.		C > T	531	Ser > Leu
CF158	25,00	R	R		n.e.			n.e.			n.e.		C > T	531	Ser > Leu
CF165	25,00	R	R		n.e.		G > C	315	Ser > Thr		n.e.		C > T	531	Ser > Leu
CF168	25,00	R	S		n.e.		G > C	315	Ser > Thr		n.e.		C > T	531	Ser > Leu
CF185	25,00	R	R		n.e.		G > C	315	Ser > Thr		n.e.		C > T	531	Ser > Leu

**Tabela 2:** Perfil de resistência fenotípico (determinado por BACTEC™ MGIT™ 960) e genotípico (determinado por sequenciamento) dos isolados clínicos utilizados. O sequenciamento foi realizado nos principais genes relacionados à resistência à INH e RIF.

R: Resistente

S: Sensível

n.e.: não encontrada

\*Sequenciamento da região RRDR (*Rifampicin Resistance Determining Region*)

### 3.1.2. Compostos sintéticos inéditos

Foram sintetizados 15 compostos inéditos derivados de furoxano e benzofuroxano (**Tabela 3**) pelo Laboratório de Pesquisa e Desenvolvimento de Fármacos (Lapdesf, FCFar – UNESP Araraquara). O planejamento teve como uma das características inovadoras a síntese dos novos derivados a partir da condensação da INH com diferentes núcleos furoxânicos, racionalmente desenhados através da estratégia de hibridação molecular (**figura 4**), visando à obtenção de fármacos que pudessem ter atividade por diferentes mecanismos na bactéria. Para a realização da parte experimental os compostos foram solubilizados em dimetilsulfóxido (DMSO) para o armazenamento das soluções estoque à 10 mg/mL.

**Figura 4:** Esquema geral do planejamento para a síntese dos compostos derivados furoxânicos.

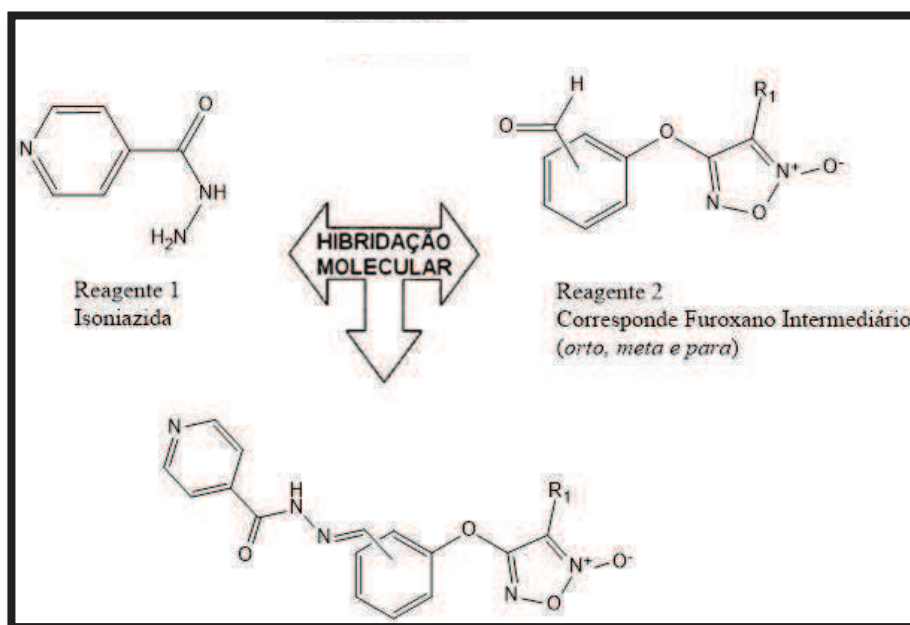
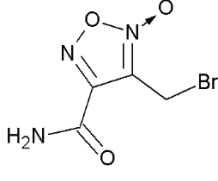
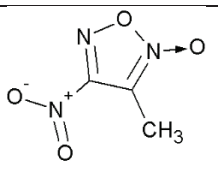
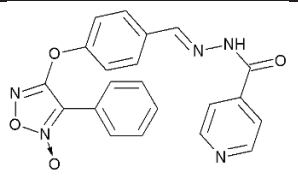
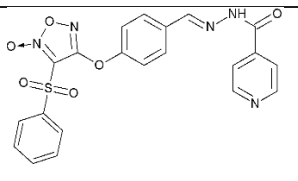
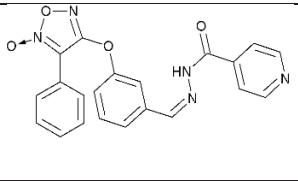
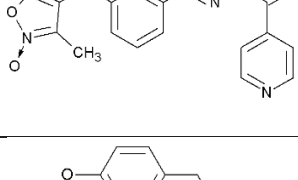
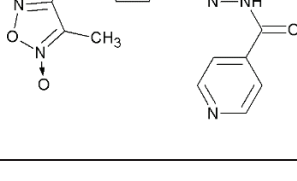
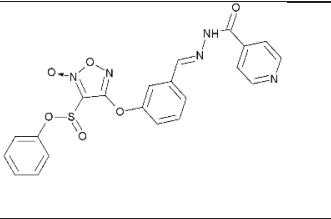
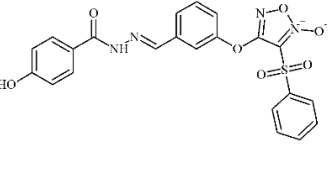
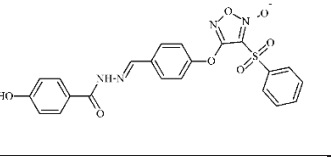
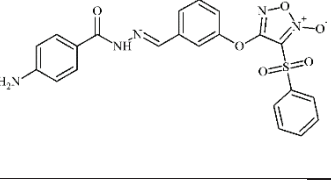
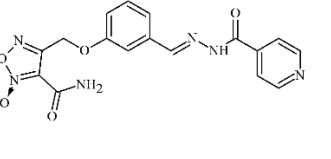
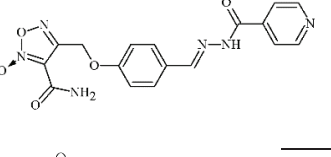
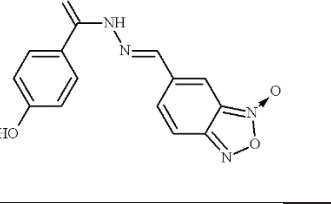
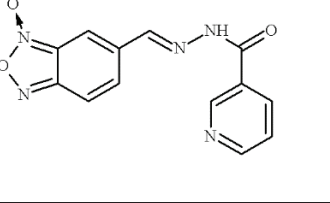


Figura 4: Esquema geral do planejamento para a síntese dos compostos derivados furoxânicos.

**Tabela 3:** Características dos compostos sintetizados e avaliados

Composto	Nome IUPAC	Sigla	Fórmula Molecular	Massa molar (g.mol <sup>-1</sup> )
	4-(bromometil)-3-carbamoil-1,2,5-oxadiazol 2-oxide	Lapdesf7 ou PJ7	C <sub>5</sub> H <sub>6</sub> BrN <sub>3</sub> O <sub>2</sub>	220,02
	3-metil-4-nitro-1,2,5-oxadiazol 2-oxide	Lapdesf8 ou PJ8	C <sub>3</sub> H <sub>3</sub> N <sub>3</sub> O <sub>4</sub>	145,07
	(E)-4-(4-((2-isonicotinoilhidrazona)metil)fenoxi)-3-fenil-1,2,5-oxadiazol 2-oxide	Lapdesf9 ou PJ9	C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>4</sub>	401,37
	(E)-4-(4-((2-isonicotinoilhidrazona)metil)fenoxi)-3-(fenilsulfonil)-1,2,5-oxadiazol 2-oxide	Lapdesf10 ou PJ10	C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>6</sub> S	465,44
	(E)-4-(3-((2-isonicotinoilhidrazona)metil)fenoxi)-3-fenil-1,2,5-oxadiazol 2-oxide	Lapdesf11 ou PJ11	C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>4</sub>	401,37
	(E)-4-(3-((2-isonicotinoilhidrazona)metil)fenoxi)-3-metil-1,2,5-oxadiazol 2-oxide	Lapdesf12 ou PJ12	C <sub>16</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	339,30
	(E)-4-(4-((2-isonicotinoilhidrazona)metil)fenoxi)-3-metil-1,2,5-oxadiazol 2-oxide	Lapdesf13 ou PJ13	C <sub>16</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	339,30

	(E)-4-(3-((2-isonicotinoilhidrazona)metil)fenoksi)-3-(fenilsulfonil)-1,2,5-oxadiazol 2-oxide	Lapdesf14 ou PJ14	C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>6</sub> S	465,44
	(E)-4-(3-((2-(4-hidroxi)benzoil)hidrazona)methyl)fenoksi)-3-(fenilsulfonil)-1,2,5-oxadiazol 2-oxide	Lapdesf15 ou PJ15	C <sub>22</sub> H <sub>16</sub> N <sub>4</sub> O <sub>7</sub> S	480,45
	(E)-4-(4-((2-(4-hidroxi)benzoil)hidrazona)methyl)fenoksi)-3-(fenilsulfonil)-1,2,5-oxadiazol 2-oxide	Lapdesf16 ou PJ16	C <sub>22</sub> H <sub>16</sub> N <sub>4</sub> O <sub>7</sub> S	480,45
	[(E)-4-(3-((2-(4-aminobenzoil)hidrazona)metil)fenoksi)-3-(fenilsulfonil)-1,2,5-oxadiazol 2-oxide	Lapdesf17 ou PJ17	C <sub>22</sub> H <sub>17</sub> N <sub>5</sub> O <sub>6</sub> S	479,46
	(E)-3-carbamoyl-4-(3-((2-isonicotinoilhidrazona)metil)fenoksi)-1,2,5-oxadiazol 2-oxide	Lapdesf18 ou PJ18	C <sub>17</sub> H <sub>14</sub> N <sub>6</sub> O <sub>5</sub>	382,33
	(E)-3-carbamoyl-4-(4-((2-isonicotinoilhidrazona)metil)fenoksi)-1,2,5-oxadiazol 2-oxide	Lapdesf19 ou PJ19	C <sub>17</sub> H <sub>14</sub> N <sub>6</sub> O <sub>5</sub>	382,33
	(E)-6-((2-(4-hidroxi)benzoil)hidrazona)methyl)benzo[c][1,2,5]oxadiazol 1-oxide	Lapdesf20 ou PJ20	C <sub>14</sub> H <sub>10</sub> N <sub>4</sub> O <sub>4</sub>	298,25
	(E)-6-((2-isonicotinoilhidrazona)metil)benzo[c][1,2,5]oxadiazol 1-oxide	Lapdesf21 ou PJ21	C <sub>13</sub> H <sub>9</sub> N <sub>5</sub> O <sub>3</sub>	283,24

**Tabela 3.** Fórmula estrutural, nome IUPAC, sigla, fórmula molecular e massa molar dos 15 compostos derivados de furoxano e benzofuroxano utilizados no estudo.

## 3.2. Métodos

### 3.2.1. Preparo das suspensões de isolados clínicos e da cepa-padrão H<sub>37</sub>Rv de *M. tuberculosis*

Todas as cepas foram cultivadas em caldo Middlebrook 7H9 (Difco™) enriquecido com OADC (BD/BBL®) e incubadas por aproximadamente 10 dias a 37°C até obtenção de uma grande quantidade de massa bacilar. A suspensão de micobactérias foi então lavada com solução salina tamponada com fosfato à 1% (tampão PBS - pH = 7,0), acrescido de Tween 80 à 0,05% e centrifugada por 15 minutos à 3150 g (≈3000 rpm), repetindo o processo por 2 vezes. O sedimento obtido foi ressuspendido em 50 mL do mesmo tampão. Essa suspensão foi alíquotada em microtubos e congelada a -80°C. O controle de qualidade (CQ) foi realizado semeando 100 µL da suspensão pura e das diluições: 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup> e 10<sup>-8</sup> em placas de 3 quadrantes contendo meio Middlebrook 7H11 acrescido de 10% de OADC, após um mínimo de 2 dias de congelamento. As placas foram incubadas em estufa a 37°C com 5% de CO<sub>2</sub> por até 20 dias para a realização da contagem das unidades formadoras de colônias/mL (UFC/mL).

### 3.2.2. Tratamento, extração e purificação de RNA

Para o tratamento, extração e posterior purificação de RNA das células, os isolados clínicos foram cultivados em 200mL de meio 7H9 (confeccionado com água tratada contendo DEPC) em 1 erlenmeyer estéril e livre de RNase (tratado com DEPC 0,1% ou forno Pasteur 180°C por 4h) até que apresentassem D.O.<sub>625nm</sub> 0,6-0,8 (correspondente à *mid-log phase*). Nesse momento uma alíquota de 50mL foi retirada e considerada como controle (t=0h). Em seguida, a cultura restante foi tratada com RIF em uma concentração de ¼ valor da CIM. Alíquotas de 50mL foram retiradas nos tempos de 24h, 48h e 72h. As alíquotas foram então transferidas para tubos cônicos de 50mL (livres de RNase) e centrifugados a 5000 rpm por 5 minutos. Os precipitados de células bacterianas foram lavados por 3 vezes com água tratada com DEPC 0,01% (Centrifugações a ≈ 5000 rpm/5 minutos). Na sequência, foram adicionados ao precipitado, pérolas de vidro e 350-600µL de tampão de lise (Lysis buffer - QIAGEN), dependendo da quantidade de bactérias obtida. Feito isso, os tubos foram submetidos

a um ciclo de lise no equipamento TissueLyser II (QIAGEN) por um período de 7 minutos e com frequência de 30s<sup>-1</sup>. Após essa etapa de lise, os tubos repousaram por 10 minutos à temperatura ambiente e na sequência foram centrifugados a 3000 rpm por 10 minutos à temperatura de 2-8°C. Ao fim da etapa de centrifugação, cerca de 350µL da fase superior de cada tubo foi transferida para um tubo de microcentrífuga (livre de RNase) e homogeneizado com 350 µL de etanol 70% gelado (preparado com água tratada com DEPC). A partir dessa etapa, a purificação de RNA foi feita com RNeasy Mini Kit (QIAGEN), de acordo com as condições padrões fornecidas pelo fabricante no manual do produto.

### 3.2.3. Transcrição reversa e RT-qPCR

Para a transcrição reversa de RNA foi utilizado o kit "High Capacity cDNA Archive" (Applied Biosystems) de acordo com as recomendações do fabricante.

Primeiramente, para RT-qPCR, foram desenhados, com auxílio do programa *Primer Express 2.0* (Applied Biosystem), iniciadores para cada transcrito selecionado, ou seja, para os genes alvos *Rv1258c*, *Rv1410c*, *Rv2459*, *Rv1217c* e *Rv1218c* envolvidos com o mecanismo de bomba de efluxo de *M. tuberculosis*, além dos controles endógenos (*rrs*, *groEL2* e *sigA*), para avaliar suas respectivas expressões nos isolados (**Tabela 4**). A quantificação da expressão dos genes envolvidos nesse processo dos isolados facilitou a compreensão do grau de resistência ao se comparar com os devidos genes de resistências já estudados por nosso grupo. O grupo de genes escolhidos pertence às famílias de *ABC-transporters* e *MFS* descritas na literatura (Braibant, Gilot e Content, 2000; De Rossi *et al.*, 2002; Da Silva *et al.*, 2011) por estarem envolvidas com as bombas de efluxo relacionadas com o mecanismo de resistência aos fármacos INH, RIF, STR, OFX e EMB.

Posteriormente, foram realizadas reações de otimização, para escolher a melhor concentração de oligonucleotídeo iniciador a utilizar na análise de cada gene alvo e controle endógeno (concentração mínima de oligonucleotídeos iniciadores possível, sem que haja alteração na eficiência de amplificação). As concentrações testadas foram 0,25µM, 0,4µM, 0,5µM e 0,7µM. O sistema de detecção utilizado foi o sistema SYBR Green, baseado no fluorocromo SYBR, fazendo uso do kit Power SYBR Green PCR Master Mix (Applied Biosystems).

As reações de otimização foram realizadas em equipamento para PCR em tempo real ABI Prism® 7500 (Applied Biosystem), em triplicatas para cada gene contendo 20ng de cDNA, 10 µl de Master Mix SYBR™ Green (Applied Biosystem) e os oligonucleotídeos iniciadores, cujas concentrações variaram como descrito acima. As condições das reações foram 10 minutos a 95°C, 40 ciclos de 15 segundos a 95°C e 1 minuto a 60°C para extensão. Ao final, foi acrescentado um passo de dissociação que compreende duas etapas de 15 segundos 95°C, intercaladas por uma etapa de 30 segundos a 60°C. A curva de dissociação tem como objetivo a verificação de ocorrência de *primer dimer*, produtos inespecíficos e de contaminações.

Uma vez realizada padronização das reações de PCR em tempo real utilizando os oligonucleotídeos específicos e confirmação da eficiência de amplificação, foram realizadas as reações com testes em triplicata para cada amostra. A diferença dos níveis de expressão de cada gene foi analisada utilizando o software Biogazelle qbase+. Esse modelo consegue levar em consideração a eficiência de amplificação dos iniciadores no cálculo da expressão relativa, permitindo a quantificação relativa da expressão quando as eficiências de amplificação dos iniciadores são diferentes entre si. O tratamento estatístico foi feito no mesmo software, utilizando o Teste t não-pareado, sempre comparando as amostras dos *time-points* com as amostras de t=0h e adotando como significativo  $p < 0,05$ .

**Tabela 4:** Oligonucleotídeos utilizados na RT-qPCR

Gene	Sequência 5' -> 3'	Temperatura de Melting (°C)	%GC	Tamanho do produto (pb)
<i>Rv1217c</i>	Primer F	GATCAGCTGGGCGATAACAC	60.6	146
	Primer R	AGTCGTAGGGTCAGCGAGAC	59.5	
<i>Rv1218c</i>	Primer F	CCAAAAGGTCTCCCTGATCTC	60.1	101
	Primer R	GAAACACGTTTTCCATCAACG	60.4	
<i>Rv2459</i>	Primer F	GGTACTGCTGTGGCTGTTTGT	60.2	144
	Primer R	ACCATCAGAGTCCCAGAAAAG	60.5	
<i>Rv1410c</i>	Primer F	CGTTGTACGGAATCTTCATCG	60.5	108
	Primer R	GCAGGCTGAAGTGGATCATT	60.2	
<i>Rv1258c</i>	Primer F	GAAATACTTCACCGACCACCA	59.8	107
	Primer R	GGATAGCCAACACGGCATAAC	60.4	
<i>rrs</i>	Primer F	GTCAAGTCATCATGCCCTTA	59.9	107
	Primer R	TGAGACCGGCTTTTAAGGATT	60.1	
<i>groEL2</i>	Primer F	GGCTACATCTCGGGTACTTC	60.0	110
	Primer R	AGCAGATCCTTGACAGTGGAC	59.3	
<i>sigA</i>	Primer F	GTGATTTCTGCTGGGATGAAG	59.5	112
	Primer R	TTGCCGATCTGTTGAGGTAG	60.3	

**Tabela 4:** Oligonucleotídeos iniciadores desenhados para o ensaio de RT-qPCR. A tabela apresenta os *primers* de genes-alvo e candidatos a controles endógenos, com suas respectivas sequências, temperaturas de Melting, porcentagem de GC e tamanho do produto formado.

### **3.2.4. Ensaio de acumulação e efluxo de brometo de etídio**

#### **3.2.4.1. Determinação da concentração ideal de brometo de etídio**

Para a determinar a concentração ideal de brometo de etídio foram utilizadas diferentes concentrações, variando de 0,125 µg/mL até 8,0 µg/mL. Um dos microtubos contendo *Mycobacterium smegmatis* mc<sup>2</sup>155 foi descongelado e cultivado em caldo Middlebrook 7H9 suplementado com 10% de OADC e incubadas a 37°C até a obtenção de uma D.O.<sub>625nm</sub> = 0,8. Ao atingir o ponto de interesse, o cultivo foi centrifugado a 3000 rpm por 10 minutos e o *pellet* formado ressuspendido em solução salina tamponada com fosfato à 1% (tampão PBS - pH = 7,0). Esta suspensão foi dividida em dois grupos, a um deles foi adicionado 0,4% de glicose e outro grupo permaneceu sem o acréscimo.

Em uma placa de 96 orifícios foram adicionados 98,5 µL das suspensões bacterianas, uma contendo glicose e outra não. Em seguida adicionados 1,5 µL de brometo de etídio diluído em água, de maneira que a alcançar diferentes concentrações finais (0,125; 0,5; 1,0; 2,0; 3,0; 4,0 e 8,0 µg/mL). Logo em seguida foi realizada a leitura da fluorescência da placa, utilizando o filtro de excitação de 530nm e o de emissão de 590nm, por um período de uma hora e 30 minutos à uma temperatura de 37°C (com variação de ±2°C) no aparelho *Synergy H4* da BioTek®. Todos os testes foram feitos em triplicata. A melhor concentração foi aquela que obteve a maior leitura de fluorescência (uma vez no interior da célula, o brometo de etídio exibe maior fluorescência do que quando está em ambiente extracelular), permitiu a viabilidade da *M. smegmatis* e não intercalou com o DNA (ácido desoxirribonucleico) do microrganismo. Os resultados obtidos foram analisados utilizando o programa GraphPad Prism 5®.

#### **3.2.4.2. Ensaio de acumulação de brometo de etídio**

Os ensaios foram realizados seguindo a metodologia proposta por Rodrigues *et al.*, 2008 (Rodrigues *et al.*, 2008) e Coldham, Webber *et al.*, 2010 (Coldham *et al.*, 2010). A cepa de *Mycobacterium smegmatis* mc<sup>2</sup>155 (ATCC 700084) foi cultivada em 50mL de meio 7H9, acrescido de 10% de OADC, na temperatura de 37°C até atingir D.O. <sub>625nm</sub>=0,8. O cultivo foi centrifugado a 3000 rpm por 10 minutos, o sobrenadante descartado e o precipitado foi ressuspendido em PBS até alcançar a D.O. <sub>625nm</sub>=0,4.

A esta suspensão foi adicionada glicose como fonte energética na concentração de 0,4%.

Os compostos derivados do furoxano e os inibidores de bomba de efluxo foram diluídos momentos antes do início do ensaio. A diluição dos compostos foi realizada de maneira que a concentração final nos orifícios da placa fosse igual ao valor da metade de suas respectivas CIM, adicionando juntamente o brometo de etídio, de modo que sua concentração final na placa fosse igual a 3,00µg/mL (metade da CIM determinada nos ensaios anteriores). A mistura de compostos com o brometo de etídio foi aplicada na placa (1,5µL) e então foi adicionada a suspensão bacteriana (98,5µL). A leitura de fluorescência foi realizada utilizando o filtro de excitação de 530nm e o de emissão de 590nm, por um período de uma hora e 30 minutos à uma temperatura de 37°C no aparelho *Synergy H4* da BioTek®. O ensaio de acúmulo foi feito em triplicata, utilizou-se como grupos de controle negativo: água destilada estéril, PBS e DMSO. O composto que melhor acumulou foi o que obteve resultado mais próximo ao do verapamil e do CCCP. Os dados obtidos do ensaio de acumulação foram avaliados utilizando a análise de one-way ANOVA e o pós-teste de Dunnett no software GraphPad Prism 5®.

#### **3.2.4.3. Ensaio de efluxo de brometo de etídio**

Seguindo a mesma metodologia definida por Rodrigues *et. al.*, 2008 e Coldham, Webber *et al*, 2010, uma cepa de *Mycobacterium smegmatis* foi descongelada, cultivada e tratada de maneira igual ao procedimento anterior. O precipitado foi resuspendido em PBS de maneira que alcançasse a D.O.  $_{625nm}=0,4$ . Em seguida separou-se 19,0 mL desta solução e foi adicionado 1,0 mL de uma mistura de verapamil com brometo de etídio de maneira que a concentração final dos dois fosse de 150,0 µg/mL e 3,0 µg/mL, respectivamente. A suspensão ficou sob agitação constante por um período de uma hora e trinta minutos à 25°C, para garantir o máximo de acúmulo de brometo de etídio no interior da bactéria sem que o mesmo intercalasse no DNA ou fosse expelido, permitindo a avaliação dos compostos derivados do furoxano como possíveis inibidores de bomba de efluxo. Após esse período de tempo, a solução foi centrifugada por 10 minutos a 3000 rpm e o precipitado resuspendido em mesmo volume de PBS contendo 0,4% de glicose, de maneira que fornecesse fonte energética à célula bacteriana para garantir as condições ideais do

funcionamento das bombas de efluxo. Em uma placa de 96 orifícios foram adicionados 98,5 µL dessa suspensão, e, em seguida, 1,5µL das soluções dos compostos derivados de furoxano, verapamil, como controle positivo (concentração final igual a metade de sua CIM), e os controles negativos (água destilada estéril e DMSO). O verapamil foi escolhido como inibidor de bomba de efluxo e como controle positivo por apresentar melhor acumulação frente ao CCCP.

A leitura de fluorescência utilizou o aparelho *Synergy H4* da BioTek®, filtro de excitação de 530nm e emissão de 590nm, por quarenta minutos à temperatura de 37°C com variação de ±3°C, permitindo as condições ideais para o funcionamento das bombas de efluxo do microrganismo. Após a leitura, os dados foram analisados utilizando o método de one-way ANOVA e o pós-teste de Dunnett pelo software GraphPad Prism 5®.

### **3.2.5. Determinação da Atividade anti-*M. tuberculosis in vitro* dos Compostos derivados de furoxânicos, dos fármacos padrão e do brometo de etídio frente aos isolados clínicos, ao *M. tuberculosis* H37Rv e ao *M. smegmatis* mc<sup>2</sup>155 (ATCC 700084)**

A CIM foi determinada empregando a metodologia descrita por Palomino e colaboradores denominada REMA (*Resazurin Microtiter Assay*) (Palomino *et al.*, 2002). Soluções estoque dos compostos foram preparadas em dimetilsulfóxido (DMSO) (Sigma®) ou água e diluídas em caldo Middlebrook 7H9 suplementado com OADC, para obter a concentração final de cada composto no intervalo de 0,098 à 25,000 µg/mL e um volume de 100µL por poço. Para os ensaios de halotolerância NaCl foi adicionado ao meio de cultura de modo a atingir concentrações finais de 125mM, 250mM e 1000mM. Todo o procedimento na placa de 96 orifícios foi feito com o auxílio da automação do equipamento *Precision*<sup>TM</sup> XS (BioTek). Uma alíquota de 100µL de cada cultura de *M. tuberculosis* ou da bactéria de interesse a uma concentração de aproximadamente 2x10<sup>5</sup> UFC/mL foi adicionada a cada orifício da placa. Na sequência, a microplaca foi incubada por 7 dias, no caso de *M. tuberculosis*, ou 2 dias, no caso de *M. smegmatis*, à 37°C com 5% CO<sub>2</sub>. Ao final do período de incubação foram aplicados 30µL de resazurina em cada orifício na concentração de 0,01% e incubada por 24h. A fluorescência foi medida e a CIM definida como a menor concentração do composto resultando na inibição ≥ a 90% da multiplicação

microbiana. Como controles, a CIM da RIF foi determinada em cada ensaio. Cada experimento foi realizado em triplicata. Um esquema geral da placa de REMA pode ser visto na **figura 5**.

**Figura 5:** Esquema do ensaio de REMA

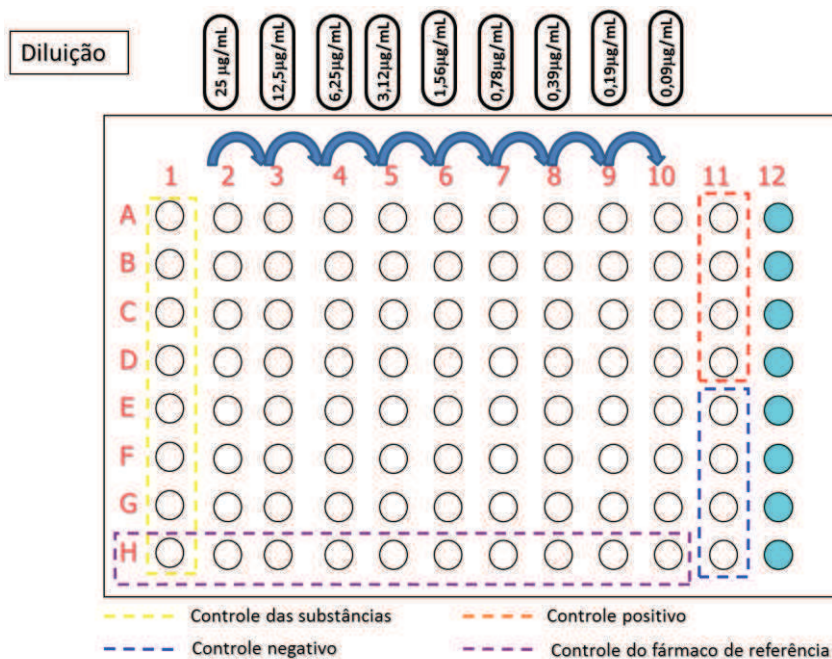


Figura 5: Esquema do ensaio de REMA.

### 3.2.6. Ensaio de liberação de NO (óxido nítrico) pelos compostos derivados furoxânicos e benzofuroxânicos

O ensaio foi realizado como reportado por Singh e colaboradores (Singh *et al.*, 2008). *Mycobacterium tuberculosis* BCG foi cultivado em 7H9 até D.O.  $_{625nm}=0,2$ . Na sequência a cultura foi ressuspensa até D.O.  $_{625nm}=0,8$  e incubada sob agitação por 2h a 37°C na presença de 10µM DAF-FM DA (diacetato de diaminofluoresceína, Sigma®). Após esse tempo a cultura foi lavada 3 vezes com meio de 7H9 sem OADC para a remoção de DAF-FM DA livre no meio. Na sequência, a cultura foi re-incubada por mais 1h nas condições anteriores para permitir um estado fisiológico adequado antes da exposição aos compostos nas respectivas concentrações de uso. DMSO e água foram utilizadas como controles negativos de liberação de NO e DETA/NO (Sigma®) foi utilizado como controle positivo. Tal ensaio foi realizado considerando o fato de que, seja por mecanismo direto de *stress* oxidativo, ou por nitratação de bombas de efluxo, a atividade sugerida por esses compostos passa pela liberação de NO. A

base metodológica para o ensaio consiste no fato de que DAF-FM DA tem a capacidade de emitir fluorescência na presença de NO devido à formação de derivados benzotriazóis. As leituras foram feitas utilizando os filtros de excitação e emissão de 495nm e 515nm respectivamente, em um ensaio cinético de 24h, com intervalos de leitura a cada 30 minutos.

### **3.2.7. Ensaio de *Microarray***

Para o ensaio de *microarray*, a cepa *M. tuberculosis* H<sub>37</sub>Rv foi tratada com os compostos Lapdesf10, Lapdesf21 e DMSO (controle negativo) durante 4 horas, nas concentrações finais de 2xCIM, sempre respeitando o limite de no máximo 1% de DMSO em cultivo. A bactéria foi cultivada em tubos Falcon em triplicata para cada tratamento, com volume final de 20mL em cada Falcon. O tratamento foi iniciado após o cultivo bacteriano atingir D.O.<sub>600nm</sub>=1.0. A etapa de extração de RNA foi feita exatamente como já descrita anteriormente.

#### **3.2.7.1. Síntese de cDNA marcado com Cy3 e Cy5**

Para cada amostra foi preparada uma reação para marcação com Cy3 e outra para marcação com Cy5 (isso ocorre pois é necessário realizar a técnica de *dye-swap*, uma vez que Cy3 e Cy5 possuem diferentes eficiências de incorporação no cDNA). Resumidamente, 1µg de RNA foi adicionado a um 1µL de oligonucleotídeos randômicos (3µg/µL) e água, de modo a completar 11µL de volume final. A combinação foi colocada em um banho seco a 70°C por 2 minutos, rapidamente resfriada em gelo e, na sequência brevemente centrifugada (8000 rpm / 5 segundos). Feito isso foram adicionados para cada combinação: 5µL do *First Strand Buffer* (5x – Invitrogen), 2,5µL de DTT (100mM), 2,3µL de dNTPs (5mM dA/G/TTP e 2mM dCTP), 1,7µL de Cy3 ou Cy5 dCTP (GE Healthcare) e 2,5µL da enzima SuperScript III (200 U/ µL). Na sequência, as amostras foram incubadas no escuro a 25°C por 10 minutos e depois a 50°C por 90 minutos.

#### **3.2.7.2. Purificação dos cDNAs marcados com Cy3 e Cy5**

Nesta etapa, foram misturadas as reações referentes a cada setor da lâmina. Por exemplo, a idéia era analisar a diferença na expressão gênica da bactéria tratada com o composto e da bactéria tratada com DMSO. Para isso, foi então misturada a reação

do composto Lapdesf10 marcado com Cy3 com a reação do DMSO marcado com Cy5 (sendo que esta mistura foi futuramente aplicada em um setor da lâmina). Para a realização do *dye swap* foi misturada a reação do composto Lapdesf10 marcado com Cy5 com a reação do DMSO marcado com Cy3, que futuramente foi aplicada em outro setor da lâmina de *microarray*. Após as misturas das reações com seus respectivos pares de comparação, ocorreu a purificação da amostra final utilizando o kit *Qiagen MinElute Purification* (número de catálogo 28004), de acordo com as instruções do fabricante.

### **3.2.7.3. Hibridização**

Para hibridização, em um microtubo novo foram adicionados: 18µL da mistura dos cDNAs marcados um com Cy3 e outro com Cy5, 4,5µL do *Agilent 10x Blocking Agent* e 22,5µL do *Agilent 2x Hybridisation Buffer*. Os microtubos foram incubados a 95°C por 5 minutos e centrifugados a 13000 rpm por 1 minuto. Na sequência foram pipetados 40µL da mistura de hibridização em cada campo demarcado na lamínula (1 campo representa 1 *array*, sendo que cada lâmina possui 8 *arrays*). Com cuidado, a lâmina de *array* (Agilent AMADID 0275543 – G4427A) foi colocada sobre a lamínula, evitando a formação de bolhas. O sistema foi colocado em uma base própria, fixado com força para não haver movimentação e na sequência incubado por 12 horas a 65°C com rotação vertical de 20 rpm. Após as 12 horas de incubação, foi feita a lavagem do sistema, utilizando os tampões *Oligo aCGH Wash Buffer 1, 2 e 3* (Agilent Technologies), de acordo com as instruções do fabricante. Após a lavagem as lâminas de *Microarray* foram escaneadas utilizando o *Agilent High Resolution Microarray Scanner* e o resultado foi interpretado através do software *GeneSpring 13.1* (Agilent Technologies).

## **4. Resultados e Discussão**

### **4.1. Ensaios de RT-qPCR e avaliação da expressão de bombas de efluxo em isolados clínicos frente ao tratamento com RIF**

Como descrito no item Material e Métodos, foi realizada uma etapa longa e necessária de otimização dos ensaios com a seleção de *primers* e padronização das concentrações dos mesmos a serem utilizadas, para a efetiva validação dos

experimentos. Para cada gene alvo e cada candidato a gene endógeno foram feitas 4 diluições de *primers* (0,25µM, 0,40µM, 0,50µM e 0,70µM), com o objetivo de encontrar a menor concentração de iniciadores capaz de permitir uma amplificação visível no meio da reação (ciclo 15 ao ciclo 30, ideal para a análise dos resultados) e específica, ou seja, com a formação de único produto, evitando a formação de dímeros de *primers* ou quaisquer outros produtos, o que pôde ser avaliado pela curva de *melting*. **Tabela 5** e **figura 6** apresentam dados da padronização, tais como concentração ideal dos *primers* e curvas-padrão com as respectivas eficiências de amplificação de cada gene pesquisado.

**Tabela 5:** Eficiências de amplificação e dados de padronização dos *primers*

Gene	Slope	Eficiência (%)	Threshold	Quantidade de cDNA (pontos da curva) (ng)	[Primers] (µM)
<i>Rv1217c</i>	-3,388	97,32	0,175101	2,5-5,0-10,0-20,0-40,0	0,25
<i>Rv1218c</i>	-3,584	90,12	0,272790	2,5-5,0-7,5-10,0-20,0	0,25
<i>Rv1410c</i>	-3,107	109,81	0,235314	2,5-5,0-10,0-20,0-40,0	0,25
<i>Rv1258c</i>	-3,234	103,79	0,662081	2,5-5,0-10,0-20,0-40,0	0,40
<i>Rv2459</i>	-3,396	96,99	0,272790	2,5-5,0-10,0-20,0-40,0	0,25
<i>sigA</i>	-3,382	97,55	0,151045	2,5-5,0-10,0-20,0-40,0	0,25
<i>groEL2</i>	-3,500	93,08	0,424981	2,5-5,0-10,0-20,0-40,0	0,25

**Tabela 5:** Tabela com as eficiências de amplificação de cada gene a ser estudado, com as respectivas quantidades de cDNA validadas para o ensaio.

**Figura 6:** Curvas-padrão demonstrando eficiência de amplificação dos genes pesquisados.

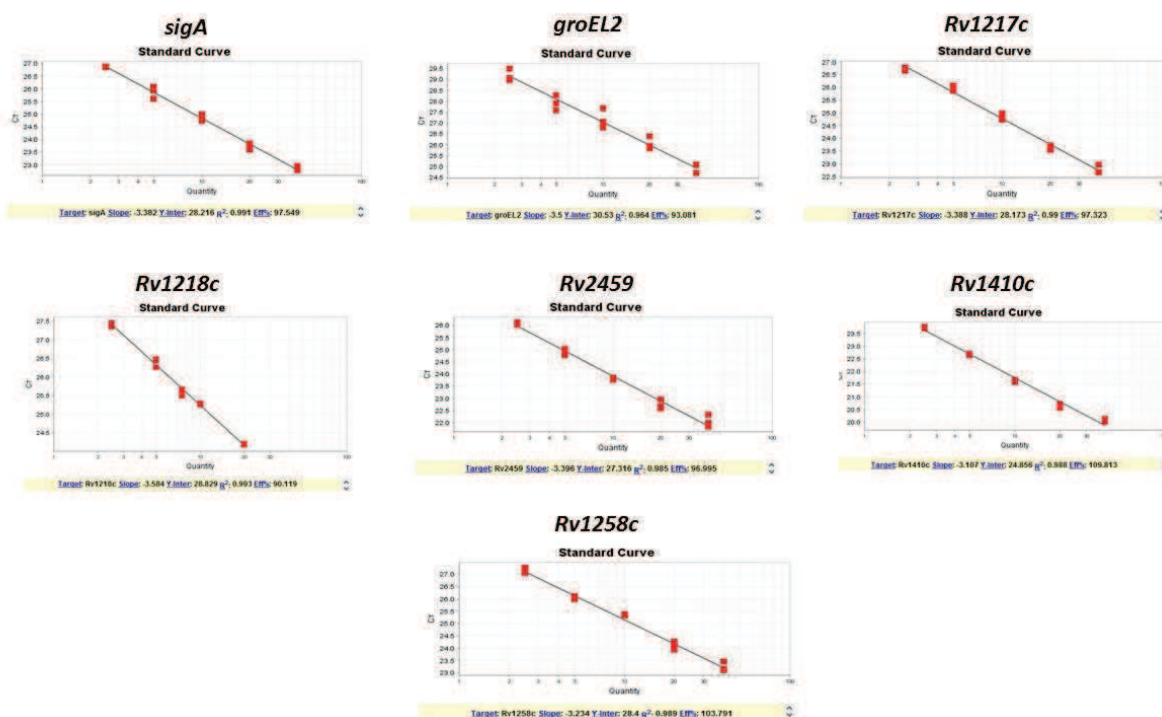


Figura 6: Curvas-padrão demonstrando eficiência de amplificação dos genes pesquisados.

O gene *rrs* apresentou problemas na validação e foi excluído dos ensaios, uma vez que se tratava apenas de um candidato a controle endógeno. Tal exclusão não representou problemas devido à existência dos outros dois candidatos (*groEL2* e *sigA*), cuja validação é bem descrita na literatura (Sharma *et al.*, 2010; Gonzalo-Asensio *et al.*, 2008; Wang *et al.*, 2013).

Terminada então a etapa de padronização, com a otimização dos *primers* e validação dos genes, partiu-se para a avaliação da expressão das bombas de efluxo selecionadas frente ao tratamento com RIF em 12 isolados clínicos, além da cepa padrão H<sub>37</sub>Rv. Os ensaios foram feitos após o tratamento dos isolados clínicos e do H<sub>37</sub>Rv com RIF, na concentração de ¼ da CIM de cada isolado, com 4 tempos de coleta: 0h, 24h, 48h e 72h. Os resultados são exibidos a seguir nas **Figuras de 7-19 e tabelas de 6-18**.

Embora de maneira heterogênea, os resultados apresentados nos 12 isolados e na cepa-padrão indicam alterações transcricionais para alguns dos genes pesquisados, como uma resposta quase imediata do bacilo ao tratamento. O trabalho baseou-se na idéia comportamental de cada isolado clínico quando desafiado ao quimioterápico, uma vez que, cada isolado possui as suas particularidades, de acordo com as condições já enfrentadas nos pacientes. Portanto, em um primeiro momento, o foco da tese foi avaliar como cada bacilo responde a um tratamento com RIF ao longo das primeiras horas de contato com o fármaco, e não a expressão constitutiva dos genes de efluxo.

As alterações mais drásticas em expressão, e por consequência, as de maior destaque no trabalho, foram as alterações sofridas pelo gene *Rv1258c* em dois isolados (CF76 e CF185). Para o isolado CF76, após 72h do tratamento, houve um aumento médio entre as triplicatas biológicas de 16,18 vezes ( $\pm 1,02$ ) na expressão do gene citado com relação à amostra inicial (t=0h). Para o isolado CF185 foram necessárias as mesmas 72h para que a expressão atingisse níveis médios 15,33 ( $\pm 1,11$ ) vezes maiores que os basais. Além destes isolados, o *Rv1258c* apresentou-se superexpresso, com pelo menos 2 vezes mais quantidade de RNA mensageiro em algum dos *time points* analisados, em outros 4 isolados (CF85, CF102, CF104 e CF145). Tais resultados corroboram com outros trabalhos da literatura que colocam o *Rv1258c* como o principal relacionado ao efluxo de RIF (Ainsa *et al.*, 1998; Jiang, X.

*et al.*, 2008; Siddiqi *et al.*, 2004; Rodrigues *et al.*, 2012). A superexpressão do *Rv1258c* corresponde a mais do que uma simples resposta a um determinado fármaco, sendo o responsável por um mecanismo adaptativo do bacilo até mesmo em condições *in vivo*. Já foi demonstrado essa bomba de efluxo participa e é necessária para a sobrevivência do bacilo no ambiente intramacrofágico (Adams *et al.*, 2011; Morris *et al.*, 2005; Schnappinger *et al.*, 2003), além de, como reforçado anteriormente, aumentar a tolerância extra e intracelularmente à RIF.

Considerando os perfis fenotípicos de resposta à RIF (levando em consideração principalmente a CIM), de maneira surpreendente, o isolado clínico CF102 (único sensível para as técnicas de REMA e BACTEC, além da cepa-padrão H<sub>37</sub>Rv) foi um dos que apresentou um maior aumento na expressão do gene *Rv1258c*, chegando a 5,24 ( $\pm 0,48$ ) vezes após 72h de tratamento com RIF, quando comparado ao tempo 0h. No desenho experimental, o esperado era que o mecanismo de resposta, com posterior aumento na transcrição de genes de efluxo fosse mais eficiente em bactérias com maior nível de resistência. Tal resultado pode ser explicado considerando o fato de que a resistência dos outros isolados é devida principalmente às mutações cromossômicas, tendo o efluxo, portanto, um papel reduzido nesse mecanismo. Outra explicação para o ocorrido é o fato de que mesmo sendo sensível à RIF, CF102 trata-se de um isolado clínico proveniente de um centro de referência e que provavelmente em algum momento já teve contato com o fármaco, o que pode ter facilitado essa resposta exacerbada. Essa linha de raciocínio corrobora com estudo anterior da literatura (Calgin *et al.*, 2013), no qual, embora não se tenha utilizado a estratégia de tratamento com o fármaco, foi observado que isolados clínicos sensíveis e resistentes podem apresentar os mesmos níveis de superexpressão de genes de efluxo, ou melhor dizendo, sem uma diferença significativa.

Como resultado deste estudo, destaca-se a importância do encontro da superexpressão do gene *Rv2459*, o qual apenas recentemente foi reportado como relacionado à uma possível tolerância à RIF (Coelho *et al.*, 2015). Esse transportador da família MFS, já foi descrito em estudos prévios participando da resposta imediata ao *stress* induzido por INH ou EMB (Gupta, A. K. *et al.*, 2010). No presente trabalho, os isolados clínicos CF76, CF78, CF85, CF110 e CF185 apresentaram aumento da expressão desse gene, principalmente o isolado CF110 com aumento médio de 6,65 ( $\pm 0,52$ ) vezes com relação ao tempo inicial. Já o isolado CF76 apresentou como

resposta um aumento médio de 5,12 ( $\pm 0,31$ ) vezes. De uma maneira geral, a resposta relacionada à essa bomba de efluxo surgiu mais tardiamente, com as alterações mais drásticas sendo observadas após 72h do tratamento. A inespecificidade desse transportador já havia sido abordada no estudo anteriormente citado (Gupta, A. K. *et al.*, 2010), uma vez que o mesmo também teve sua expressão alterada mediante ao tratamento com estreptomicina, embora de maneira bem mais discreta (aumento de 2 vezes). Outros transportadores já demonstraram ampla capacidade de transporte de mais de um quimioterápico, tais quais *drxB* (Li *et al.*, 2015), *Rv1819c* e *Rv3728* (Gupta, A. K. *et al.*, 2010), tendo sido descritos como responsáveis por efluxo tanto de INH quanto de RIF. A hipótese é de que *Rv2459* esteja participando na aquisição de uma baixa resistência à RIF em alguns isolados, fato semelhante ao ocorrido com *drxA*, o que acaba por gerar controvérsias entre alguns estudos da área (Li *et al.*, 2015; Pang *et al.*, 2013).

Considerando as bombas de efluxo analisadas da família dos *ABC transporters* (*Rv1217c-Rv1218c*), os resultados foram bem significativos para 7 dos 12 (58,33%) isolados clínicos analisados. O fato é que esses dois genes constituem um único sistema de efluxo, com duas cópias de *Rv1218c* (funcionando como domínios de ligação de nucleotídeos), e uma cópia de *Rv1217c*, fusionadas por dois domínios de membrana (Braibant, Gilot e Content, 2000) . Apesar de constituírem um único complexo, a variação na expressão dos genes é independente.

O resultado de maior destaque provém do isolado CF76 com uma expressão média 7,29 ( $\pm 0,46$ ) vezes maior que o tempo inicial para o *Rv1217c*, após 72h de tratamento. O diferencial para esses transportadores, foi uma resposta mais rápida de forma geral, com alterações significativas aparecendo já nos tempos de 24h e 48h. Por exemplo, para o mesmo CF76 as alterações já foram nítidas no período de 24h, com um aumento de 4,13 ( $\pm 0,31$ ) vezes na expressão pós-tratamento. CF104 apresentou um perfil semelhante em 24h, aumentando a expressão de *Rv1217c* em 4,04 ( $\pm 0,69$ ) vezes e de 6,30 ( $\pm 0,47$ ) em 72h. Outro isolado, CF185, atingiu níveis de expressão também significativos, com aumento de 5,65 vezes ( $\pm 0,46$ ) após 72h. O gene *Rv1218c*, por sua vez, apresentou padrões mais constantes, mas com alguns destaques, como a expressão aumentada em CF185 após 72h de tratamento, com 6,82 ( $\pm 0,50$ ) vezes mais expresso que o tempo inicial. Outros índices de superexpressão do gene foram encontrados para o isolado CF110 (3,77  $\pm$  0,27) e

CF114 ( $2,83 \pm 0,27$ ). Os resultados encontrados corroboram com trabalhos prévios, como o de Wang e colaboradores (Wang *et al.*, 2013), que apesar de utilizar a estratégia de quantificação constitutiva da expressão desses genes (ou seja, sem tratamento), evidenciou a importância do aumento desses genes na caracterização de isolados como MDR. Além disso, os resultados confirmam RIF como um possível substrato desse sistema de efluxo, discordando de um dado prévio do trabalho de Balganesch (Balganesch *et al.*, 2012). A controvérsia foi provavelmente gerada pois no estudo anteriormente citado, a influência de *Rv1218c* no efluxo de RIF foi estudada através da construção de um mutante *knockout* ( $\Delta Rv1218c$ ) e testes de susceptibilidade a fármacos. A não diminuição da CIM da RIF frente ao mutante provavelmente se deveu ao fato de existirem outras bombas de efluxo capazes de realizar a extrusão do fármaco, com base na inespecificidade que as mesmas apresentam para os substratos.

A bomba de efluxo *Rv1410c*, mostrou-se importante para dois isolados avaliados, CF78 e CF185, com aumento de 4,00 ( $\pm 0,28$ ) para o primeiro em 48h e de 10,29 ( $\pm 0,80$ ) para o segundo após 72h do tratamento. Os resultados indicam também a importância desse sistema como resposta ao *stress* causado pela RIF, embora em um grau menor para a população estudada se comparado às outras bombas de efluxo presentes no estudo. Em estudos prévios (Kardan Yamchi *et al.*, 2015; Jiang, X. *et al.*, 2008), esse sistema de efluxo assumiu papel principal na diferenciação entre isolados MDR e isolados pan-sensíveis, evidenciando as particularidades e importância do estudo de cada população de forma separada, o que no futuro pode influenciar as decisões para a terapia.

Para alguns dos genes estudados nesse trabalho, foi observada, em alguns momentos, uma diminuição da expressão no decorrer do tempo. A hipótese para tal fato, é que a bactéria passe a expressar de forma prioritária apenas os genes de transportadores que sejam convenientes para a mesma em detrimento de outros, evitando um gasto desnecessário de energia. Aliado a esse fato, RIF aparentemente é um substrato para muitos dos hipotéticos transportadores e *M. tuberculosis*, reforçando a heterogeneidade de resposta entre os isolados.

Analisando os dados fenotípicos de forma conjunta com os dados de expressão, o isolado CF76 foi o mais responsivo ao tratamento, apresentando elevada expressão de 4 dos 5 genes avaliados (única exceção para o *Rv1410c*). Esses resultados

apontam para uma contribuição significativa no perfil de resistência do isolado, uma vez que o mesmo se apresenta como um dos mais resistentes ao tratamento (CIM > 25,00µg/mL). Desse modo é possível concluir que, ainda que de maneira secundária, a alta expressão de bombas de efluxo atua de maneira sinérgica para determinar o grau de resistência de um isolado, mesmo em presença de mutações na região RRDR como o principal fator.

No contexto da presente tese, os resultados dos isolados que apresentam perfil de resistência sem carregar mutações na região RRDR merecem uma atenção especial, pois podem evidenciar mais claramente o papel do efluxo na resistência micobacteriana. Nesta categoria encontram-se os isolados CF78, CF110, CF114 e CF145. O isolado CF78 apresenta uma resistência fenotípica à RIF considerada baixa, uma vez que a CIM é 2,00µg/mL, levando à conclusão que essa aparente tolerância pode ter sido causada por um aumento discreto na expressão das bombas de efluxo, principalmente Rv1410c. Isolados CF110, CF114 e CF145, apresentam resistência fenotípica considerada moderada, e com perfis de expressão dos genes estudados muito similares. Apesar de não apresentarem um aumento exorbitante na expressão de um ou outro gene, CF110 e CF114 mostraram um aumento moderado (de 2 a 6 vezes após o tratamento com RIF) para 3 e 4 bombas de efluxo, respectivamente. CF145 demonstrou capacidade de aumento na expressão, embora também de forma moderada, para todos os genes estudados. Os resultados apresentados indicam a influência dos transportadores estudados na aquisição de resistência micobacteriana frente à RIF. Obviamente, outro importante fator para resistência, a composição da parede celular de cada isolado, não foi abordado no estudo, o que não diminui a importância dos resultados de expressão gênica.

Destaca-se que os resultados encontrados quanto ao papel das bombas de efluxo podem ter uma implicação considerável na clínica. Uma vez que muitos desses transportadores apresentam uma atividade inespecífica para substratos, ou seja, podem atuar na eliminação de mais de um fármaco do ambiente celular, deve existir ao se efetuar o tratamento, uma atenção na escolha dos fármacos e na ordem de utilização dos mesmos na terapia. Já foi descrito na literatura, relatos na indução de tolerância à ofloxacina (OFL) em isolados tratados previamente com RIF (Louw *et al.*, 2011). O fato pode ser explicado devido a expressão elevada principalmente de

*Rv1258c*, ocasionada pela exposição à RIF, sendo que este transportador é capaz de atuar também no efluxo de OFL (Jiang, Xin *et al.*, 2008).

Compilando tais resultados, conclui-se que: (1) RIF é um substrato compatível para ambos os tipos de transportadores, sejam pertencentes à classe dos *ABC transporters* ou MFS; (2) bombas de efluxo podem participar, embora como um mecanismo secundário (gerando baixa resistência), da emergência de cepas MDR em nossa população de isolados clínicos; (3) *Rv2459* pode ser confirmado como um transportador de RIF do ambiente intracelular para o ambiente extracelular; (4) existe uma heterogeneidade de respostas dos isolados da população frente ao tratamento, com isolados clínicos sensíveis também demonstrando capacidade de aumentar a expressão de genes de efluxo, colocando essa via de resistência provavelmente como 1º mecanismo de tolerância a surgir em micobactérias; e (5) a adição de fármacos inibidores de efluxo constitui-se em uma terapia adjuvante muito coerente, considerando a linha de que o tratamento nunca é monoterápico e deve sempre atingir a bactéria na maior quantidade de alvos possível.

**Figura 7:** Expressão de genes de efluxo para isolado clínico CF76

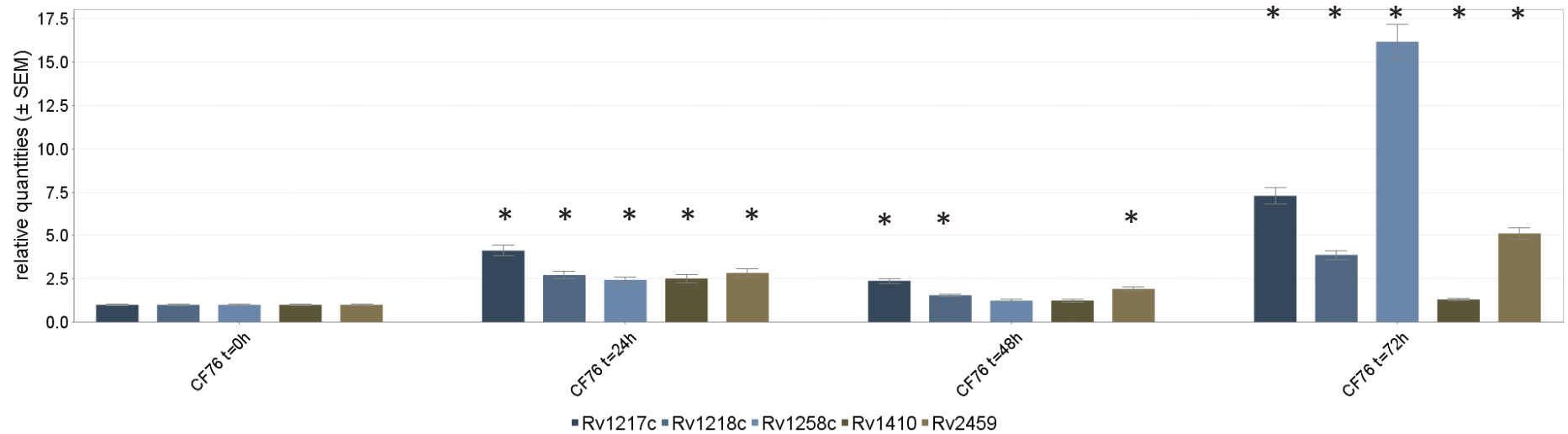


Figura 7: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF76 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 6:** Expressão de genes de efluxo para isolado clínico CF76

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF76 t=0h	1,00 ± 0,06	1,00 ± 0,05	1,00 ± 0,06	1,00 ± 0,05	1,00 ± 0,06
CF76 t=24h	4,13 ± 0,31	2,72 ± 0,21	2,44 ± 0,18	2,52 ± 0,22	2,84 ± 0,24
CF76 t=48h	2,38 ± 0,14	1,55 ± 0,08	1,24 ± 0,07	1,24 ± 0,09	1,92 ± 0,10
CF76 t=72h	7,29 ± 0,46	3,88 ± 0,26	16,18 ± 1,02	1,31 ± 0,08	5,12 ± 0,31

**Tabela 6:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF76 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 8:** Expressão de genes de efluxo para isolado clínico CF78

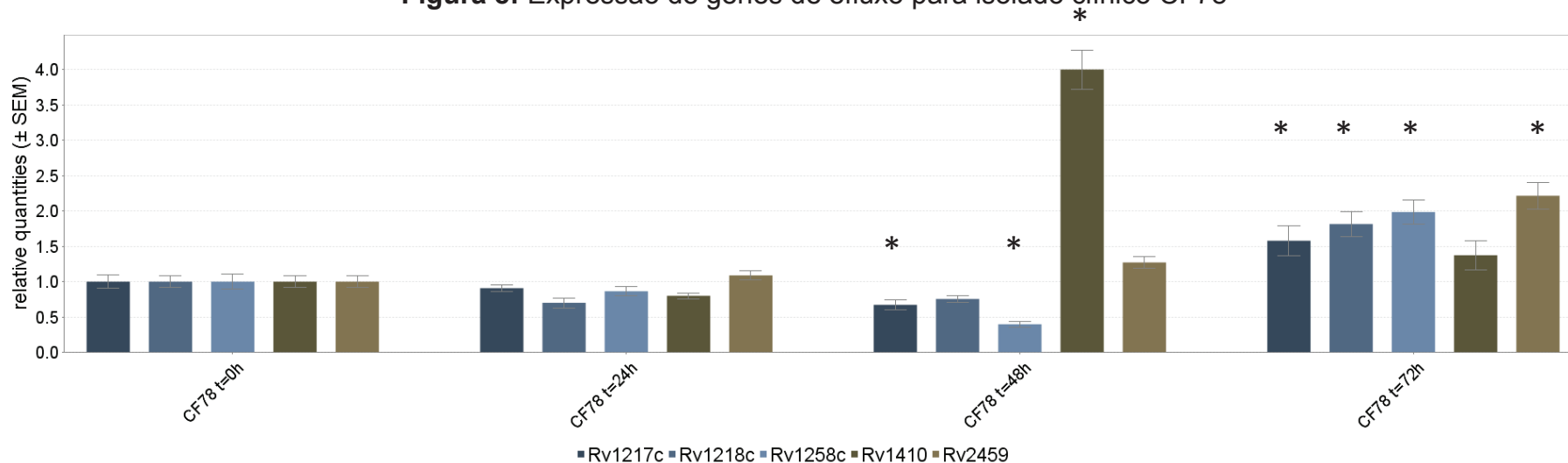


Figura 8: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF78 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 7:** Expressão de genes de efluxo para isolado clínico CF78

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF78 t=0h	1,00 ± 0,09	1,00 ± 0,08	1,00 ± 0,10	1,00 ± 0,08	1,00 ± 0,08
CF78 t=24h	0,91 ± 0,05	0,70 ± 0,07	0,86 ± 0,07	0,80 ± 0,04	1,09 ± 0,06
CF78 t=48h	0,67 ± 0,07	0,75 ± 0,05	0,40 ± 0,04	4,00 ± 0,28	1,27 ± 0,08
CF78 t=72h	1,58 ± 0,21	1,81 ± 0,18	1,98 ± 0,17	1,37 ± 0,20	2,22 ± 0,19

**Tabela 7:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF78 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 9:** Expressão de genes de efluxo para isolado clínico CF85

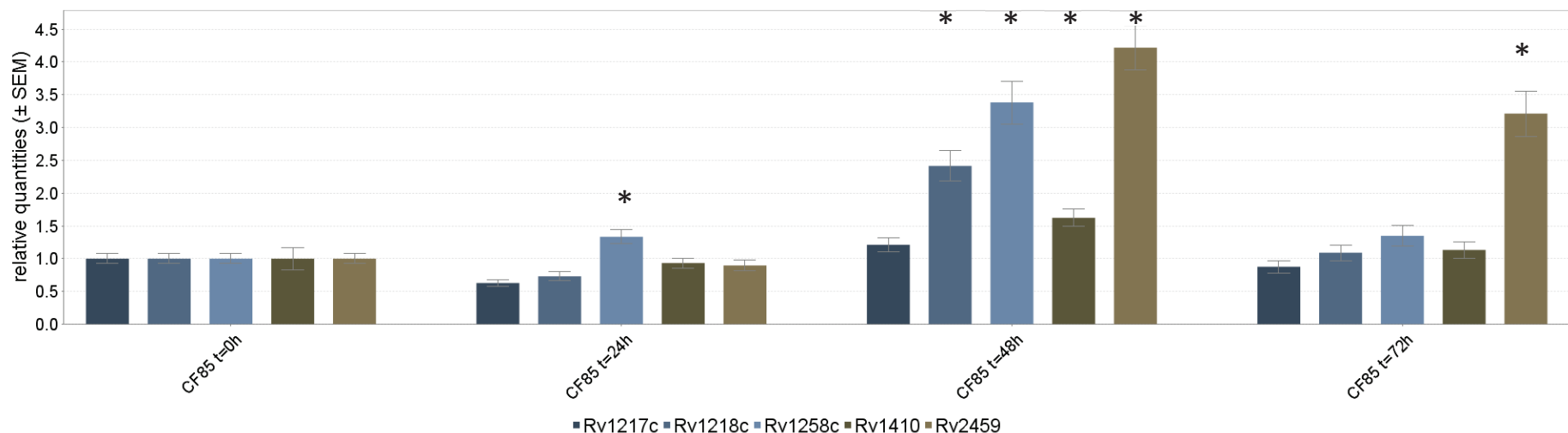


Figura 9: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF85 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 8:** Expressão de genes de efluxo para isolado clínico CF85

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF85 t=0h	1,00 ± 0,08	1,00 ± 0,07	1,00 ± 0,07	1,00 ± 0,17	1,00 ± 0,07
CF85 t=24h	0,63 ± 0,05	0,73 ± 0,07	1,33 ± 0,11	0,93 ± 0,07	0,90 ± 0,08
CF85 t=48h	1,21 ± 0,11	2,41 ± 0,23	3,38 ± 0,33	1,62 ± 0,13	4,22 ± 0,34
CF85 t=72h	0,88 ± 0,09	1,09 ± 0,12	1,35 ± 0,15	1,13 ± 0,12	3,21 ± 0,34

**Tabela 8:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF85 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 10:** Expressão de genes de efluxo para isolado clínico CF93

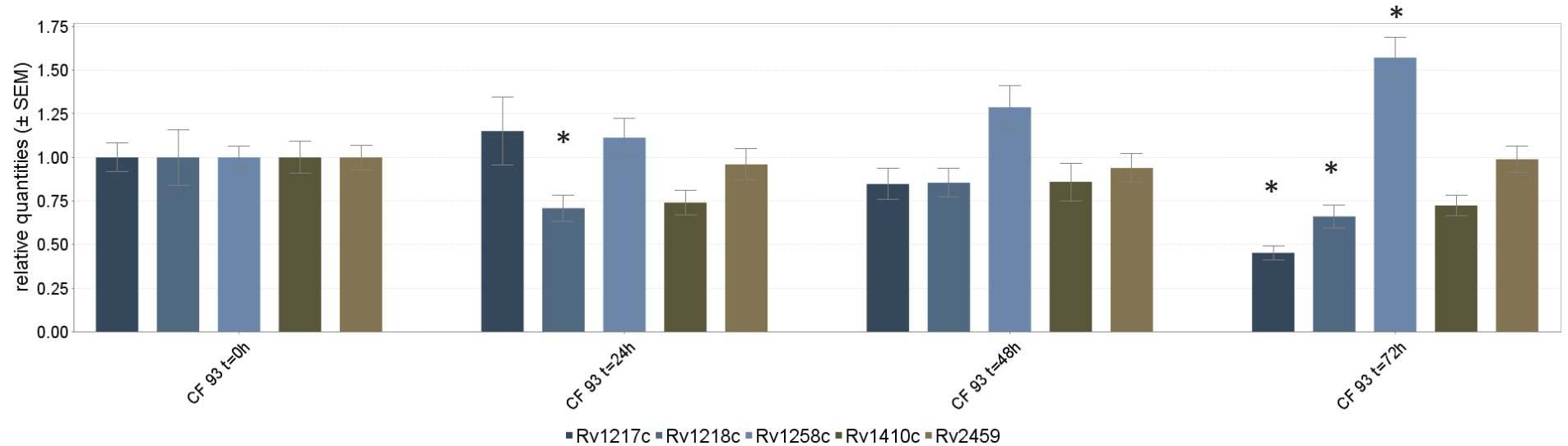


Figura 10: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF93 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 9:** Expressão de genes de efluxo para isolado clínico CF93

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF93 t=0h	1,00 ± 0,08	1,00 ± 0,16	1,00 ± 0,06	1,00 ± 0,09	1,00 ± 0,07
CF93 t=24h	1,15 ± 0,20	0,71 ± 0,08	1,11 ± 0,11	0,74 ± 0,07	0,96 ± 0,09
CF93 t=48h	0,85 ± 0,09	0,85 ± 0,08	1,29 ± 0,12	0,86 ± 0,11	0,94 ± 0,08
CF93 t=72h	0,45 ± 0,04	0,66 ± 0,07	1,57 ± 0,11	0,72 ± 0,06	0,99 ± 0,07

**Tabela 9:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF93 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 11:** Expressão de genes de efluxo para isolado clínico CF102

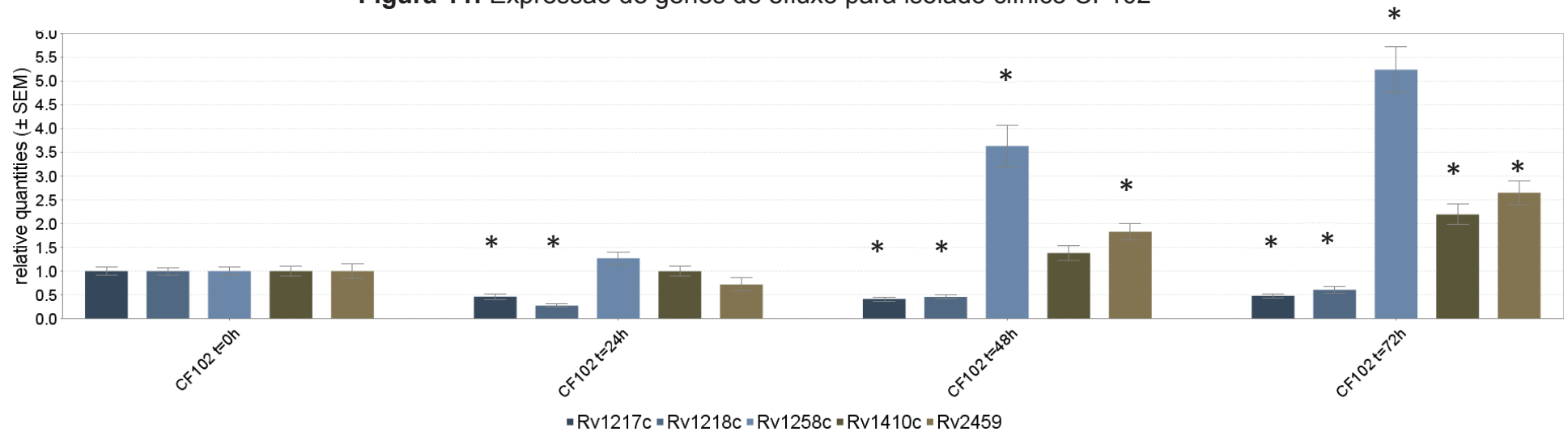


Figura 11: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF102 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 10:** Expressão de genes de efluxo para isolado clínico CF102

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF102 t=0h	1,00 ± 0,08	1,00 ± 0,08	1,00 ± 0,09	1,00 ± 0,10	1,00 ± 0,15
CF102 t=24h	0,46 ± 0,06	0,28 ± 0,03	1,27 ± 0,13	1,00 ± 0,10	0,72 ± 0,14
CF102 t=48h	0,41 ± 0,04	0,46 ± 0,05	3,63 ± 0,44	1,38 ± 0,15	1,83 ± 0,17
CF102 t=72h	0,48 ± 0,05	0,61 ± 0,07	5,24 ± 0,48	2,19 ± 0,21	2,65 ± 0,25

**Tabela 10:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF102 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 12:** Expressão de genes de efluxo para isolado clínico CF104

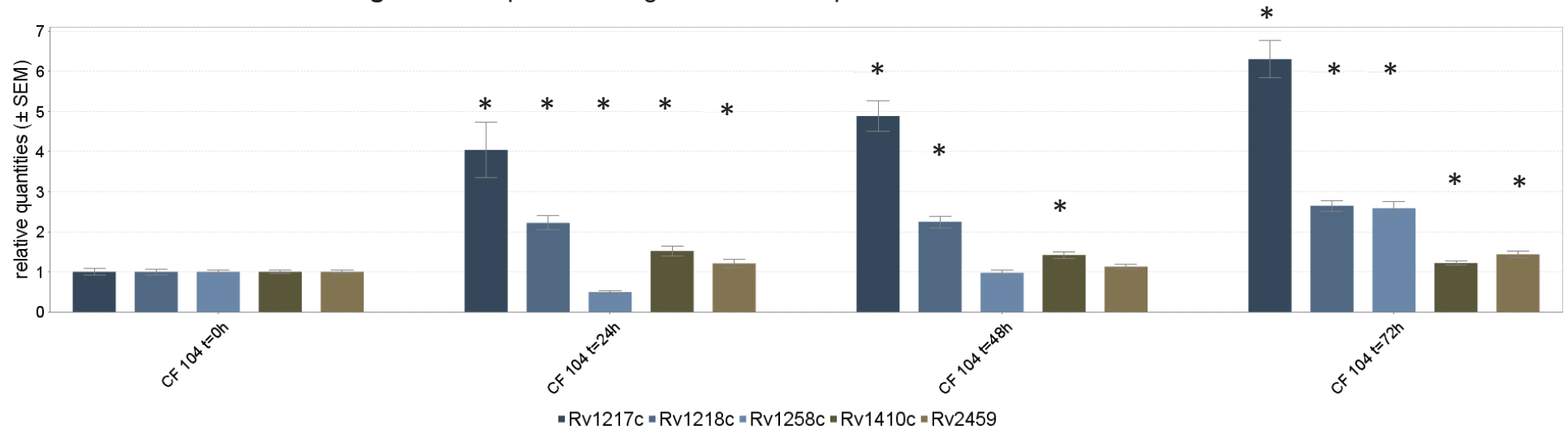


Figura 12: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF104 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 11:** Expressão de genes de efluxo para isolado clínico CF104

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF104 t=0h	1,00 ± 0,08	1,00 ± 0,07	1,00 ± 0,04	1,00 ± 0,04	1,00 ± 0,05
CF104 t=24h	4,04 ± 0,69	2,22 ± 0,17	0,50 ± 0,04	1,52 ± 0,12	1,21 ± 0,10
CF104 t=48h	4,89 ± 0,39	2,25 ± 0,14	0,98 ± 0,06	1,42 ± 0,08	1,13 ± 0,07
CF104 t=72h	6,30 ± 0,47	2,65 ± 0,14	2,59 ± 0,17	1,22 ± 0,06	1,44 ± 0,08

**Tabela 11:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF104 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 13:** Expressão de genes de efluxo para isolado clínico CF110

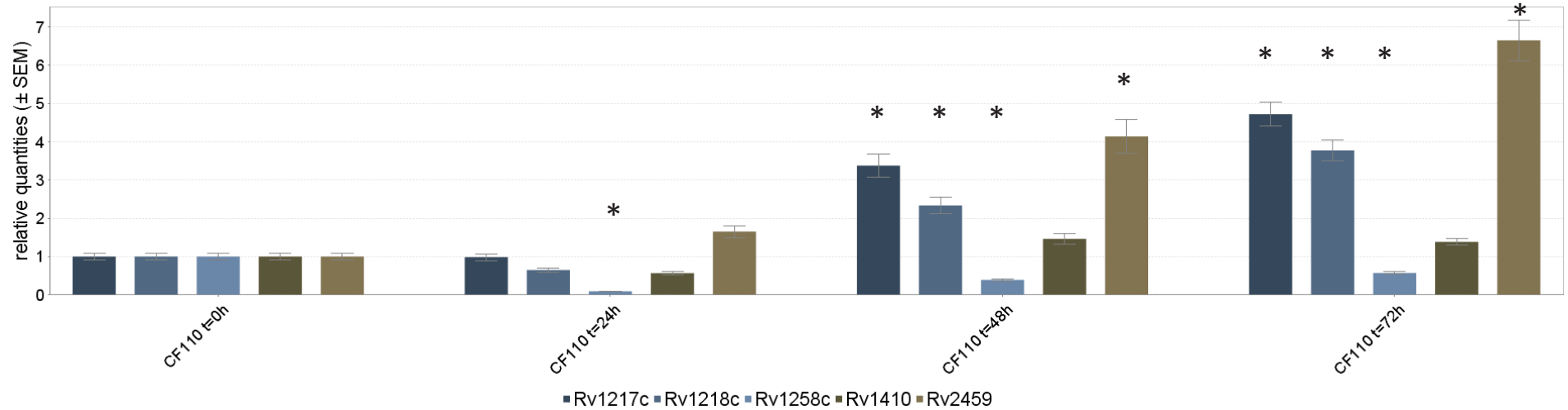


Figura 13: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF110 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 12:** Expressão de genes de efluxo para isolado clínico CF110

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF110 t=0h	1,00 ± 0,09	1,00 ± 0,08	1,00 ± 0,08	1,00 ± 0,09	1,00 ± 0,09
CF110 t=24h	0,98 ± 0,09	0,65 ± 0,06	0,09 ± 0,01	0,57 ± 0,05	1,65 ± 0,15
CF110 t=48h	3,38 ± 0,30	2,34 ± 0,21	0,39 ± 0,04	1,46 ± 0,14	4,14 ± 0,44
CF110 t=72h	4,72 ± 0,32	3,77 ± 0,27	0,57 ± 0,04	1,38 ± 0,09	6,65 ± 0,52

**Tabela 12:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF110 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 14:** Expressão de genes de efluxo para isolado clínico CF114

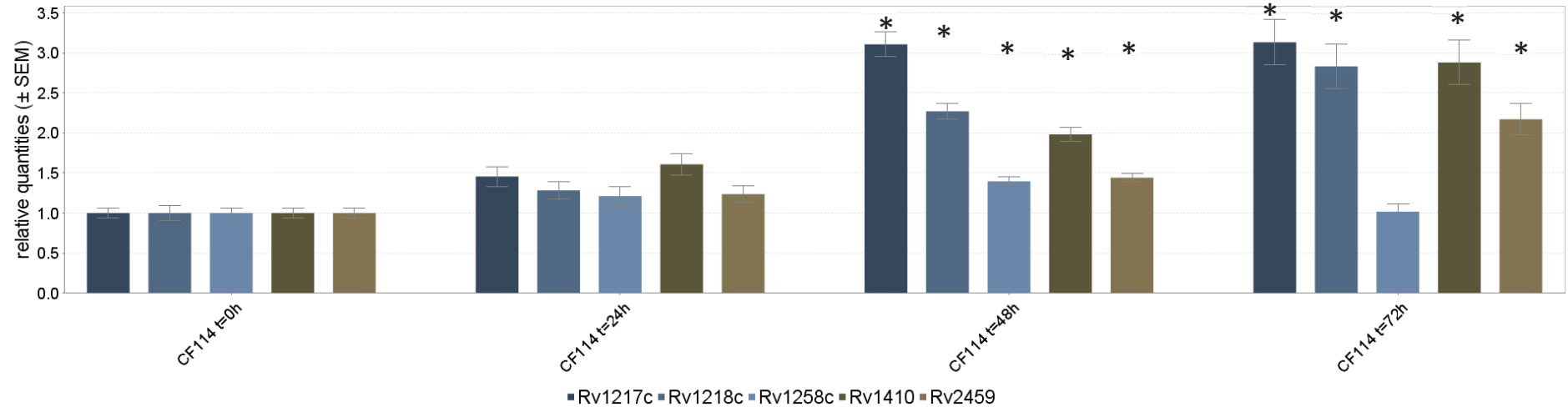


Figura 14: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF114 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 13:** Expressão de genes de efluxo para isolado clínico CF114

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF114 t=0h	1,00 ± 0,06	1,00 ± 0,09	1,00 ± 0,06	1,00 ± 0,06	1,00 ± 0,06
CF114 t=24h	1,46 ± 0,12	1,28 ± 0,11	1,21 ± 0,12	1,61 ± 0,13	1,24 ± 0,10
CF114 t=48h	3,11 ± 0,15	2,27 ± 0,10	1,40 ± 0,06	1,98 ± 0,08	1,44 ± 0,05
CF114 t=72h	3,13 ± 0,28	2,83 ± 0,27	1,02 ± 0,10	2,88 ± 0,28	2,17 ± 0,20

**Tabela 13:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF114 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 15:** Expressão de genes de efluxo para isolado clínico CF145

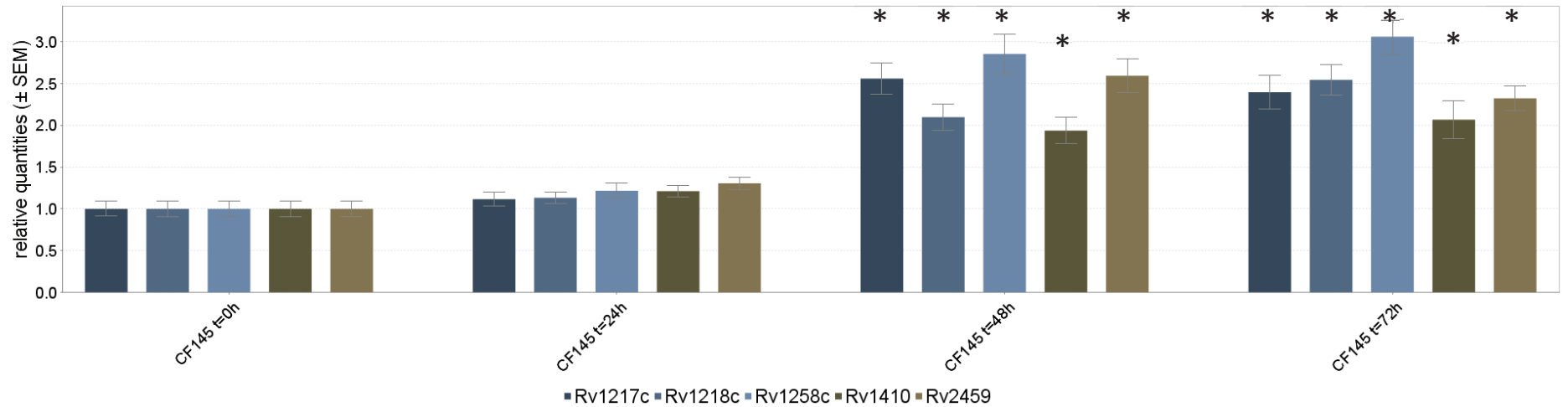


Figura 15: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF145 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 14:** Expressão de genes de efluxo para isolado clínico CF145

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF145 t=0h	1,00 ± 0,09	1,00 ± 0,09	1,00 ± 0,09	1,00 ± 0,09	1,00 ± 0,09
CF145 t=24h	1,12 ± 0,08	1,13 ± 0,07	1,22 ± 0,09	1,21 ± 0,07	1,31 ± 0,08
CF145 t=48h	2,56 ± 0,19	2,10 ± 0,16	2,85 ± 0,24	1,94 ± 0,16	2,59 ± 0,20
CF145 t=72h	2,40 ± 0,20	2,54 ± 0,18	3,06 ± 0,21	2,07 ± 0,23	2,32 ± 0,15

**Tabela 14:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF145 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 16:** Expressão de genes de efluxo para isolado clínico CF158

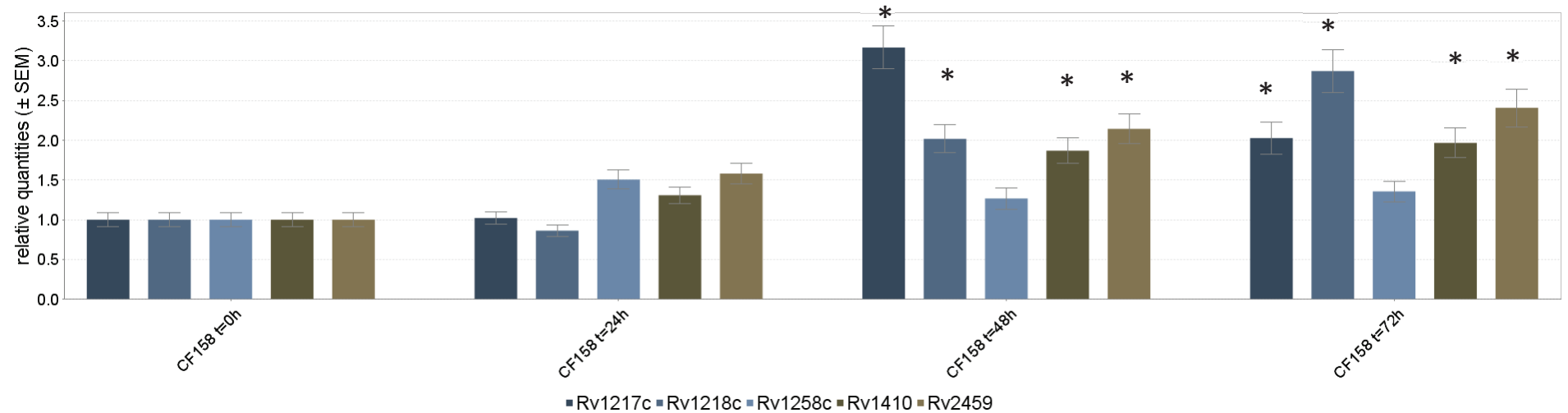


Figura 16: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF158 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 15:** Expressão de genes de efluxo para isolado clínico CF158

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF158 t=0h	1,00 ± 0,09	1,00 ± 0,09	1,00 ± 0,09	1,00 ± 0,09	1,00 ± 0,09
CF158 t=24h	1,02 ± 0,08	0,86 ± 0,07	1,51 ± 0,12	1,31 ± 0,10	1,58 ± 0,13
CF158 t=48h	3,17 ± 0,27	2,02 ± 0,18	1,27 ± 0,14	1,87 ± 0,16	2,14 ± 0,19
CF158 t=72h	2,03 ± 0,20	2,87 ± 0,27	1,36 ± 0,13	1,97 ± 0,18	2,41 ± 0,24

**Tabela 15:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF158 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 17:** Expressão de genes de efluxo para isolado clínico CF168

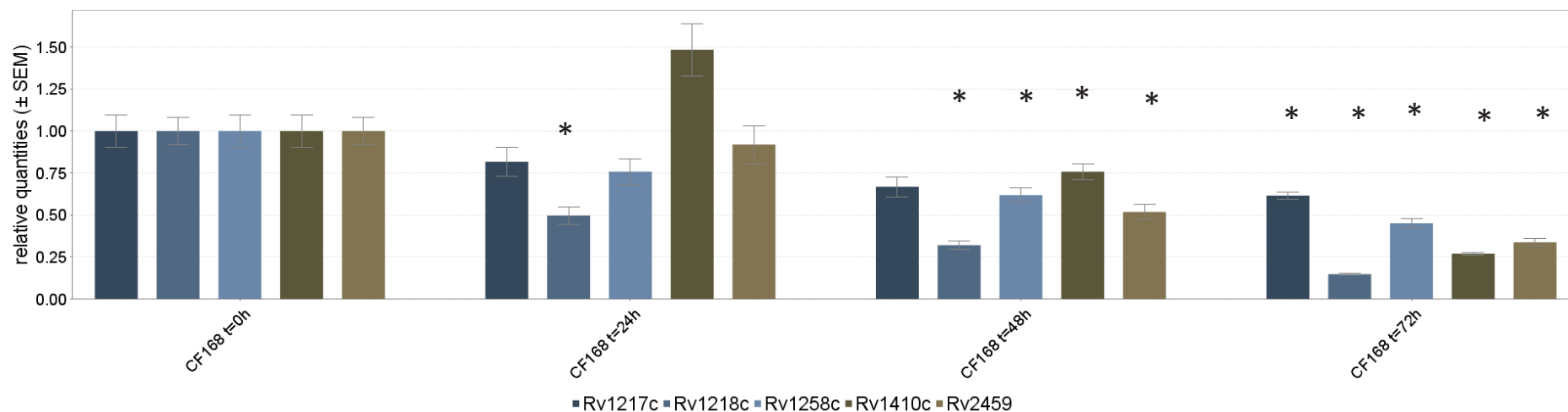


Figura 17: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF168 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 16:** Expressão de genes de efluxo para isolado clínico CF168

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF168 t=0h	1,00 ± 0,09	1,00 ± 0,08	1,00 ± 0,09	1,00 ± 0,09	1,00 ± 0,08
CF168 t=24h	0,82 ± 0,08	0,50 ± 0,05	0,76 ± 0,08	1,48 ± 0,15	0,92 ± 0,11
CF168 t=48h	0,67 ± 0,06	0,32 ± 0,02	0,62 ± 0,04	0,76 ± 0,05	0,52 ± 0,05
CF168 t=72h	0,62 ± 0,02	0,15 ± 0,05	0,45 ± 0,03	0,27 ± 0,05	0,34 ± 0,02

**Tabela 16:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF168 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 18:** Expressão de genes de efluxo para isolado clínico CF185

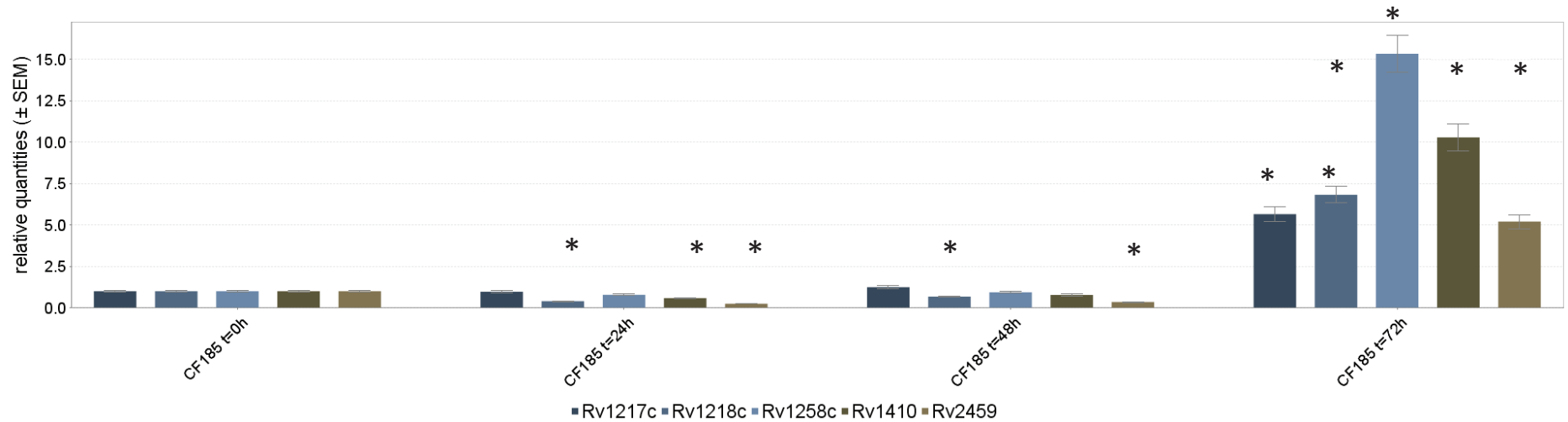


Figura 18: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF185 após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 17:** Expressão de genes de efluxo para isolado clínico CF185

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
CF185 t=0h	1,00 ± 0,06	1,00 ± 0,05	1,00 ± 0,05	1,00 ± 0,05	1,00 ± 0,05
CF185 t=24h	0,97 ± 0,07	0,40 ± 0,02	0,78 ± 0,04	0,58 ± 0,03	0,24 ± 0,01
CF185 t=48h	1,24 ± 0,08	0,68 ± 0,04	0,94 ± 0,06	0,78 ± 0,05	0,35 ± 0,02
CF185 t=72h	5,65 ± 0,46	6,82 ± 0,50	15,33 ± 1,11	10,29 ± 0,80	5,20 ± 0,42

**Tabela 17:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* do isolado CF185 ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

**Figura 19:** Expressão de genes de efluxo para *M. tuberculosis* H<sub>37</sub>Rv

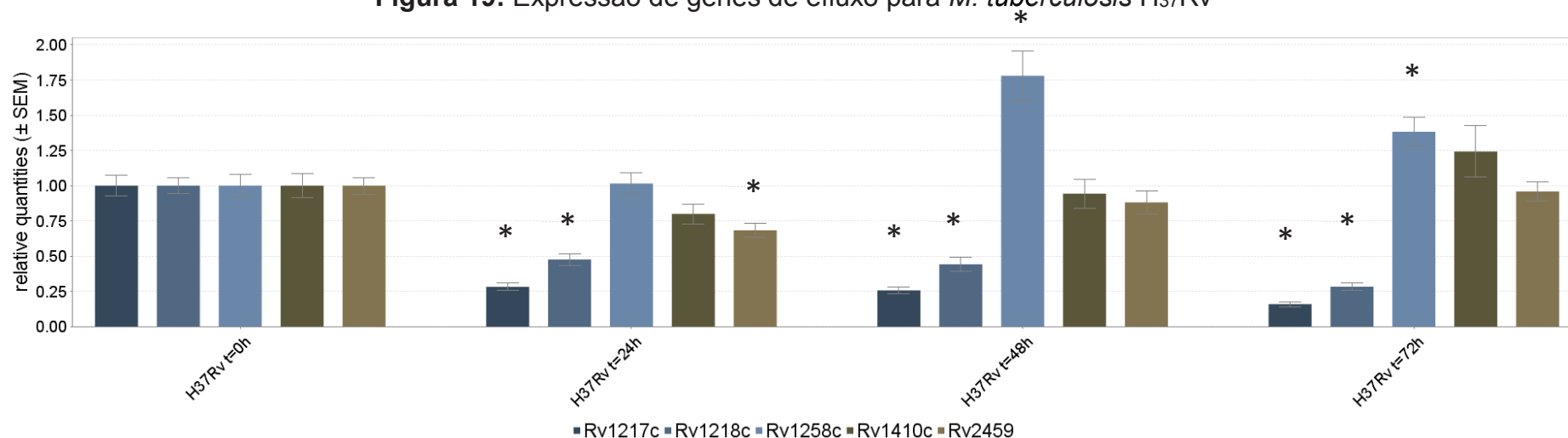


Figura 19: Expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* da cepa padrão H<sub>37</sub>Rv após o tratamento com RIF (1/4 da CIM) em relação ao tempo inicial (t=0h).

**Tabela 18:** Expressão de genes de efluxo para *M. tuberculosis* H<sub>37</sub>Rv

Amostra	Quantidade relativa da expressão em relação ao tempo inicial (t=0h)				
	<i>Rv1217c</i> ± SEM	<i>Rv1218c</i> ± SEM	<i>Rv1258c</i> ± SEM	<i>Rv1410</i> ± SEM	<i>Rv2459</i> ± SEM
H37Rv t=0h	1,00 ± 0,07	1,00 ± 0,06	1,00 ± 0,08	1,00 ± 0,08	1,00 ± 0,06
H37Rv t=24h	0,28 ± 0,03	0,48 ± 0,04	1,02 ± 0,07	0,80 ± 0,07	0,68 ± 0,05
H37Rv t=48h	0,26 ± 0,03	0,44 ± 0,05	1,78 ± 0,17	0,94 ± 0,10	0,88 ± 0,08
H37Rv t=72h	0,16 ± 0,02	0,28 ± 0,03	1,38 ± 0,10	1,24 ± 0,18	0,96 ± 0,07

**Tabela 18:** Valores em número de vezes da expressão relativa dos genes *Rv1217c*, *Rv1218c*, *Rv1258c*, *Rv1410c* e *Rv2459* da cepa padrão H<sub>37</sub>Rv ao longo do tempo (0h, 24h, 48h, 72h), após o tratamento com RIF.

## 4.2. Compostos derivados furoxânicos e benzofuroxânicos

### 4.2.1. Atividade contra cepa-padrão (H37Rv), isolados clínicos resistentes e citotoxicidade em células VERO

Os 15 compostos derivados furoxânicos foram submetidos a ensaios de atividade (cálculo da CIM) contra cepa padrão H<sub>37</sub>Rv e isolados clínicos resistentes já previamente caracterizados (ver **Tabela 2**), além de ensaio de citotoxicidade em células VERO (IC<sub>50</sub>) para os cálculos dos respectivos índices de seletividade (IS). Os dados relacionados à cepa padrão H<sub>37</sub>Rv e células VERO são apresentados na **Tabela 19**, enquanto que os resultados obtidos com os isolados clínicos resistentes são apresentados na **Tabela 20**.

**Tabela 19:** Resultados de atividade e citotoxicidade dos derivados furoxânicos utilizando a cepa-padrão *M. tuberculosis* H<sub>37</sub>Rv ATCC 27294 e células VERO.

Composto	Sigla	Massa molar (g/mol)	CIM (µg/mL)	CIM (µM)	IC <sub>50</sub> (µg/mL)	IC <sub>50</sub> (µM)	IS
C <sub>5</sub> H <sub>6</sub> BrN <sub>3</sub> O <sub>2</sub>	PJ7	220,02	0,70	3,18	3,90	17,72	5,58
C <sub>3</sub> H <sub>3</sub> N <sub>3</sub> O <sub>4</sub>	PJ8	145,07	0,38	2,50	3,90	26,88	10,40
C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>4</sub>	PJ9	401,37	4,74	11,80	>500,00	>1245,73	>105,00
C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>6</sub> S	PJ10	465,44	0,48	1,02	15,60	33,51	32,84
C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>4</sub>	PJ11	401,37	>25,00	>62,30	250,00	622,87	*
C <sub>16</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	PJ12	339,30	>25,00	>73,68	>500,00	>1473,62	*
C <sub>16</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	PJ13	339,30	>25,00	>73,68	500,00	1473,62	*
C <sub>21</sub> H <sub>15</sub> N <sub>5</sub> O <sub>6</sub> S	PJ14	465,44	0,75	1,62	15,60	33,51	20,71
C <sub>22</sub> H <sub>16</sub> N <sub>4</sub> O <sub>7</sub> S	PJ15	480,45	6,02	12,52	11,20	23,31	1,86
C <sub>22</sub> H <sub>16</sub> N <sub>4</sub> O <sub>7</sub> S	PJ16	480,45	5,20	10,82	13,30	27,68	2,56
C <sub>22</sub> H <sub>17</sub> N <sub>5</sub> O <sub>6</sub> S	PJ17	479,46	5,37	11,19	9,90	20,65	1,84
C <sub>17</sub> H <sub>14</sub> N <sub>6</sub> O <sub>5</sub>	PJ18	382,33	>25,00	65,39	>500,00	>1307,77	*
C <sub>17</sub> H <sub>14</sub> N <sub>6</sub> O <sub>5</sub>	PJ19	382,33	>25,00	65,39	>500,00	>1307,77	*
C <sub>14</sub> H <sub>10</sub> N <sub>4</sub> O <sub>4</sub>	PJ20	298,25	>25,00	83,82	>500,00	>1676,44	*
C <sub>13</sub> H <sub>9</sub> N <sub>5</sub> O <sub>3</sub>	PJ21	283,24	5,84	20,62	84,30	297,63	14,43

**Tabela 19:** Resultados de atividade dos compostos furoxânicos frente à cepa padrão H<sub>37</sub>Rv, expressos em CIM, IC<sub>50</sub> em ensaios com células VERO e respectivos valores de IS (índice de seletividade obtido pela divisão do valor de IC<sub>50</sub> pelo valor da CIM). Ensaios foram realizados em triplicata.

**Tabela 20:** Resultados de atividade dos derivados furoxânicos em 5 isolados clínicos de *M. tuberculosis*.

			Atividade dos compostos em isolados clínicos resistentes									
			CF46		CF97		CF104		CF110		CF152	
			$\mu\text{g.mL}^{-1}$	$\mu\text{M}$	$\mu\text{g.mL}^{-1}$	$\mu\text{M}$	$\mu\text{g.mL}^{-1}$	$\mu\text{M}$	$\mu\text{g.mL}^{-1}$	$\mu\text{M}$	$\mu\text{g.mL}^{-1}$	$\mu\text{M}$
Compostos	PJ7	$\text{C}_5\text{H}_6\text{BrN}_3\text{O}_2$	12,98	58,99	1,40	6,36	9,86	44,81	4,70	21,36	5,78	26,27
	PJ8	$\text{C}_3\text{H}_3\text{N}_3\text{O}_4$	1,44	9,92	0,38	2,62	1,84	12,68	0,87	5,99	1,92	13,23
	PJ9	$\text{C}_{21}\text{H}_{15}\text{N}_5\text{O}_4$	*	*	1,99	4,96	19,79	49,3	5,57	13,87	9,75	24,29
	PJ10	$\text{C}_{21}\text{H}_{15}\text{N}_5\text{O}_6\text{S}$	5,32	11,43	1,44	3,09	6,12	13,14	6,86	14,73	3,26	7,00
	PJ11	$\text{C}_{21}\text{H}_{15}\text{N}_5\text{O}_4$	*	*	*	*	*	*	*	*	*	*
	PJ12	$\text{C}_{16}\text{H}_{13}\text{N}_5\text{O}_4$	*	*	*	*	*	*	*	*	*	*
	PJ13	$\text{C}_{16}\text{H}_{13}\text{N}_5\text{O}_4$	*	*	*	*	*	*	*	*	*	*
	PJ14	$\text{C}_{21}\text{H}_{15}\text{N}_5\text{O}_6\text{S}$	19,04	40,90	3,35	7,19	18,29	39,29	14,36	30,85	9,91	21,29
	PJ15	$\text{C}_{22}\text{H}_{16}\text{N}_4\text{O}_7\text{S}$	*	*	4,13	8,59	18,95	39,44	21,50	44,75	25,00	52,03
	PJ16	$\text{C}_{22}\text{H}_{16}\text{N}_4\text{O}_7\text{S}$	*	*	8,61	17,92	*	*	*	*	*	*
	PJ17	$\text{C}_{22}\text{H}_{17}\text{N}_5\text{O}_6\text{S}$	*	*	7,31	15,24	*	*	*	*	*	*
	PJ18	$\text{C}_{17}\text{H}_{14}\text{N}_6\text{O}_5$	*	*	*	*	*	*	*	*	*	*
	PJ19	$\text{C}_{17}\text{H}_{14}\text{N}_6\text{O}_5$	*	*	*	*	*	*	*	*	*	*
	PJ20	$\text{C}_{14}\text{H}_{10}\text{N}_4\text{O}_4$	*	*	*	*	*	*	*	*	*	*
	PJ21	$\text{C}_{13}\text{H}_9\text{N}_5\text{O}_3$	*	*	14,03	49,53	*	*	*	*	*	*

\*CIM não encontrada no intervalo testado (0,098 à 25,000 $\mu\text{g/mL}$ ).

**Tabela 20:** Resultados expressos em CIM dos 15 compostos derivados furoxânicos frente a 5 isolados clínicos (CF46, CF97, CF104, CF110 e CF152) com diferentes perfis de resistência (**Tabela 2**). Ensaios realizados em triplicata.

Os resultados obtidos nos ensaios de atividade dos compostos furoxânicos demonstram um panorama muito favorável para nove das quinze novas moléculas testadas contra a cepa padrão. Porém, um outro aspecto que deve ser levado em consideração é quanto a inocuidade dessas moléculas às células de mamíferos, o que pôde ser avaliada inicialmente através do ensaio de citotoxicidade em células VERO (*screening in vitro*), para a determinação do IC<sub>50</sub>. Após o ensaio da citotoxicidade foi determinado o índice de seletividade (IS), como descrito por Protopopova e colaboradores (Protopopova *et al.*, 2005), e considerados como promissores os compostos com IS $\geq$ 10 (Orme, 2001). Dessa forma, as moléculas PJ8, PJ9, PJ10, PJ14 e PJ21 obtiveram os melhores perfis para a continuidade dos ensaios.

Além de serem promissores biologicamente, em um primeiro momento, os processos de síntese dos compostos foram considerados extremamente satisfatórios, ou seja, moléculas planejadas de modo a serem mais ativas apresentaram maior atividade nos ensaios. Por exemplo, a molécula PJ10 apresentou maior atividade do que a PJ9 por ter grupamento sulfonil ligado ao Carbono da posição 3 do anel furoxânico (o que retira a densidade eletrônica do mesmo) tornando esse carbono

mais susceptível ao ataque nucleofílico dos grupamentos tióis de cisteínas das bactérias, ao invés do grupo fenil encontrado no PJ9. Moléculas como PJ12 e PJ13 não apresentaram atividade provavelmente por apresentarem como substituinte um grupamento metila na posição C-3, que é um doador de densidade eletrônica. Tal alteração diminui a possibilidade de ataque pelos grupamentos tióis. Essa concepção está intimamente ligada com a idéia inicial do trabalho, de que a doação de NO seria o mecanismo essencial para a atividade dessas moléculas, fato que não se comprovou com os experimentos posteriores.

Embora estes compostos inéditos tragam em suas estruturas a subunidade de INH (exceção do PJ7 e PJ8), uma vez que foram sintetizados utilizando o mecanismo de hibridação molecular, a atividade promissora demonstrada frente a isolados resistentes à INH sugere um mecanismo de ação diferente do fármaco de primeira-linha. Outro fato que reforça essa teoria é justamente a atividade demonstrada pelos compostos PJ7 e PJ8, que trazem na estrutura apenas a unidade furoxânica, tendo sido utilizados como precursores para a síntese das outras moléculas da série. É evidente que para o caso de cepas sensíveis à INH, a atividade pode ocorrer por intermédio de ambas as subunidades, atendendo as expectativas da racionalidade empregada na síntese. Mesmo que os novos compostos (derivados furoxânicos) não apresentem, comparativamente, maior atividade que a dos fármacos já utilizados na terapia, seus resultados devem ser ressaltados pois a bactéria provavelmente ainda não possui mecanismos de resistência desenvolvidos a eles, como uma resposta adaptativa

Quando a comparação é realizada com compostos híbridos de N-acil hidrazonas de furoxanila (Hernandez *et al.*, 2013) os resultados são semelhantes, indicando que estes compostos e a estratégia de síntese dos mesmos podem estar na linha de frente para o tratamento da TB em poucos anos.

Aspectos que reforçam a importância do investimento no estudo dos derivados furoxânicos podem ser aqui destacados, como por exemplo, o fato de que no combate à tuberculose é preciso lidar com um microrganismo extremamente ardiloso, que é capaz de adquirir mutações para sobreviver até mesmo nos ambientes mais hostis, manifestando resistência às moléculas de primeira e segunda escolha do tratamento. Exemplos demonstrando essa capacidade são abundantes na literatura: resistência à isoniazida, mediada por mutações nos genes *katG* (Rouse *et al.*, 1996; Cockerill *et al.*,

1995; Sreevatsan, Stockbauer, *et al.*, 1997; Hazbon *et al.*, 2006), *inhA* (Sreevatsan *et al.*, 1996; Miyata *et al.*, 2011; Jnawali *et al.*, 2013), *oxyR-ahpC* (Deretic *et al.*, 1995; Pym, Saint-Joanis e Cole, 2002; Dhandayuthapani, Mudd e Deretic, 1997), *ndh* (Lee, Teo e Wong, 2001; Cardoso *et al.*, 2007) e *kasA* (Mdluli *et al.*, 1998; Ramaswamy e Musser, 1998; Lee *et al.*, 1999), resistência à rifampicina por alterações gênicas em *rpoβ* (Zaczek *et al.*, 2009; Ramaswamy e Musser, 1998; Mohammed, Ahmed e Ahmed, 2013; Makadia *et al.*, 2012), perda de sensibilidade ao etambutol por mutações em *embB* (Yoon *et al.*, 2012; Shi, D. *et al.*, 2011) e pirazinamida por mutações em *pncA* (Scorpio *et al.*, 1997; Sreevatsan, Pan, *et al.*, 1997) e *rpsA* (Shi, W. *et al.*, 2011). Além dessas táticas das quais o bacilo faz uso, pode-se citar também alterações em genes ligados a proteínas ribossomais (mediando resistência aos aminoglicosídeos) (Zaunbrecher *et al.*, 2009; Georghiou *et al.*, 2012; Reeves *et al.*, 2013) e genes relacionados à girases (principal alvo das fluoroquinolonas) (Ginsburg, Grosset e Bishai, 2003; Takiff *et al.*, 1994).

Diante dos bons resultados obtidos com alguns desses compostos e da necessidade urgente por novos fármacos anti-TB (devido à habilidade do bacilo acima explicada), ensaios posteriores foram realizados na tentativa de elucidação do mecanismo de ação dos mesmos, com os resultados descritos na sequência.

#### **4.2.2. Ensaios de acumulação e efluxo de brometo de etídio na presença dos derivados furoxânicos em *M. smegmatis* mc<sup>2</sup> 155**

Para avaliação da capacidade de atuação como inibidores de bombas de efluxo em micobactérias, os compostos foram submetidos a ensaios de acumulação e efluxo de brometo de etídio em *M. smegmatis* mc<sup>2</sup> 155. Considerando que esses ensaios foram feitos em outra espécie de bactéria foi necessário medir a atividade de cada um dos compostos selecionados (PJ8, PJ9, PJ10, PJ14 e PJ21), além dos controles (verapamil, reserpina e CCCP) e do brometo de etídeo contra essa bactéria. Tal fato se fez necessário para que nos ensaios fossem utilizadas concentrações sub-inibitórias dos compostos (1/2 CIM), de modo a manter a bactéria viável para o experimento. A **tabela 21** mostra esses resultados de atividade:

**Tabela 21:** Resultados de atividade dos derivados furoxânicos em *M. smegmatis mc<sup>2</sup>155*

Composto	CIM (µg/mL)	CIM (µM)
PJ8	1.19	8.20
PJ9	10.54	26.26
PJ10	1.51	3.24
PJ14	4.38	9.41
PJ21	12.50	44.13
Verapamil	300.00	652.75
Reserpina	300.00	492.91
CCCP	22.03	107.66
Brometo de etídeo	5.91	14.99

**Tabela 21:** Atividade dos compostos, controles e brometo de etídeo contra a bactéria *M. smegmatis mc<sup>2</sup>155*.

Definidas as concentrações a serem utilizadas de cada composto, foram realizadas as cinéticas de acumulação e efluxo de brometo de etídeo, como demonstram as figuras 20 e 21.

**Figura 20:** Ensaio de acúmulo de EtBr

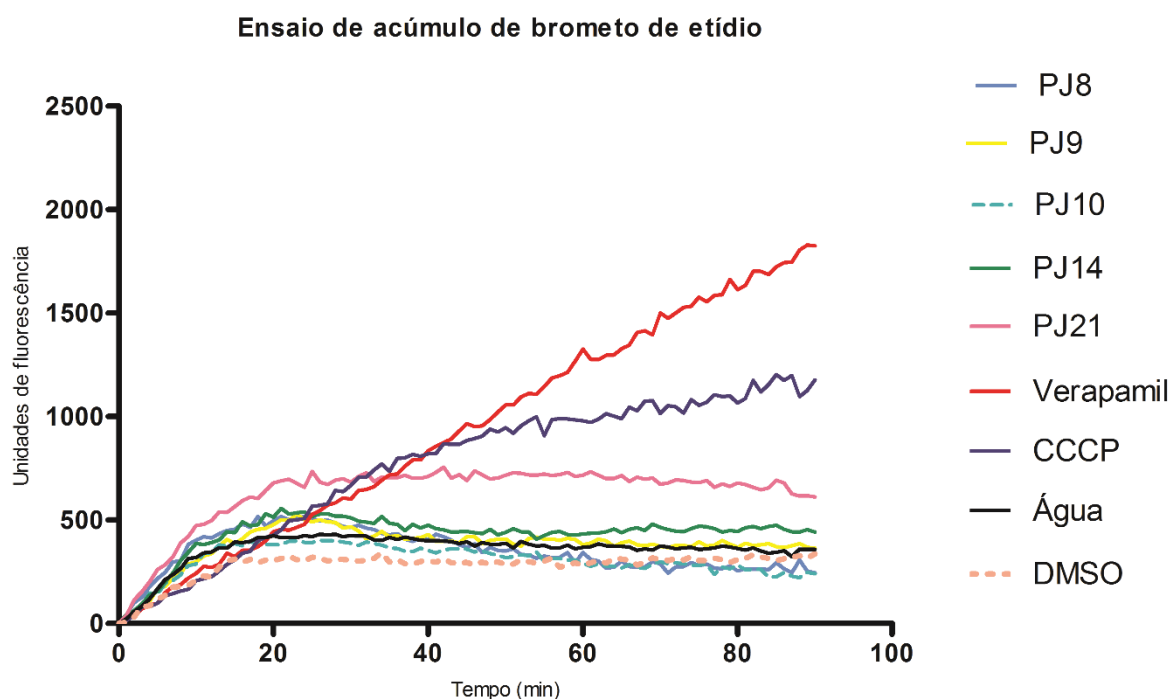


Figura 20: Ensaio de acúmulo de EtBr em *M. smegmatis mc<sup>2</sup>155* na presença dos compostos derivados furoxânicos (PJ8, PJ9, PJ10, PJ14 e PJ21) e dos controles positivos e negativos (verapamil, CCCP, água e DMSO).

**Figura 21:** Ensaio de efluxo de EtBr

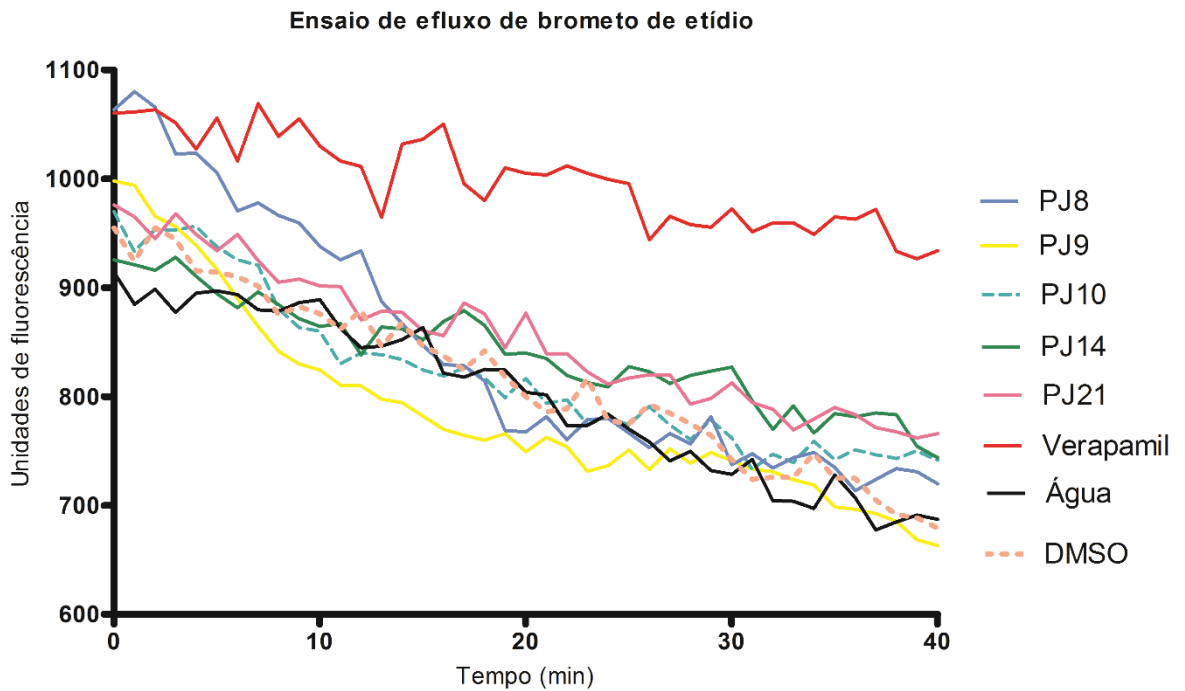


Figura 21: Ensaio de efluxo de EtBr em *M. smegmatis* mc<sup>2</sup>155 na presença dos compostos derivados furoxânicos (PJ8, PJ9, PJ10, PJ14 e PJ21) e dos controles positivos e negativos (verapamil, CCCP, água e DMSO).

A **Figura 20** mostra os resultados do ensaio de acumulação de brometo de etídeo (EtBr) no interior de *M. smegmatis* mc<sup>2</sup>155 após o tratamento com os compostos. Pode-se observar que com exceção do composto PJ21, todos os outros compostos apresentaram leituras semelhantes ao controle negativo (DMSO), indicando que não atuam, pelo menos na concentração testada (1/2 CIM), inibindo as bombas dessa bactéria. Mesmo o composto PJ21, que apresentou diferenças com relação ao controle negativo, chegando até mesmo a ter um padrão semelhante a um dos controles positivos (CCCP), não foi capaz de manter esse perfil, permitindo o efluxo de EtBr a partir do minuto 20, em contraste com os controles positivos.

Na **Figura 21** é representada a cinética de efluxo do EtBr na presença dos compostos derivados furoxânicos. Neste ensaio a bactéria é submetida a um período inicial para acumulação do EtBr em seu interior, e na sequência, com a troca da solução tampão, adição de glicose (0,4%) e dos compostos candidatos a inibidores de efluxo, é avaliada a capacidade do EtBr de permanecer no interior da bactéria. Quando ocorre inibição do efluxo, a fluorescência permanece alta, refletindo a

presença de EtBr intracelular. Como pode ser avaliado na figura, nenhum dos compostos apresentou capacidade de manutenção do EtBr próxima do verapamil (controle positivo), com rápida queda na fluorescência, indicando o perfeito funcionamento dos sistemas transportadores. É importante ressaltar que a fluorescência inicial de cada composto partiu de valores diferentes devido à etapa inicial de acumulação, o que não influenciou no resultado final, já que nenhum dos compostos apresentou valor de  $|\Delta F|$  (  $|\text{Fluorescência final} - \text{Fluorescência inicial}|$  ) menor ou próximo do verapamil.

Os resultados diferem do comportamento encontrado para moléculas propostas como EPIs na literatura, como tioridazina, clorpromazina (Rodrigues *et al.*, 2008), piperina (Jin *et al.*, 2011) e moléculas fenilpropanóides encontradas em plantas (Roy *et al.*, 2012). É possível observar que tais moléculas não apresentam a potência do verapamil na inibição, mas que possuem uma capacidade de reter o EtBr no interior das células em um grau mediano.

Os resultados apontam para um mecanismo de ação dos compostos não relacionado com a inibição do efluxo, embora ainda exista a possibilidade da ação dos mesmos ser de alguma maneira específica para uma família de transportadores que não realizam o efluxo de EtBr. Tal observação fundamenta-se na ideia de que para alguns microrganismos como *Staphylococcus aureus*, o efluxo de EtBr é feito principalmente via NorA (Rodrigues *et al.*, 2008), uma bomba de efluxo da família MFS, sendo que os relatos de nitração de bombas de efluxo por derivados fenilfuroxânicos ocorreram em transportadores pertencentes à família dos ABC transporters (Fruttero *et al.*, 2010).

O fato é que os ensaios de acumulação/efluxo de EtBr teriam sido muito informativos se fossem observadas leituras próximas aos controles positivos, porém a negatividade do mesmo não é indício suficiente para a elucidação de como essas novas moléculas agem no bacilo causador da TB, indicando apenas que o principal mecanismo de atividade dos mesmos não está relacionado com inibição de efluxo.

#### **4.2.3. Ensaio de liberação de NO pelos compostos derivados furoxânicos**

Considerando a complexidade e a quantidade de experimentos realizados na tese, foi necessário reduzir o número de compostos para alguns dos testes, sendo escolhidos um composto de cada classe (PJ10 – derivado furoxânico e PJ21 –

derivado benzofuroxânico), de acordo com os parâmetros de atividade e citotoxicidade reportados anteriormente e julgando que essas moléculas poderiam ser representativas para todo o grupo.

O ensaio de liberação de NO tem como fundamento técnico transformação química ocorrida com a diaminofluoresceína baseada na reatividade das unidades amina dessa molécula com NO na presença de oxigênio. A nitrosação ocorrida leva à formação de uma molécula triazólica altamente fluorescente que oferece a vantagem de especificidade e sensibilidade para detecção de NO (Nakatsubo *et al.*, 1998).

A importância de constatar a liberação de NO pelas novas moléculas reside no fato de que esta molécula desempenha um papel essencial na resposta do sistema imunológico ao bacilo, além do que já foi discutido previamente a respeito do suposto mecanismo de ação dos furoxanos. NO é um importante mediador produzido por macrófagos durante a infecção por *M. tuberculosis*, e dados na literatura apontam que baixas concentrações do mesmo inibem a atividade da bactéria em processos anabólicos como síntese de DNA, RNA e proteínas. Este fato induz a bactéria a entrar em um estado latente, que é extremamente crítico para a terapia, devido à relativa inércia aos fármacos (Voskuil *et al.*, 2003). Após a infecção os macrófagos aumentam a produção da enzima óxido nítrico sintase (iNOS) e conseqüentemente de óxido nítrico, em uma tentativa de criar um ambiente extremamente hostil para o patógeno. O ambiente, repleto de espécies reativas de oxigênio pode levar ao chamado estresse oxidativo, com conseqüências para a micobactéria, tais como: (1) danos a componentes celulares como lipídios, proteínas e material genético; (2) inibição da multiplicação bacteriana; (3) ativação da resposta inflamatória/antimicrobiana dos macrófagos e (4) modulação da apoptose (Kurthkoti e Varshney, 2012). Dessa forma foi avaliado se de fato as inéditas moléculas furoxânicas poderiam auxiliar no processo, doando NO ao ambiente para causar o *stress* oxidativo.

Os compostos PJ10 e PJ21 foram testados no ensaio, com o objetivo de compará-los, em duas concentrações: 44µM (CIM do PJ21 em *M. bovis* BCG) e 13µM (CIM do PJ10 em *M. bovis* BCG). DETA/NO foi incluído no ensaio como controle positivo para a liberação de NO, enquanto DMSO e água foram incluídos como controles negativos (**Figura 22**).

**Figura 22:** Liberação de NO pelos derivados furoxânicos

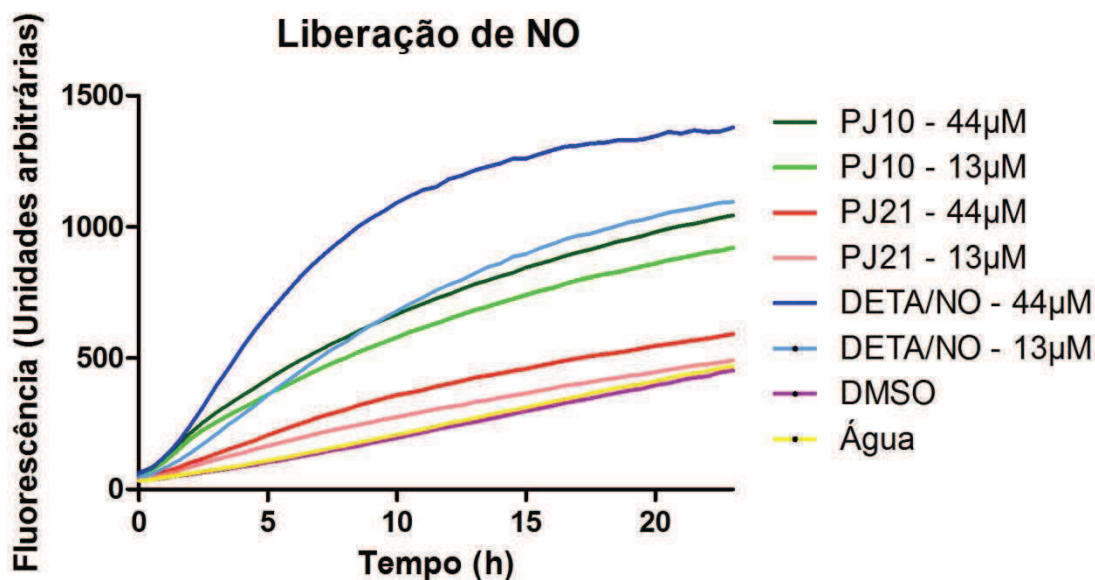


Figura 22: Gráfico de liberação de NO pelos derivados furoxânicos (PJ10 e PJ21), controle positivo (DETA/NO) e controles negativos (DMSO e água) em um tempo total de 24h. Os ensaios foram feitos em triplicata biológica.

A escolha das concentrações de cada composto a ser testado, foi com o objetivo de traçar uma comparação direta entre eles. Caso o *stress* oxidativo gerado pelo NO liberado fosse o principal mecanismo de ação dos compostos, seria esperado observar índices de NO semelhantes quando comparadas as CIM dos dois compostos (13µM para PJ10 e 44 µM para PJ21).

Os resultados demonstram PJ10 com uma capacidade de liberação de óxido nítrico maior que PJ21 (mesmo quando em menor concentração), condizente com os resultados de atividade de ambos. PJ21 na concentração de 13µM (0,3 x CIM) apresentou leituras de fluorescência muito próximas aos controles negativo, indicando praticamente uma inexistência na liberação de NO. Os resultados indicam que a liberação de NO pode participar da atividade das moléculas, mas como um mecanismo assessorio, não desempenhando o papel principal. O controle positivo DETA/NO apesar da massiva liberação de NO não apresentou atividade contra o bacilo até a concentração testada (aproximadamente 153 µM) (dados não mostrados). Tal constatação ainda reforça a ideia de que muito provavelmente a quantidade de

NO liberada pelos compostos no interior da bactéria não seja suficiente para causar danos severos ao seu metabolismo, mais uma vez destacando a vertente de que este não é o mecanismo principal de ação.

#### 4.2.4. Ensaio de *microarray*

Diante das dificuldades para elucidar como os compostos derivados furoxânicos agem no *M. tuberculosis*, com a não confirmação das hipóteses iniciais, foi empreendido um ensaio de *microarray* de modo a observar as diferenças transcricionais ocorridas na bactéria após o tratamento com os compostos. Para isso, a bactéria foi exposta aos compostos testados (PJ10 e PJ21) em uma concentração de 2xCIM, além de também serem expostas ao DMSO como controle negativo, em todos os casos por um período de 4h. As comparações foram feitas de maneira separada, de modo a comparar em uma lâmina as diferenças entre o tratamento com PJ10 e DMSO, e em outra lâmina comparar o tratamento com PJ21 e DMSO. Como observado na **figura 23**, 960 genes apresentaram diferenças significativas na transcrição após o tratamento com PJ10 em relação ao tratamento com DMSO e 723 genes apresentaram diferenças significativas na transcrição após o tratamento com PJ21 em relação ao tratamento com DMSO. Foram considerados apenas genes com alterações de pelo 2 vezes na quantidade de RNA mensageiro.

**Figura 23:** Diagrama representando genes diferencialmente expressos após tratamento com compostos furoxânicos

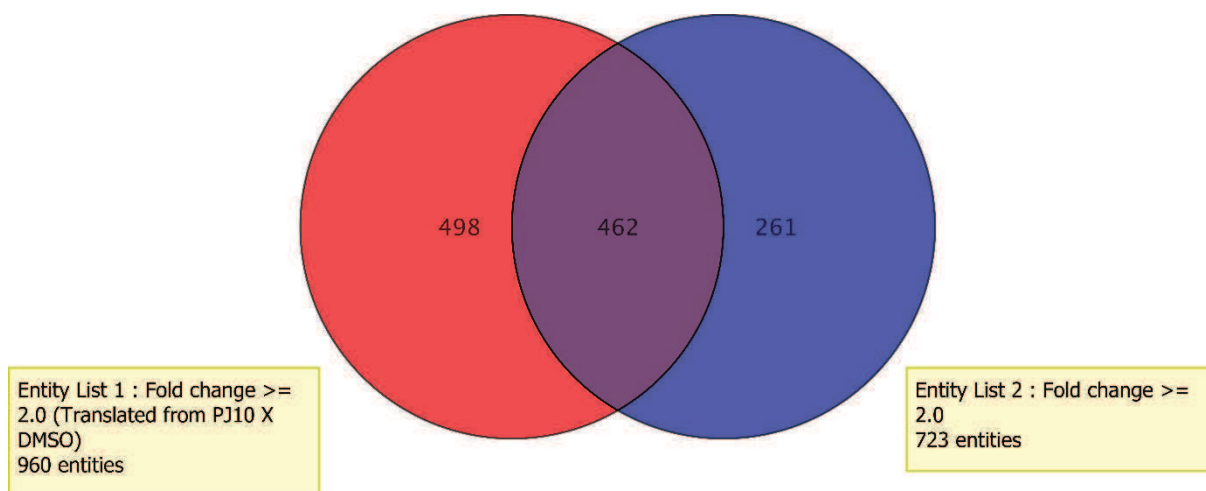


Figura 23: Diagrama de Venn com número de genes diferencialmente expressos pelo tratamento com os compostos PJ10 e PJ21.

Considerando a dificuldade em se avaliar separadamente cada um dos 1221 genes com alteração na transcrição, e considerando que os compostos possuem suas similaridades, optou-se pela avaliação dos genes que foram alterados em ambos os tratamentos (com PJ10 e PJ21). Para facilitar ainda mais a visualização geral desses 462 genes e as principais vias afetadas na bactéria, recorreu-se à ferramenta iTUBY2 (<http://pathways.embl.de/iTuby/>), na qual é possível a visualização do mapa metabólico da bactéria, destacando os genes afetados.

Logo após a utilização da ferramenta, foi notório o destaque para alterações em genes que codificavam para proteínas ribossomais, fato este confirmado pelas **figuras 24 e 25**.

**Figura 24:** Ilustração das proteínas ribossomais alteradas pelo tratamento com PJ10 e PJ21.

## Ribosomal Proteins

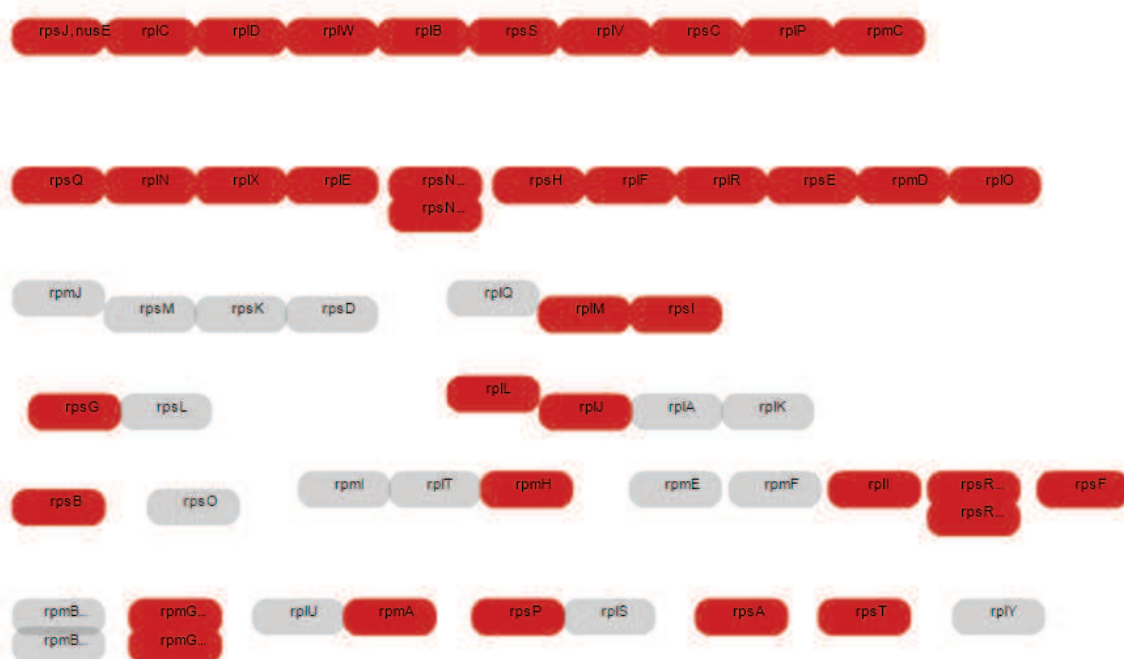


Figura 24: Figura retirada da ferramenta iTUBY2 (<http://pathways.embl.de/iTuby/>), mostrando alterações de transcrição nos genes que codificam para proteínas ribossomais. A cor vermelha indica um aumento no número de RNAs mensageiros transcritos a partir desses genes.

De um total de 57 proteínas ribossomais, 39 apresentaram diferenças de duas vezes ou mais na expressão de seus genes de origem, após o tratamento com os derivados furoxânicos. Apesar de muitas das alterações estarem apenas perto do

valor de *cut-off* (2 vezes), o fato deve ser destacado pois realça alterações de um grande número de proteínas envolvidas em uma via específica. Os resultados apontaram ainda para um maior efeito na via após o tratamento com o composto PJ21, com alterações de 7,80 vezes na expressão de *rpsJ*, 7,35 para *rpsS* e 6,48 para *rpsC*. Essas proteínas apresentam funções específicas na bactéria: RpsJ é uma proteína constituinte do RNAr 30S da bactéria, envolvida na ligação do RNAt nos ribossomos e biossíntese de RNAr, por modular a eficiência da terminação do processo de transcrição (Gopal *et al.*, 2001). RpsS também é parte do RNAr 30S estando associado em complexo com a proteína ribossomal S13, importantes nas etapas de translocação (Cukras *et al.*, 2003). RpsC está relacionada à ligação do RNAt-metionina, etapa necessária para o início do processo de tradução.

A ocorrência de alterações nessa via para ambas as moléculas, poderia sugerir apenas um estado de alerta para uma bactéria tentando lidar com um *stress*. O fato é que a análise foi feita em um tempo inicial curto (principalmente para uma bactéria de metabolismo lento), apenas 4h após o tratamento, o que sugere que essa ação dos compostos na maquinaria de transcrição/tradução ocorre de forma precoce, levantando a hipótese do mecanismo de ação estar relacionado ao impedimento ou complicação desses processos. Outros resultados que corroboram com a hipótese são a diminuição na quantidade de RNAm transcritos (*downregulation*) a partir dos genes *rpoE* (9,17 vezes menos para PJ10 e 3,19 vezes menos para PJ21), *sigB* (6,57 vezes menos para PJ10 e 8,68 vezes menos para PJ21), *sigE* (9,09 vezes menos para PJ10 e 5,85 vezes menos para PJ21). Tais genes e alguns outros que sofreram *downregulation* são fatores de transcrição conhecidos e de importância em *M. tuberculosis* para a iniciação do processo de transcrição.

Resultados semelhantes aos encontrados nesse estudo foram reportados por Boshoff e colaboradores (Boshoff *et al.*, 2004) em um grande trabalho que buscou elucidar as respostas transcriptômicas do bacilo causador da TB frente à várias condições de *stress* e moléculas inibidoras de metabolismo. No trabalho de Boshoff, o mesmo perfil aqui descrito para as moléculas PJ10 e PJ21 foi encontrado após o tratamento com aminoglicosídeos, sendo relatado uma diminuição nos transcritos de genes relacionados à síntese de macromoléculas e um aumento nos transcritos de genes relacionados à arquitetura ribossomal e aparato traducional, até mesmo como uma tentativa compensatória por parte da bactéria.

Ainda na elucidação do provável mecanismo de ação dos compostos, alguns resultados de menor importância podem ser destacados: (1) o tratamento com PJ10 levou a um aumento na quantidade transcritos a partir do gene *iniA* (3,21 vezes), *iniB* (5,82 vezes) e *iniC* (3,05 vezes). Esses genes são induzidos na presença de INH (*isoniazid inducible gene*) (Alland *et al.*, 2000), indicando que a bactéria é capaz de “sentir” a subunidade de INH presente em PJ10, e reforçando a hipótese de que embora este não seja o mecanismo de ação principal/único da molécula (pois foi ativa contra isolados resistentes à INH), a subunidade de INH pode ser importante no combate aos bacilos sensíveis. Outro resultado a ser destacado (2) foi a ausência de diferenças na expressão de genes relacionados ao *dosR*, um conhecido regulon em *M. tuberculosis* relacionado à resposta ao *stress* oxidativo e nitrosilativo (Takii, 2015). Esse resultado, corrobora com os dados de liberação de NO pelos compostos no ambiente intracelular, indicando que provavelmente *stress* oxidativo/nitrosilativo não possui papel central na ação dos compostos.

**Figura 25:** Heatmap mostrando alteração no número de transcritos a partir de genes de proteínas ribossomais.

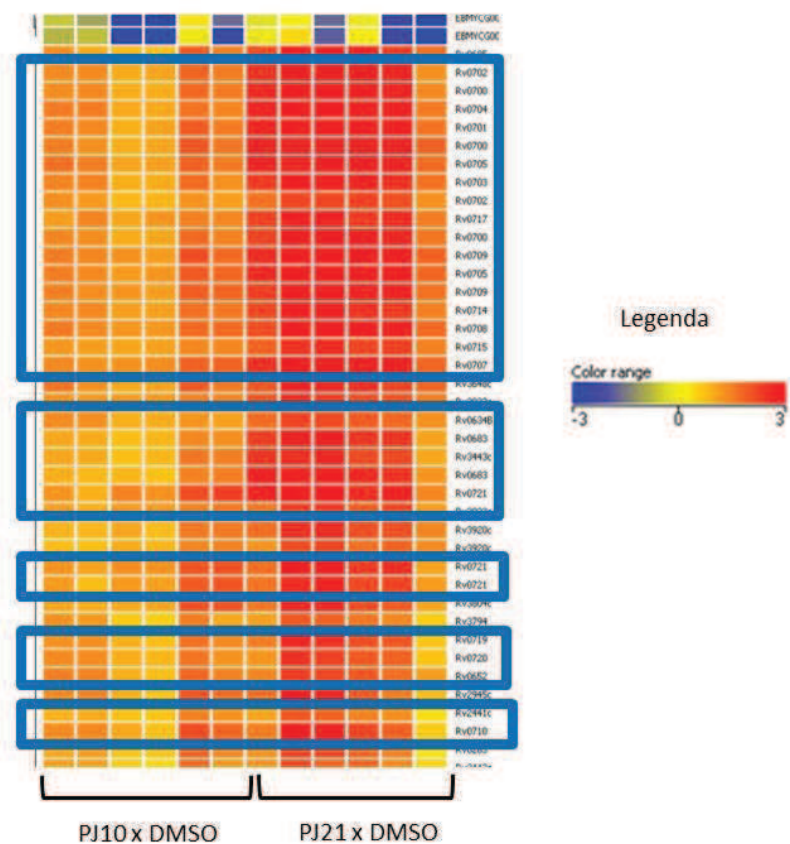


Figura 25: Heatmap gerado com o software GeneSpring 13.1 (Agilent Technologies) para demonstrar as alterações na transcrição dos genes. Destacados dentro dos quadros azuis estão os genes que codificam para proteínas ribossomais.

Compilando esses dados, concluímos que os derivados furoxânicos podem ser moléculas com grande futuro na terapia contra TB, atuando de maneira semelhante aos aminoglicosídeos (em geral fármacos de 2<sup>a</sup> linha para TB, como canamicina, amicacina e capreomicina). Essa atuação em maquinaria de transcrição/tradução ainda pode ser inédita, uma vez que todo o processo é extenso, e, dessa forma, os furoxânicos poderiam atuar em etapas diferentes dos aminoglicosídeos, evitando a existência de uma possível resistência cruzada. Obviamente, os resultados de *microarray* ainda serão validados por ensaios de RT-qPCR e ensaios mais específicos para avaliar transcrição e tradução na presença dos compostos estão em andamento, não contemplados nesse trabalho apenas por questão de tempo.

### 4.3. Halotolerância em *Mycobacterium tuberculosis*

Para avaliar a influência da concentração de NaCl na atividade dos fármacos em *M. tuberculosis* foram testados 12 fármacos de 1<sup>a</sup> e 2<sup>a</sup> linha para o tratamento: isoniazida (INH), etambutol (ETA ou EMB), etionamida (ETH), rifampicina (RIF), D-cicloserina (DCS), canamicina (KAN), estreptomicina (STR), gentamicina (GEN), amicacina (AMK), ciprofloxacina (CIP), gatifloxacina (GAT) e moxifloxacina (MOX). A atividade dos fármacos foi avaliada inicialmente pela técnica do REMA, com a utilização de meios de cultura (7H9) contendo diferentes concentrações de NaCl (10mM, 125mM, 250mM e 1000mM).

Após 1 semana de incubação da bactéria na presença dos fármacos e nas concentrações de NaCl de 125mM, 250mM e 1000mM, constatou-se após adição de resazurina, morte bacteriana frente a todos os fármacos em todas as concentrações de NaCl testadas.

Considerando que em situações *in vivo* a bactéria é capaz de lidar com tais concentrações de NaCl, hipotetizou-se que talvez fosse necessário um período de pré-adaptação ao *stress* osmótico antes da exposição aos fármacos. Com isso, antes do ensaio de REMA, inóculos bacterianos foram expostos por 1 semana nas diferentes concentrações de NaCl (10mM, 125mM, 250mM), para sua adaptação. Na sequência foram submetidos ao teste de susceptibilidade aos fármacos. Os valores de CIM obtidos são apresentados na **figura 26**.

**Figura 26:** CIM<sub>90</sub> dos fármacos testados nas diferentes concentrações de NaCl

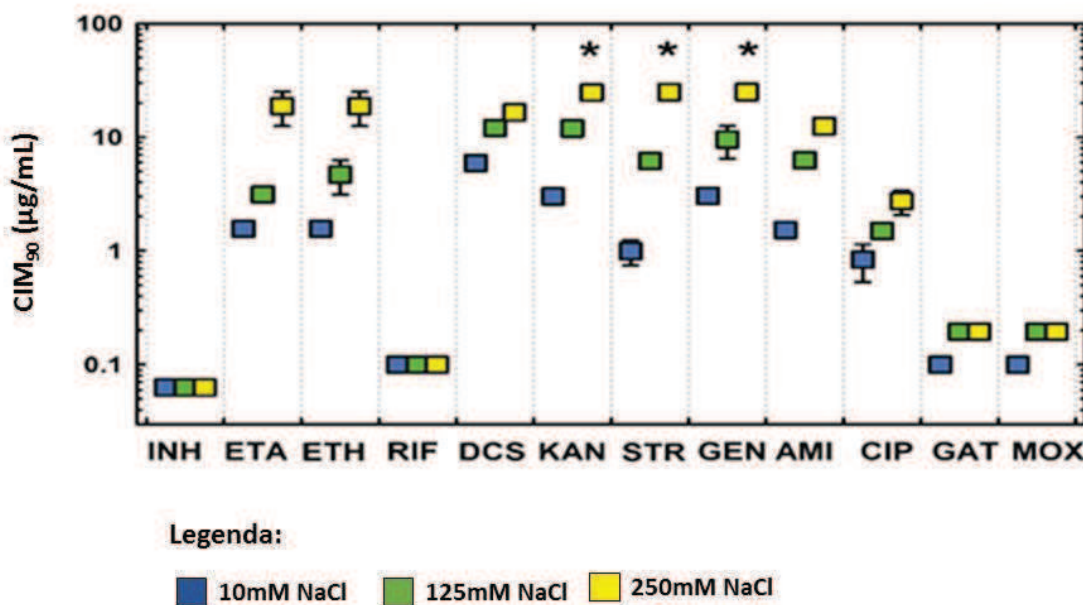


Figura 26: Gráfico com as concentrações inibitórias mínimas dos fármacos testados com suas variações conforme ocorre a mudança da concentração de NaCl no meio de cultura.

Na fase de adaptação em 1000mM de NaCl, a multiplicação bacteriana foi praticamente interrompida, impedindo a realização do ensaio subsequente nesta concentração salina. Nas demais concentrações de NaCl, não houve alteração nos valores das CIM<sub>90</sub> para os fármacos INH e RIF. Esse resultado indica que as mudanças não são causadas por efeitos na taxa de multiplicação bacteriana, pois se assim fosse, as CIM<sub>90</sub> de ambas teriam sido alteradas com o aumento do stress osmótico.

Resultados surpreendentes foram encontrados para os fármacos etambutol, etionamida e para os aminoglicosídeos. Para etambutol e etionamida os valores de CIM<sub>90</sub> aumentaram praticamente 25 vezes quando comparadas as incubações bacterianas nas concentrações salinas de 10mM (CIM<sub>90</sub> 1,56µg/mL) e 250mM (CIM<sub>90</sub> 25,00µg/mL). Comparativamente, nas mesmas concentrações salinas, para canamicina o valor saltou de 3,01 µg/mL para 24,71µg/mL, com um aumento de aproximadamente 8 vezes na CIM<sub>90</sub>. Estreptomicina, gentamicina e amicacina, apresentaram respectivamente, alterações de 25 vezes (de 0,99µg/mL para

24,92 $\mu$ g/mL), de 8 vezes (de 3,05 $\mu$ g/mL para 25,00 $\mu$ g/mL) e também de 8 vezes (de 1,53 $\mu$ g/mL para 12,50 $\mu$ g/mL). D-cicloserina apresentou uma alteração mais discreta, de aproximadamente 3 vezes (de 5,92 $\mu$ g/mL para 16,53 $\mu$ g/mL), enquanto para as fluoroquinolonas as diferenças foram menores, porém não menos importantes. Ciprofloxacina teve seu valor de CIM<sub>90</sub> aumentado em 3 vezes (de 0,84 $\mu$ g/mL para 2,74 $\mu$ g/mL), enquanto gatifloxacina e moxifloxacina em 2 vezes (ambas de 0,10 $\mu$ g/mL para 0,19 $\mu$ g/mL). Em quase todos os casos em que houveram alterações (exceção GAT e MOX), os valores da CIM<sub>90</sub> dos fármacos testados em meio com 125mM de NaCl foram intermediários aos valores encontrados nos testes a 10mM e 250mM, indicando que de fato os aumentos observados foram devido ao também aumento da concentração de NaCl.

Para comprovar que este efeito não estava relacionado à alguma particularidade da técnica, como interação do sal com revelador por exemplo, foi realizada a contagem de unidades formadoras de colônia (UFC) para várias concentrações do fármaco canamicina, escolhido por ter apresentado uma das maiores diferenças entre as concentrações de NaCl (**figura 27**).

**Figura 27:** Efeito da concentração de NaCl no meio de cultura para a contagem de UFC após teste de susceptibilidade à canamicina.

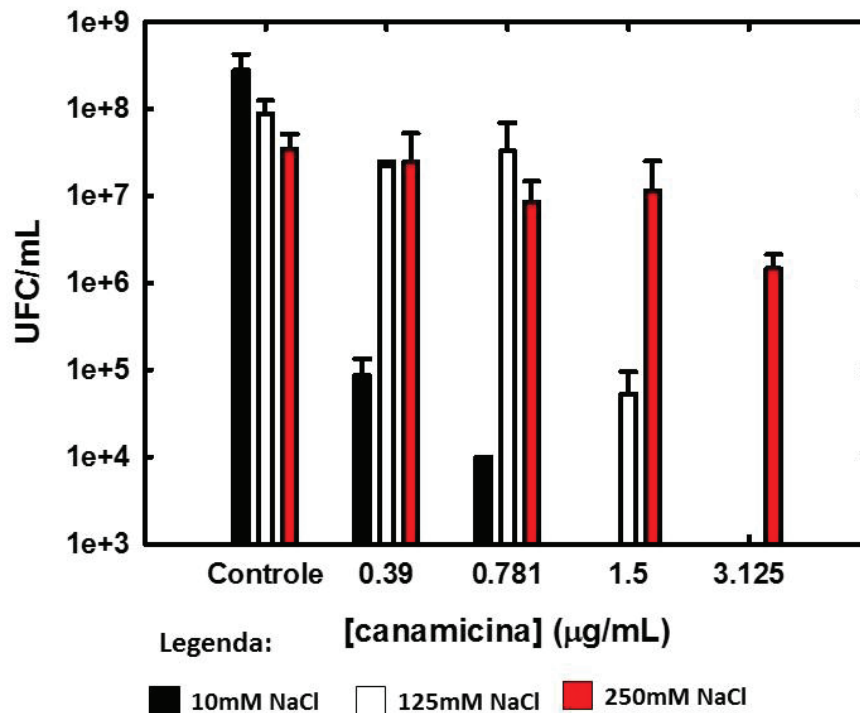


Figura 27: Gráfico para com contagem de UFC/mL para as diferentes concentrações de canamicina (eixo X) nos meios com diferentes concentrações de NaCl.

Como é possível observar no gráfico, uma concentração de 0,39µg/mL de canamicina foi capaz de reduzir, para a bactéria pré-adaptada à 10mM de NaCl, a contagem bacilar da ordem de 10<sup>8</sup>UFC/mL para a ordem de 10<sup>5</sup>UFC/mL, após uma exposição de 7 dias. Para a bactéria pré-adaptada à 250mM de NaCl, não houve praticamente redução alguma na mesma concentração de canamicina, mantendo-se na ordem 10<sup>7</sup>UFC/mL. A submissão à concentração de 1,50µg/mL de canamicina foi capaz de eliminar todos os bacilos pré-adaptados à 10mM, sendo que para os pré-adaptados à 250mM a ordem 10<sup>7</sup>UFC/mL foi mantida. Para a concentração máxima testada, de 3,12µg/mL foi observada a eliminação até mesmo dos bacilos pré-adaptados à 125mM de NaCl, sendo que para os pré-adaptados à 250mM houve apenas a redução de 1log<sub>10</sub>, caindo para a ordem de 10<sup>6</sup>UFC/mL.

Tais resultados indicam que o período de pré-adaptação com *stress* osmótico é essencial para que a bactéria desenvolva certo grau de tolerância a determinados antimicrobianos. Como descrito no trabalho de Masip (Masip, Veeravalli e Georgiou, 2006) com *E. coli*, o mesmo talvez possa ocorrer com *M. tuberculosis*, a existência de 2 fases após exposição ao *stress*: uma fase extremamente prejudicial à bactéria, com danos à inúmeras vias e uma 2ª fase, na qual ela inicia o acúmulo de metabólitos e substâncias osmoprotetoras que permitirão sua adaptação. A exposição da bactéria aos antimicrobianos de forma imediata (como realizado no 1º experimento), pode atingi-la exatamente nessa 1ª fase prejudicial, aumentando os danos e, conseqüentemente levando à total eliminação.

Para provar que tais efeitos não são exclusivos de uma cepa laboratorial padrão (*M. tuberculosis* H37Rv), o experimento foi realizado em 5 isolados clínicos, e os resultados encontram-se na **tabela 22**.

**Tabela 22:** CIM<sub>90</sub> dos fármacos avaliados em 5 isolados clínicos com diferentes concentrações de NaCl no meio de cultura

Isolado Clínico	[NaCl]	CIM <sub>90</sub> (µg/mL)							
		ETH	KAN	STR	GEN	AMI	CIP	GAT	MOX
CF46	10mM	6,20	1,60	3,10	1,60	0,80	0,40	0,10	0,10
	125mM	6,20	4,70	25,00	6,25	3,10	1,20	0,20	0,20
	250mM	12,50	12,50	>25,00	12,50	12,50	1,20	0,40	0,40
CF81	10mM	1,60	3,10	12,50	3,10	1,60	0,40	0,10	0,10
	125mM	6,20	4,70	>25,00	4,70	1,60	1,90	0,30	0,30
	250mM	25,00	18,70	>25,00	>25,00	18,70	6,20	1,20	1,60
CF85	10mM	2,30	9,40	1,60	3,10	3,10	1,20	0,10	0,10
	125mM	3,10	6,20	3,10	6,20	3,10	1,20	0,20	0,20
	250mM	18,70	12,50	12,50	12,50	12,50	2,30	1,00	0,40
CF102	10mM	6,20	3,10	0,80	3,10	1,60	0,80	0,10	0,10
	125mM	6,20	6,20	3,10	6,20	4,70	0,80	0,10	0,10
	250mM	12,50	12,50	6,20	12,50	6,20	0,80	0,20	0,10
CF158	10mM	N.D.	3,00	0,30	1,50	0,80	1,50	N.D.	0,20
	125mM	N.D.	5,00	2,20	5,20	2,50	1,60	N.D.	0,30
	250mM	N.D.	21,00	22,80	22,90	21,00	8,10	N.D.	1,00

N.D.: não determinado

**Tabela 22:** Tabela com as CIM<sub>90</sub> de 5 isolados clínicos avaliadas em meio de cultura 7H9 com diferentes concentrações de NaCl.

Os resultados indicam para os isolados clínicos, a mesma tendência observada na cepa laboratorial padrão, ou seja, aumento no valor da CIM<sub>90</sub> (na maioria dos fármacos testados), com o aumento da concentração salina no meio de cultura. Estas

observações apresentam indícios de que a halotolerância esteja mediando também tolerância à fármacos.

Na sequência buscou-se avaliar se as mudanças ocorridas na bactéria responsáveis pela tolerância eram devidas a mutações cromossomais ou se eram resultado de resistência fenotípica. De uma maneira mais clara, buscou-se avaliar se a etapa de pré-adaptação estaria selecionando mutantes naturalmente resistentes ao *stress* osmótico, ou se estava permitindo ao bacilo criar uma situação de real adaptação. Para essa avaliação desenhou-se um experimento com a inclusão de mais 1 semana de adaptação, conforme exemplificado na **figura 28** e com resultados para gatifloxacina e canamicina na **figura 29**.

**Figura 28:** Experimento para avaliação de resistência fenotípica ou seleção de mutantes resistentes ao stress osmótico

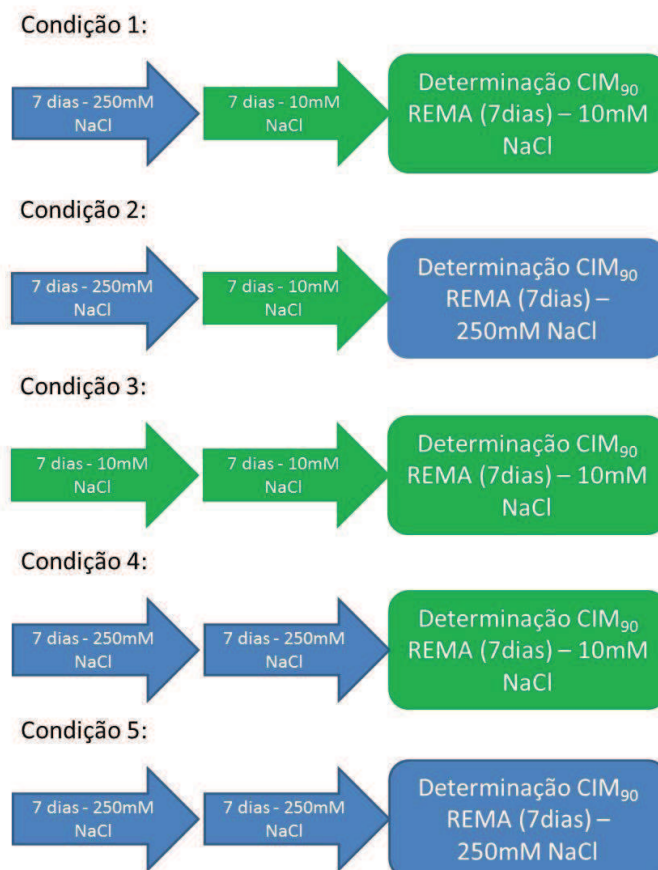


Figura 28: Experimento para avaliação de resistência fenotípica ou seleção de mutantes resistentes ao stress osmótico.

O fundamento desse experimento baseia-se na comparação direta entre as condições 1 e 3, com as demais servindo como controle. Se a tolerância aos fármacos é mediada por uma resistência fenotípica, a CIM<sub>90</sub> da condição 1 deve ser exatamente igual a da condição 3. Caso a tolerância aos fármacos seja produto da seleção de um mutante resistente ao *stress* osmótico, a CIM<sub>90</sub> da condição 1 deve ter valor maior que o da condição 3, uma vez que esse mutante teria sido selecionado no primeiro período de adaptação (250mM de NaCl por 7 dias).

**Figura 29:** CIM90 de gatifloxacina e canamicina perante às diferentes condições de adaptação ao stress osmótico.

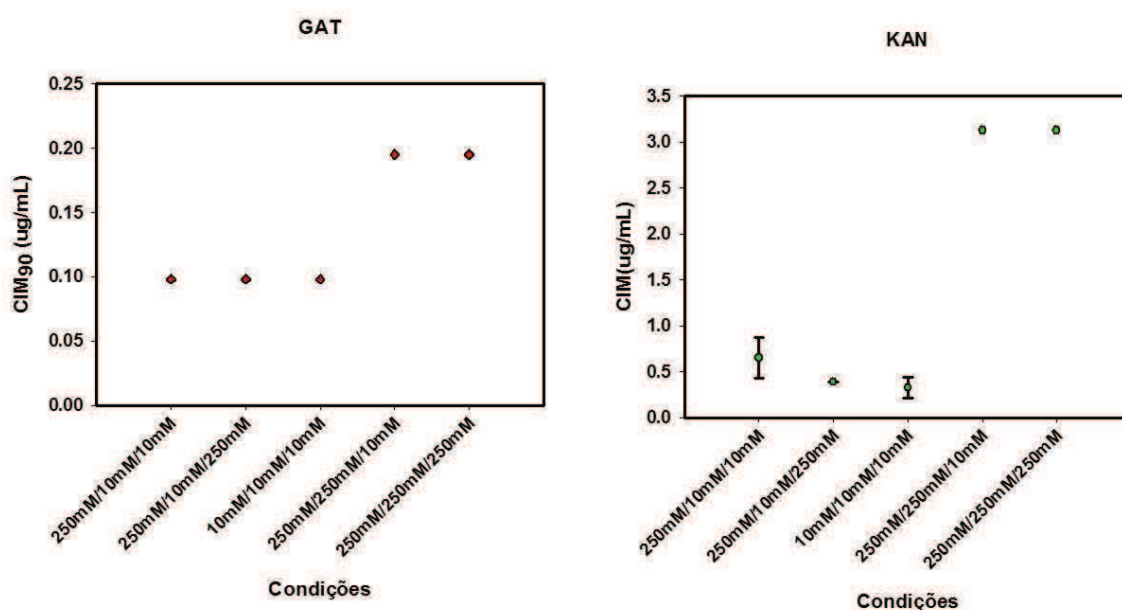


Figura 29: CIM90 de gatifloxacina e canamicina perante às diferentes condições de adaptação ao stress osmótico.

Como é possível observar nos gráficos, os resultados das CIM<sub>90</sub> de gatifloxacina e canamicina foram exatamente iguais para as condições 1 e 3, indicando que a tolerância é mediada por fenômenos fenotípicos. Os resultados indicam que o período de adaptação importante para a determinação da CIM<sub>90</sub> é o 2º período, imediatamente antes do ensaio de REMA. A idéia é que a bactéria lança mão das transformações de que necessita de acordo com a concentração de NaCl a que é submetida.

Vale ressaltar que essa frente da presente Tese é apenas parte de um grande projeto, que incluiu também ensaios de proteômica e metabolômica em busca da elucidação do mecanismo de como a halotolerância pode mediar resistência à fármacos em tuberculose. Tais ensaios não estão contemplado nesse trabalho, uma vez que não foram realizados pelo candidato em questão, porém o manuscrito já se encontra sob revisão na revista *ACS Infectious Disease*. De acordo com esses ensaios complementares, os indícios apontam para uma remodelagem da parede celular da bactéria quando exposta ao *stress*, o que alteraria também seus perfis de resistência aos fármacos.

Apesar de não contemplar o trabalho como um todo, os resultados obtidos nessa frente da Tese permitem conclusões de grande importância, principalmente nos novos rumos da terapia e dos ensaios nas etapas *in vitro* da descoberta de novos fármacos.

Pode-se concluir portanto que, durante o seu ciclo de infecção e desenvolvimento da doença, a bactéria apresenta diferentes perfis de sensibilidade a fármacos, de acordo com o ambiente em que se encontra. O cloreto de sódio é essencial na determinação desses perfis, uma vez que uma bactéria que se adapte a um ambiente com maior concentração do mesmo, pode desenvolver tolerância aos fármacos utilizados. Essa inicial tolerância, representada por um tratamento que acaba utilizando concentrações sub-inibitórias do fármaco, pode se tornar a longo prazo uma resistência mediada pela aquisição de mutações cromossômicas, fato esse extremamente problemático no contexto mundial da doença. Sendo assim, os protocolos de teste de novos fármacos e conduta terapêutica devem ser revistos, considerando a importância que o NaCl possui nas particularidades de cada microambiente frequentado pelo *M. tuberculosis*.

## 5. Conclusões

As conclusões da Tese apresentada são:

### Frente 1:

- RIF é um substrato compatível para os transportadores *ABC transporters* ou MFS;
- Bombas de efluxo participam da emergência de cepas resistentes na população estudada;
- *Rv2459* é uma bomba de efluxo participante da extrusão de RIF;
- Os perfis de resistência de isolados clínicos não determinam uma resposta programada para a expressão de bombas de efluxo. Isolados sensíveis fenotipicamente podem apresentar alta expressão de alguns sistemas de efluxo, o que coloca esse tipo de atividade como um dos primeiros mecanismos de resistência a se manifestar em *M. tuberculosis*;
- A adição de fármacos inibidores de efluxo constitui-se em uma terapia adjuvante muito coerente, considerando a linha de que o tratamento nunca é

monoterápico e deve sempre atingir a bactéria na maior quantidade de alvos possíveis.

## **Frente 2:**

- Compostos derivados furoxânicos e benzofuroxânicos podem ser promissores para a terapia da TB;
- Os compostos presentes nesse estudo não possuem atividade como inibidores de bombas de efluxo;
- O mecanismo principal de ação desses compostos é independente da subunidade de isoniazida, uma vez que os mesmos apresentaram atividade contra isolados clínicos sabidamente resistentes à isoniazida. Porém, como revelado pelo ensaio de *microarray*, a subunidade de isoniazida desempenha atividade em isolados clínicos sensíveis, como observado para PJ10, desempenhando uma atividade sinérgica;
- O principal mecanismo de ação dos compostos independe do óxido nítrico como unidade essencial de um suposto *stress* oxidativo. O NO liberado pode ter pequena participação como adjuvante, porém os ensaios demonstraram que a quantidade liberada ainda não é suficiente para pôr em cheque o metabolismo bacteriano;
- Compostos estudados podem apresentar mecanismo de atividade semelhante aos aminoglicosídeos, atuando em maquinaria de transcrição e tradução, como revelado pelo ensaio de *microarray*.

## **Frente 3:**

- O *Mycobacterium tuberculosis* apresenta perfis de sensibilidade a fármacos diferentes de acordo com o microambiente em que se encontra;
- A concentração de cloreto de sódio no ambiente é um fator primordial para determinar a tolerância da bactéria a fármacos;
- É necessário um período de pré-adaptação do bacilo a altas concentrações de NaCl para que o mesmo desenvolva essa tolerância e a mesma ocorre por alterações fenotípicas e não por mutações cromossomais;

- A bactéria torna-se “mais tolerante” aos fármacos da terapia anti-TB (exceção INH e RIF) após adaptar-se a ambientes de *stress* osmótico;
- Considerando que o macrófago é o principal nicho do bacilo causador da TB, e que a concentração de NaCl no mesmo é de aproximadamente 250mM (25 vezes maior que nos meios de cultura padrão para testes com a bactéria), os protocolos de teste de novos fármacos e conduta terapêutica devem ser revisados. Tal atitude justifica-se, pois, os novos fármacos devem ser testados nas condições mais próximas possíveis do que ocorre *in vivo*, permitindo uma acurácia maior na escolha de compostos promissores. Pelo lado terapêutico, as doses devem ser ajustadas considerando a possível tolerância mediada pelas diferentes concentrações de NaCl nos ambientes frequentados pelo bacilo.

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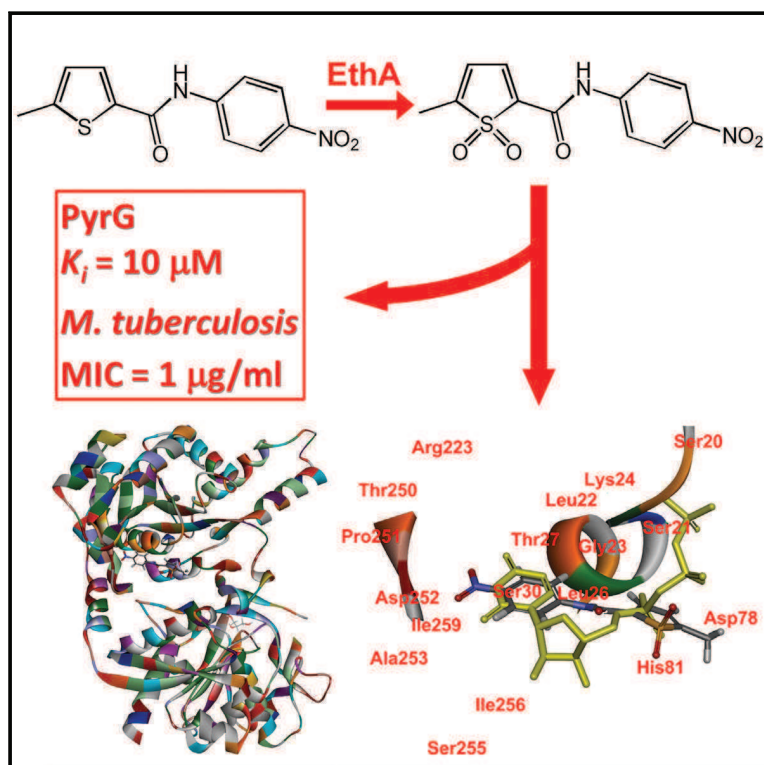
# CAPÍTULO II

**Artigos e trabalhos publicados durante o  
período de Doutorado**

# Chemistry & Biology

## Thiophenecarboxamide Derivatives Activated by EthA Kill *Mycobacterium tuberculosis* by Inhibiting the CTP Synthetase PyrG

### Graphical Abstract



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### In Brief

CTP synthetase PyrG, essential in *Mycobacterium tuberculosis*, could represent a new potential drug target. With a multidisciplinary approach, Mori et al. identify two compounds killing growing and dormant mycobacteria through PyrG inhibition, and define their mechanism of action.

### Highlights

- Two compounds activated by EthA kill *M. tuberculosis* through PyrG inhibition
- EthA metabolite is active against PyrG and *M. tuberculosis* growth
- Definition of the mechanism of activation and validation of PyrG as a new drug target

### Accession Numbers

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4ZDJ  
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# Thiophenecarboxamide Derivatives Activated by EthA Kill *Mycobacterium tuberculosis* by Inhibiting the CTP Synthetase PyrG

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## SUMMARY

To combat the emergence of drug-resistant strains of *Mycobacterium tuberculosis*, new antitubercular agents and novel drug targets are needed. Phenotypic screening of a library of 594 hit compounds uncovered two leads that were active against *M. tuberculosis* in its replicating, non-replicating, and intracellular states: compounds 7947882 (5-methyl-*N*-(4-nitrophenyl)thiophene-2-carboxamide) and 7904688 (3-phenyl-*N*-[(4-piperidin-1-ylphenyl)carbamothioyl]propanamide). Mutants resistant to both compounds harbored mutations in *ethA* (*rv3854c*), the gene encoding the monooxygenase EthA, and/or in *pyrG* (*rv1699*) coding for the CTP synthetase, PyrG. Biochemical investigations demonstrated that EthA is responsible for the activation of the compounds, and by mass spectrometry we identified the active metabolite of 7947882, which directly inhibits PyrG activity. Metabolomic studies revealed that pharmacological inhibition of PyrG strongly perturbs DNA and RNA biosynthesis, and other metabolic processes

requiring nucleotides. Finally, the crystal structure of PyrG was solved, paving the way for rational drug design with this newly validated drug target.

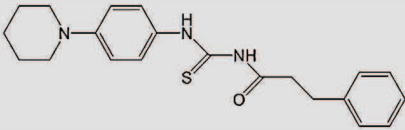
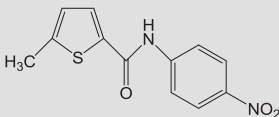
## INTRODUCTION

Tuberculosis (TB) remains a leading cause of infectious mortality worldwide, killing approximately 1.5 million people each year. Drug-resistant strains of *Mycobacterium tuberculosis* threaten global TB management, with an estimated 450,000 cases being multidrug resistant, defined as resistant to rifampin and isoniazid. A subset of these cases, approximately 10%, is also resistant to the second-line drug classes, fluoroquinolones, and injectable aminoglycosides, and is referred to as extensively drug resistant (WHO, 2014).

Defining the pharmacological target(s) of antitubercular drugs under development and finding new compounds with greater potency are both important aspects in the search for agents that are effective against drug-sensitive and drug-resistant *M. tuberculosis* strains (Lechartier et al., 2014). Several current antimycobacterial agents are prodrugs requiring some form of cellular activation before they can bind to their specific targets



**Table 1. Activity In Vitro in Latent and Replicating *M. tuberculosis* Growth and Activity Ex Vivo of the Two Selected Compounds**

Compound ID	Structure	H37Rv MIC ( $\mu\text{g/ml}$ )	ss18b IC <sub>50</sub> /IC <sub>90</sub> ( $\mu\text{g/ml}$ )	Intracellular IC <sub>50</sub> /IC <sub>90</sub> ( $\mu\text{g/ml}$ )
7904688		0.5	2.5/20	0.175/0.625
7947882		0.5	2.5/10	0.625/1.25

and, in such cases, resistance can be mediated by mutations that prevent the activation step. Therefore, understanding the mode of activation not only helps to decipher the mechanisms of drug resistance, but may also facilitate the development of analogs that do not require activation (Dover et al., 2007).

In this work, by screening a library of compounds with known antitubercular activity, established by the National Institute of Allergy and Infectious Diseases (NIAID) (Ananthan et al., 2009; Goldman and Laughon, 2009; Maddry et al., 2009), a new series of molecules was found, displaying a very low minimum inhibitory concentration (MIC) value (0.5  $\mu\text{g/ml}$ ), that includes compounds 7947882 and 7904688. Through the isolation of *M. tuberculosis*-resistant mutants, genetic validation, and biochemical and structural studies, the main mechanisms of activation and resistance of these new antitubercular compounds have been characterized. The combined data indicate that 7947882 and 7904688 are prodrugs activated by the EthA monooxygenase, which then target PyrG, a cytidine triphosphate (CTP) synthetase catalyzing the ATP-dependent amination of uridine triphosphate (UTP) to form the essential pyrimidine nucleotide CTP (Long and Pardee, 1967). CTP synthetase is thus a tractable new TB drug target.

## RESULTS AND DISCUSSION

### Screening of NIAID Library

A library of 594 compounds, selected by high-throughput screening (HTS) against *M. tuberculosis* H37Rv (Ananthan et al., 2009; Goldman and Laughon, 2009; Maddry et al., 2009), was tested for activity against non-replicating *M. tuberculosis* using the streptomycin-starved 18b (ss18b) model (Sala et al., 2010; Zhang et al., 2012). Two promising compounds were identified: a 5-methyl-*N*-(4-nitrophenyl)thiophene-2-carboxamide (7947882) and a 3-phenyl-*N*-[(4-piperidin-1-yl)phenyl]carbamothioyl]propanamide (7904688). Both compounds also showed activity against replicating and intracellular *M. tuberculosis* H37Rv (Table 1). Moreover, the molecules were not cytotoxic to HepG2, A549, Raw, and Huh7 cell lines at concentrations below 40  $\mu\text{g/ml}$ . Compounds were re-purchased from Chembridge Chemical Store (<http://www.hit2lead.com/>) and the results were confirmed.

### Isolation and Characterization of *M. tuberculosis*-Resistant Mutants

To characterize the mechanism of action of 7947882 and 7904688, several spontaneous *M. tuberculosis* mutants resistant

to the compounds were isolated. The spontaneous mutants exhibited the same resistance levels to both drugs (10  $\mu\text{g/ml}$ , 20 $\times$  MIC) (Table 2). Illumina whole-genome sequencing of all mutants revealed mutations either in *ethA* (*rv3854c*), encoding a monooxygenase responsible for ethionamide (ETH) activation (Baulard et al., 2000), and/or *pyrG* (*rv1699*), encoding the CTP synthetase, which performs the ATP-dependent amination of UTP to form CTP as the final step of the pyrimidine nucleotide biosynthetic pathway (Endrizzi et al., 2004) (Table 2). Notably, *M. tuberculosis* mutants resistant to compound 7947882 carried different point mutations in *ethA*, resulting in either an amino acid substitution or a truncated protein. In addition, these mutants all harbored the same mutation in the *pyrG* gene: T557G (Val186Gly). By contrast, no mutations in *ethA* were found in *M. tuberculosis* mutants resistant to compound 7904688, but these all carried the Val186Gly substitution in PyrG (Table 2).

Since *pyrG*, unlike *ethA*, is predicted to be an essential gene in *M. tuberculosis* (Sassetti et al., 2001), it was hypothesized that EthA could be required to activate 7947882 and 7904688 compounds, while the target of the activated metabolites might be PyrG. The finding that all strains harboring a mutation in *ethA* showed cross-resistance to ETH, whereas strains mutated only in *pyrG* remained ETH sensitive, reinforced this hypothesis (Table 2).

### EthA Is an Activator of 7947882 and 7904688 Compounds

To verify whether EthA is responsible for the activation of 7947882 and 7904688, the *ethA* gene was cloned in the expression vector pSODIT-2, and *M. tuberculosis* H37Rv cells were transformed with the corresponding recombinant plasmid. A statistically significant shift in the MIC of the transformants was observed with respect to the control; overexpression of *ethA* in *M. tuberculosis* H37Rv increased the sensitivity to 7947882 and 7904688 (Table S1). Moreover, the overexpression of wild-type *ethA* restored the sensitivity to 7947882 in *M. tuberculosis* 82.14 mutant cells, carrying a mutation in *ethA* (Table S1).

To prove that both compounds were activated by EthA, a recombinant form of the *M. tuberculosis* enzyme was expressed in *Escherichia coli* and purified, and its activity toward the two compounds as substrates was assayed. EthA was active toward both 7947882 and 7904688, with  $k_{\text{cat}}$  values of  $2.9 \pm 0.08$  and  $2.4 \pm 0.15 \text{ min}^{-1}$  and  $K_m$  values of  $0.037 \pm 0.002$  and  $0.055 \pm 0.004 \text{ mM}$  for 7947882 and 7904688, respectively. Moreover, both compounds were better substrates for EthA than ETH,

**Table 2. Main Features of *M. tuberculosis* Mutants Resistant to 7947882 and 7904688**

<i>M. tuberculosis</i> Strains	MIC ( $\mu\text{g/ml}$ )			WGS Sequencing Results (Amino Acid Change)	
	7947882	7904688	ETH	<i>ethA</i>	<i>pyrG</i>
H37Rv	0.5	0.5	1	–	–
82.14	>40	>40	10	T133C (W45R)	T557G (V186G)
82.19	>40	>40	10	T386C (L129P)	T557G (V186G)
82.22	>40	>40	10	$\Delta$ T-94	T557G (V186G)
88.7	5–10	10	0.5	–	T557G (V186G)
88.10	5–10	10	0.5	–	T557G (V186G)
81.10 <sup>a</sup>	>40	>40	10	$\Delta$ 1109–1137	–

See Table S1.

<sup>a</sup>Laboratory collection.

showing  $\sim$ 10-fold higher affinity ( $K_m$  for ETH 0.34 mM), similar to that for phenylacetone, the best EthA substrate found so far ( $K_m$  0.06 mM and  $k_{cat}$   $0.027\text{ s}^{-1}$ ) (Fraaije et al., 2004). The body of genetic and biochemical data strongly suggests that these two compounds are prodrugs that need EthA activation.

#### 7947882 and 7904688 Do Not Affect PyrG Enzyme Activity but Require EthA Activation

To check whether compounds 7947882 and 7904688 were able to inhibit PyrG, their effect on the enzyme activity was evaluated. For this purpose, wild-type PyrG and the V186G mutant protein were produced in *E. coli*, purified, and characterized. *M. tuberculosis* PyrG shows catalytic constants ( $k_{cat}$   $21.9 \pm 0.5\text{ s}^{-1}$  and  $K_m$   $0.18 \pm 0.01\text{ mM}$  toward ATP;  $k_{cat}$   $22.9 \pm 0.9\text{ s}^{-1}$  and  $K_m$   $0.14 \pm 0.01\text{ mM}$  toward UTP) very similar to those of other bacterial CTP synthetases (Anderson, 1983; Long and Pardee, 1967; Willemoës et al., 2005). The PyrG mutant V186G was still active, but partially impaired, displaying reduced  $k_{cat}$  values toward both substrates ( $1.5 \pm 0.11$  and  $1.6 \pm 0.08\text{ s}^{-1}$  for ATP and UTP, respectively). Moreover, the mutant enzyme showed a  $K_m$  value for ATP that was about 10-fold higher than that of the wild-type protein ( $1.46 \pm 0.18\text{ mM}$ ), whereas the affinity for UTP was unchanged.

Since this mutation is associated with resistance to 7947882 and 7904688 (Table 2), it was conceivable that the ATP-binding site was involved in binding the inhibitors. For this reason, the effects of the two compounds were tested on wild-type PyrG at a final concentration of 200  $\mu\text{M}$ . As expected for molecules that need to be activated by EthA, the compounds were ineffective toward PyrG in all the conditions tested.

Thus, to confirm that EthA produces metabolites that might act on PyrG, the EthA enzymatic reaction was performed with either 7947882 or 7904688 in the presence of PyrG, and the activity of the latter enzyme was monitored during the course of the reaction. The blank control was performed omitting reduced

nicotinamide adenine dinucleotide phosphate (NADPH) to hinder the EthA-catalyzed reaction, and under these conditions PyrG maintained full activity for up to 6 hr of incubation. By contrast, in the presence of an actively working EthA, PyrG lost full activity within 4 hr when incubated with 7947882, and about 80% of its activity in 6 hr when incubated with 7904688 (Figures 1A and 1C).

At the end of incubation, to remove EthA as well as any unbound compounds, PyrG was re-purified by Ni-NTA (nitrilotriacetic acid) chromatography and dialyzed. Whereas PyrG from the blank reaction preserved its activity, the enzyme incubated in the full reaction remained completely inactive. Moreover, in the UV-Vis spectrum of PyrG incubated with EthA and 7947882, an additional peak appeared at 330 nm (Figure 1B). This peak, which was not present in the PyrG spectrum from blank reactions without NADPH, is characteristic of 7947882, thus demonstrating that, in contrast to its prodrug, the EthA-activated metabolite is able to bind PyrG. Similarly, the spectrum of PyrG incubated with 7904688 showed the broad peak between 310 and 400 nm, typical of the compound; this peak was absent in the blank control (Figure 1D). These results demonstrated that the conversion of 7947882 and 7904688 by EthA leads to active inhibitors of PyrG.

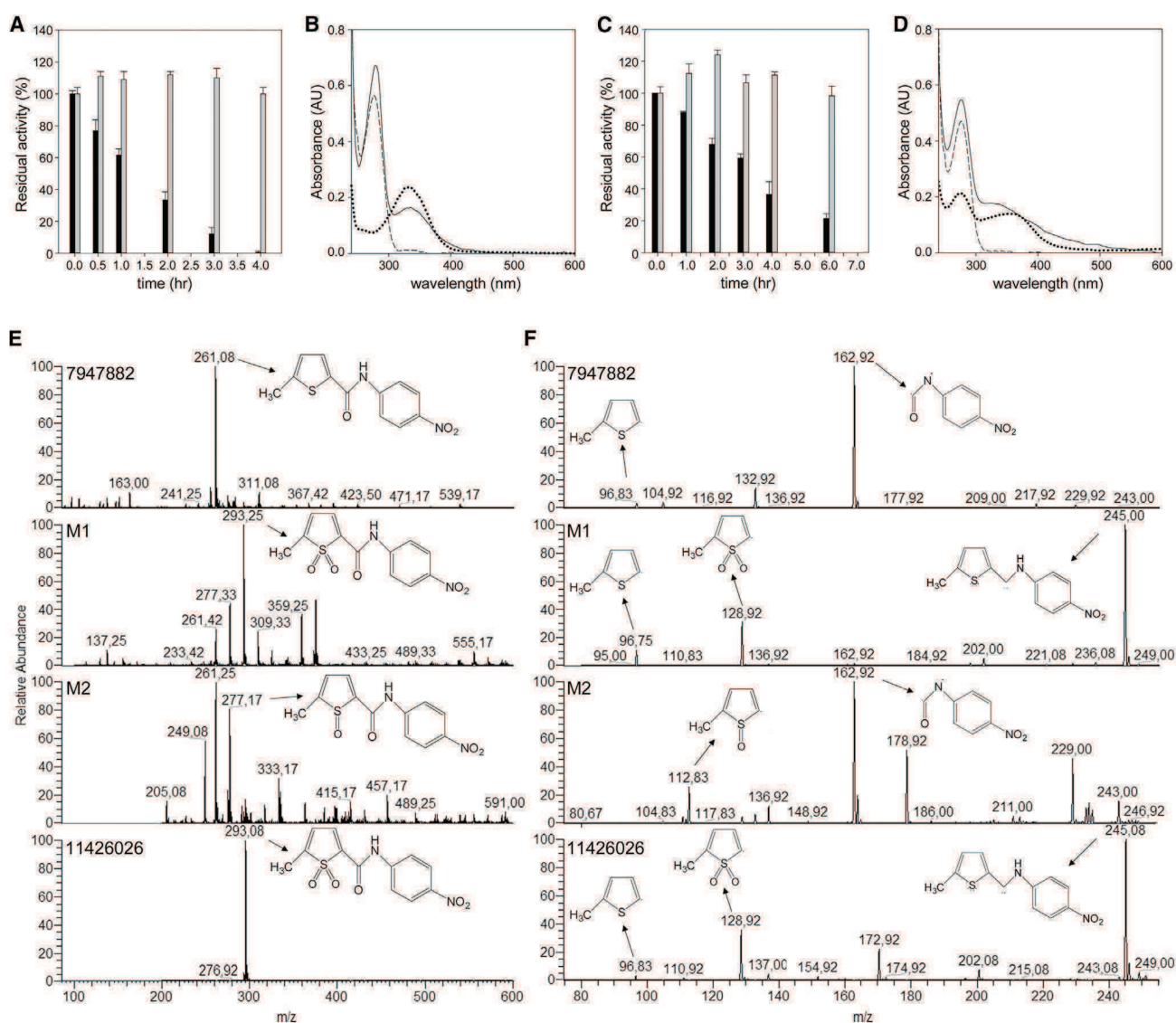
#### Identification of Active Metabolites of 7947882

EthA is known to catalyze the oxygenation of the thioamide moiety of ETH, leading to the formation of S-oxide and S-dioxide products (Vannelli et al., 2002), as well as the oxygenation of the sulfide group of methyl(*p*-tolyl)sulfide (Fraaije et al., 2004). Thus, it is conceivable that EthA might catalyze a similar reaction on the thiophene moiety of 7947882. To confirm this hypothesis, we attempted to identify the active metabolite(s) of the 7947882 prodrug after purification from the EthA reaction mixture.

Two main products (M1 and M2) were isolated and subjected to mass spectrometry analysis. The two isolated compounds showed  $m/z$  values of 293 and 277, respectively, which are in agreement with the S-dioxide and the S-monoxide derivatives of the 7947882 compound. Moreover, the fragmentation spectra of the metabolites showed a pattern similar to that of 7947882, in accordance with mono- and di-oxygenation of the thiophene sulfur atom of the substrate (Figures 1E and 1F). The partially purified metabolites were tested against PyrG protein and found to inhibit its enzymatic activity. Notably, the M1 product showed a higher degree of inhibition.

To better characterize the 7947882 metabolites, its S-dioxide derivative was chemically synthesized, giving rise to compound 11426026. The mass spectrum of 11426026 showed the same pattern as the M1 compound, confirming that M1 corresponds to the 7947882 S-dioxide derivative (Figures 1E and 1F). Therefore, the effects of 11426026 toward *M. tuberculosis* growth and toward PyrG activity were assessed. The MIC of 11426026 for *M. tuberculosis* H37Rv, *ethA*, and *pyrG* mutant strains was determined (Table S2). Wild-type *M. tuberculosis* and the *ethA* mutant were similarly sensitive to 11426026 (with MICs close to that of the parent compound 7947882), showing that 11426026 does not require activation by EthA, whereas the *pyrG* mutant strain was resistant, thus demonstrating that PyrG could be the target of this active metabolite.

Indeed, this was confirmed when the inhibitory activity of 11426026 for PyrG was assessed, since the compound was



**Figure 1. EthA Converts the 7947882 and 7904688 Compounds into Active PyrG Inhibitors**

(A) Inhibition of PyrG activity during the co-incubation with EthA and 7947882. Gray bars correspond to the activities of the blank controls in the absence of NADPH, and black bars represent the residual activities after incubation with working EthA.

(B) UV-Vis spectra of the re-purified PyrG after co-incubation with EthA reaction with 7947882 compound. Solid line is the spectrum of PyrG incubated with full EthA reaction; dashed line is the spectrum of PyrG from blank reaction; dotted line is the spectrum of the compound at 20 μM.

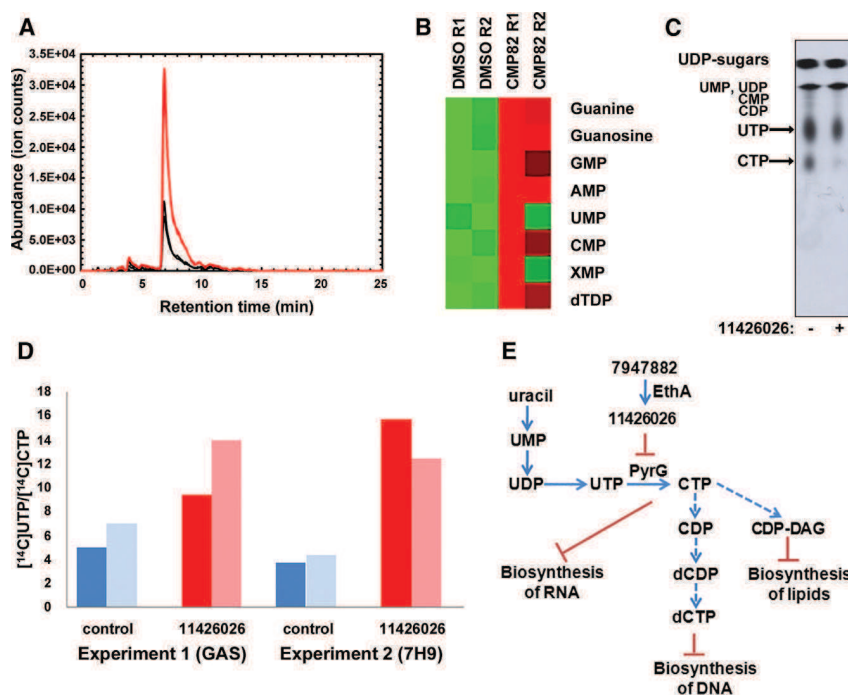
(C and D) Co-incubation of PyrG with EthA and 7904688 compound. Conditions are the same as for (A) and (B), respectively.

(E and F) Identification of in vitro EthA metabolites of 7947882 compound. Mass spectrometry analysis (from top to bottom) of the 7947882 compound, the partially purified products of EthA reaction M1 and M2, and the synthetic metabolite 11426026. (E) Full electrospray ionization mass spectrometry of the compounds recorded in negative mode. (F) Fragmentation pattern of the compounds.

See also [Figures S1](#) and [S2](#); [Table S2](#).

effective against the wild-type enzyme. Interestingly, the inhibitory effects were only found at subsaturating concentrations of ATP ( $IC_{50}$   $0.035 \pm 0.002$  mM in the presence of 0.2 mM ATP). Moreover, the compound was not active against the PyrG V186G mutant when tested under the same conditions. In fact, the estimated  $IC_{50}$  value was 44-fold higher than against the wild-type enzyme ( $1.5 \pm 0.15$  mM), at an ATP concentration of 1.5 mM, which corresponds to the  $K_m$  of the mutant for this substrate ([Figure S1A](#)).

This evidence confirms the hypothesis that 11426026 affects or binds at the ATP-binding site of PyrG, behaving as a competitive inhibitor with respect to ATP ( $K_i$   $0.010 \pm 0.002$  mM; [Figures S1B](#) and [S1C](#)). The high  $K_m$  value of the PyrG V186G mutant for ATP probably reflects the structural changes resulting from the mutation, which distorts the ATP-binding site and leading to an even lower affinity for the 11426026 derivative, thus explaining the resistance to this compound.



**Figure 2. PyrG Inhibition Affects Nucleotide Metabolism in *M. tuberculosis***

(A) Representative EIC for the AMP ion ( $(M + H)^+$ ,  $m/z = 348.07036$ ) illustrating its increased pool size in 7947882-treated *M. tuberculosis* (red lines) compared with control (black lines).

(B) Heatmap illustrating overall changes in nucleotide pool sizes in 7947882-treated *M. tuberculosis* compared with control. Data are derived from two biological replicates.

(C) Thin-layer chromatography of nucleotide extract from [ $^{14}\text{C}$ ]uracil-labeled *M. tuberculosis* H37Ra grown on glycerol-alanine-salts (GAS) medium. The figure is a representative image from three separate experiments.

(D) Ratio of radioactivity incorporated to [ $^{14}\text{C}$ ]UTP and [ $^{14}\text{C}$ ]CTP. Nucleotides were extracted from [ $^{14}\text{C}$ ]uracil-labeled *M. tuberculosis* H37Ra grown on GAS, or 7H9 media and separated by HPLC. The two columns represent duplicate 2-ml samples removed from the radiolabeled culture, processed separately.

(E) Schematic representation of the major effects of PyrG inhibition in mycobacteria.

See also Figure S3.

The same procedure was used to identify the metabolite(s) derived from 7904688. In this case only one metabolite was found, corresponding to 3-phenyl-*N*-[(4-piperidin-1-ylphenyl) carbamoyl]propanamide (Figure S2). This derivative likely arises from sequential EthA reactions on the sulfur atom of the carbamothioyl moiety (Chigwada et al., 2014). However, this last metabolite showed no effect on PyrG activity. It is conceivable that the active metabolite(s) of 7904688 might be an unstable intermediate, thus precluding its isolation.

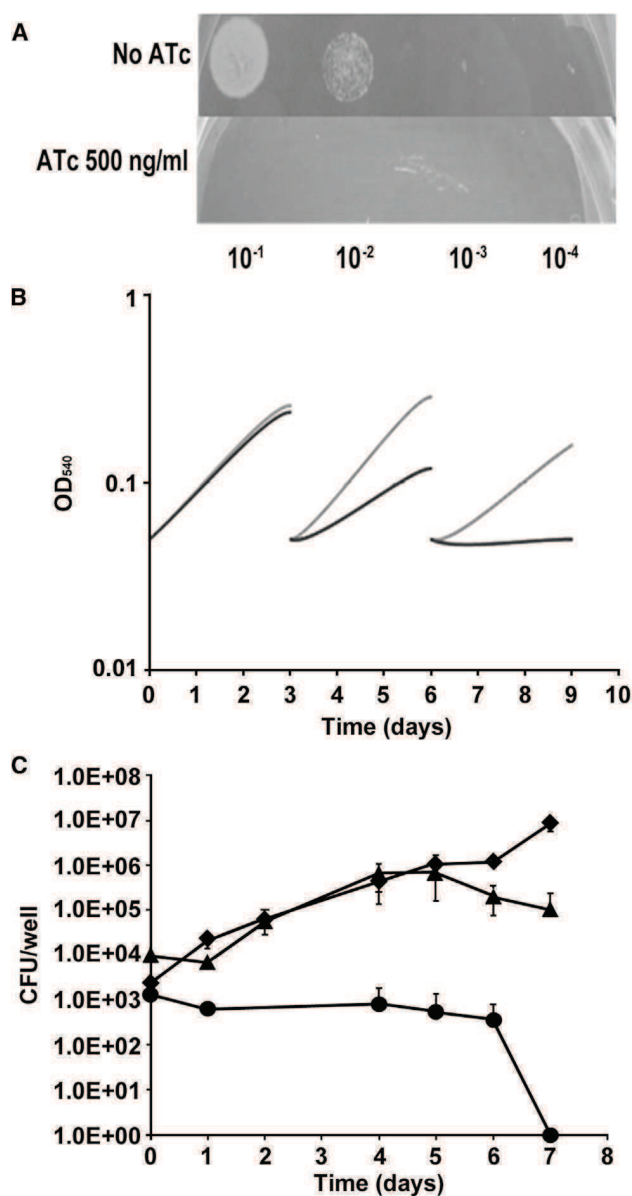
### 7947882 Inhibition of PyrG Alters Nucleotide Metabolism in *M. tuberculosis*

Since PyrG is a key enzyme involved in de novo pyrimidine biosynthesis (Meng et al., 2004), the effect of 7947882 on *M. tuberculosis* nucleotide metabolism was investigated. For this purpose, metabolomic experiments were performed with *M. tuberculosis* exposed for 24 hr to 7947882 (5 $\times$  MIC) or its solvent, DMSO. Polar metabolites were extracted and analyzed by standard methods (de Carvalho et al., 2010; Larrouy-Maumus et al., 2013) that focused on bases, nucleosides, and nucleotides. *M. tuberculosis* H37Rv cells treated with 7947882 showed a substantial increase in the abundance of all nucleotide intermediates that were detected. Figure 2A illustrates extracted ion chromatograms (EIC) obtained for AMP in *M. tuberculosis* extracts treated with either compound or DMSO alone. Compound-induced changes in abundances of the ions detected are shown in Figure 2B. Taken together, these data demonstrate that direct inhibition of PyrG decreased CTP levels, leading to disruption of the nucleotide metabolic network, characterized by increased levels of several intermediates in the biosynthesis of pyrimidines and purines.

The molecular target of thiophenecarboxamides in mycobacteria was further corroborated through metabolic studies with

[ $^{14}\text{C}$ ]uracil and the active metabolite 11426026, using *M. tuberculosis* H37Ra (MIC 4  $\mu\text{g}/\text{ml}$ ) grown in glycerol-alanine-salts (GAS) medium with or without 11426026 (16  $\mu\text{g}/\text{ml}$ ) for 1 hr, then [ $^{14}\text{C}$ ]uracil was added and radiolabeling continued for 3 hr. In the cells [ $^{14}\text{C}$ ]uracil is initially incorporated into [ $^{14}\text{C}$ ]uridine monophosphate (UMP) through the action of uracil phosphoribosyltransferase (Upp) from the pyrimidine salvage pathway (Villela et al., 2011). This is then further metabolized to the whole range of nucleotides and sugar nucleotides originating from uracil. After labeling, the cells were harvested and the nucleotide pool was extracted with diluted formic acid (Bochner and Ames, 1982). In the pilot experiment the PyrG substrate [ $^{14}\text{C}$ ]UTP was separated from the PyrG product [ $^{14}\text{C}$ ]CTP by thin-layer chromatography (TLC). An autoradiograph produced from the TLC plate clearly showed a decrease of [ $^{14}\text{C}$ ]CTP relative to [ $^{14}\text{C}$ ]UTP in treated *M. tuberculosis* compared with the control (Figure 2C). To quantify the changes, the labeling experiment was repeated under the same conditions and the nucleotides were analyzed by high-performance liquid chromatography (HPLC). Individual fractions co-eluting with the set of standards comprising UMP, uridine diphosphate (UDP), UTP, cytidine monophosphate, cytidine diphosphate, CTP, UDP-Gal, UDP-GlcNAc, and UDP-MurNAc pentapeptide were collected and quantified by measuring their radiolabel levels. Although incorporation of radioactivity into [ $^{14}\text{C}$ ]UTP and [ $^{14}\text{C}$ ]CTP was rather low in this experiment, the ratio of [ $^{14}\text{C}$ ]UTP/[ $^{14}\text{C}$ ]CTP did increase in the treated culture, as expected for PyrG inhibition (Figure 2D). Higher incorporation of [ $^{14}\text{C}$ ]uracil was achieved by using 7H9/ADC/Tween medium, thereby confirming the trend of increased [ $^{14}\text{C}$ ]UTP/[ $^{14}\text{C}$ ]CTP following 11426026 treatment (Figure 2D; Figure S3).

In conclusion, these experiments highlighted that inhibition of PyrG affects nucleotide metabolism and, thus, very likely several



**Figure 3. Essentiality of *pyrG* In Vitro and Ex Vivo**

(A) Ten microliters of a *pyrG* cKD mutant suspension containing about  $10^5$  cfu were spotted at the indicated dilutions on Middlebrook 7H10 plates ( $\pm 500$  ng/ml ATc).

(B) Bacteria were grown in 7H9 medium ( $\pm 500$  ng/ml ATc) and diluted 1:10 in fresh media ( $\pm 500$  ng/ml ATc) every 3 days.  $OD_{540\text{ nm}}$  was recorded and used to compile the growth curves. Each experiment was repeated at least twice. Gray line, *pyrG* conditional mutant grown without ATc; black line, *pyrG* conditional mutant grown with ATc.

(C) Growth of *pyrG* conditional mutant and its parental strain (control) in THP-1-derived macrophages at an MOI of 1:20 (bacteria/macrophage). The results are expressed as cfu per well. The reported values represent the average and the SE obtained from two parallel independent infections. The experiment was repeated twice using independent bacterial inocula and THP-1 cultures. ATc (200 ng/ml) was added or not to the cell culture medium. Circles, *pyrG* conditional mutant plus ATc; diamonds, control plus ATc; triangles, *pyrG* conditional mutant, no ATc.

aspects of mycobacterial physiology. In particular, the metabolic changes should interfere not only with DNA and RNA biosynthesis, but also with other metabolic processes that require nucleotides, such as fatty acid, carbohydrate and amino acid biosynthesis, cell wall biosynthesis, and cAMP- and c-di-AMP-dependent signaling (Figure 2E).

#### Validation of *PyrG* Essentiality In Vivo and Ex Vivo

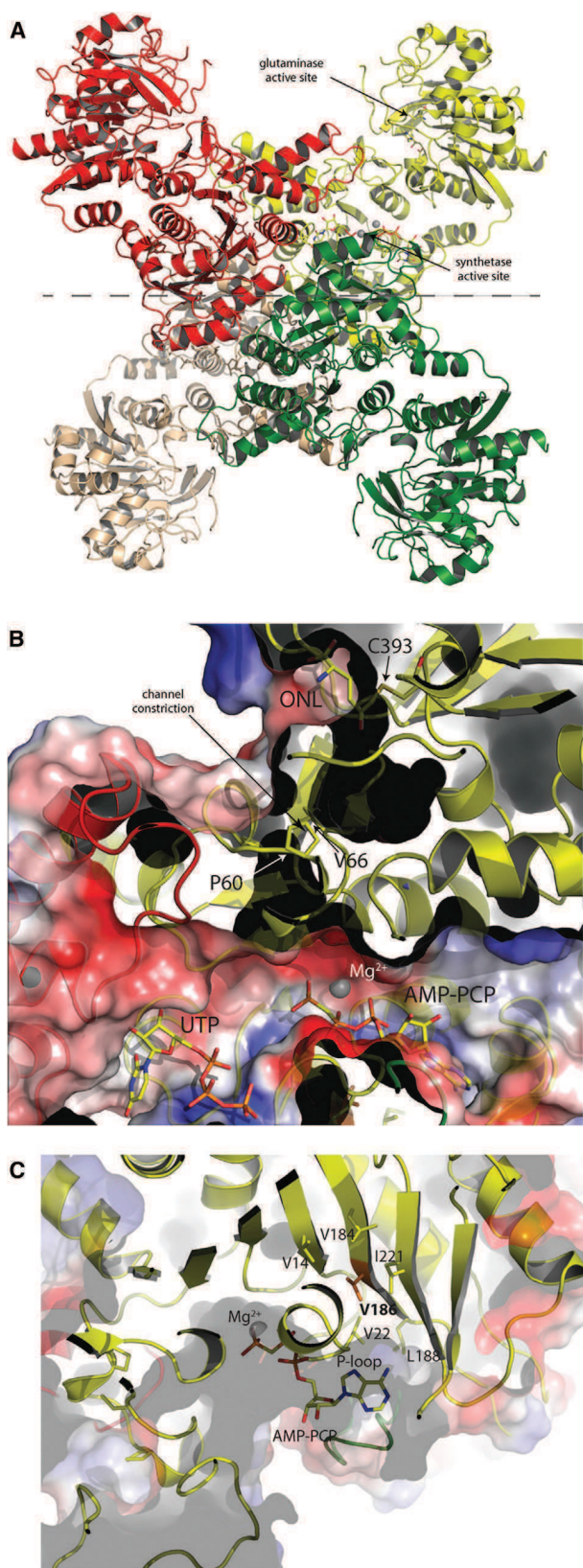
Since *PyrG* inhibition by the active metabolite of 7947882 has been unambiguously demonstrated, its validation as a drug target was further investigated. To show the essentiality of *pyrG* in *M. tuberculosis*, a conditional mutant was constructed where the *pyrG* promoter was replaced by the repressible promoter  $P_{ptr}$  in a strain carrying the TetR-PipOFF repressible system (Boldrin et al., 2010). In this conditional mutant, the expression of *pyrG* was expected to be downregulated by the addition of anhydrotetracycline (ATc) to the culture medium, thus leading to depletion of its protein product. The growth of the *pyrG* conditional mutant was evaluated on solid 7H10 and in liquid 7H9 media ( $\pm$  ATc, 500 ng/ml). In each case, this conditional mutant exhibited inhibition of growth upon ATc exposure, while its parental strain was not affected, thus clearly demonstrating that *PyrG* is essential for *M. tuberculosis* growth in vitro (Figures 3A and 3B).

*PyrG* essentiality was also verified during intracellular growth. For this purpose, THP-1-derived macrophages were infected with the *pyrG* conditional mutant or with its parental strain, and the cells were incubated in the presence or absence of ATc (200 ng/ml). While the control was able to divide intracellularly under both conditions, the *pyrG* conditional mutant grew similarly to the control only in the absence of ATc. When *pyrG* expression was downregulated by ATc, the number of viable bacteria dropped rapidly, demonstrating *pyrG* essentiality also during intracellular growth (Figure 3C). Proof that *PyrG* is essential both in vitro and ex vivo further corroborates the value of this enzyme as a drug target.

#### *PyrG* Crystal Structure

The crystal structure of *PyrG* was solved by molecular replacement on a 2.0-Å resolution data set (Table S3). This structure showed a bidomain enzyme with an N-terminal amidoligase (ALase) domain, also commonly known as the synthetase domain (residues 1–278), connected through an interdomain linker (residues 279–Pro298) to a C-terminal glutamine amidotransferase (GATase) domain (residues 299–552), both domains displaying a Rossmann-like fold (Figure 4A). This bidomain architecture is typical of amidotransferases, already observed in the other available structures of full-length bacterial CTP synthetases (Goto et al., 2004; Endrizzi et al., 2004, 2005; Lauritsen et al., 2011). On the other hand, the 34-residue C-terminal extension of *M. tuberculosis* *PyrG*, which has no predicted secondary structure or known function, could not be traced due to the lack of supporting electron density, suggesting a high degree of flexibility.

The enzyme, in complex with either UTP, at 2.0-Å resolution, or UTP plus the non-hydrolyzable ATP analog AMP-PCP and the glutamine analog 5-oxo-L-norleucine (3.5 Å; Table S3), is a homotetramer with crystallographic 222 symmetry (Figure 4A), consistent with previous studies reporting positive cooperativity



**Figure 4. Crystal Structure of *M. tuberculosis* PyrG**

(A) Tetrameric structure of *M. tuberculosis* PyrG in complex with nucleotides and analogs, i.e. either UTP or UTP/AMP-PCP/L-DON. As observed in the other available crystal structures of CTP synthetases, the N-terminal synthetase domain is positioned at the center of the tetramer while the C-terminal glutaminase domain is pointing outwards. The gray dashed line indicates the PyrG dimers (yellow/red versus green/brown) as they can be found in the apo structure.

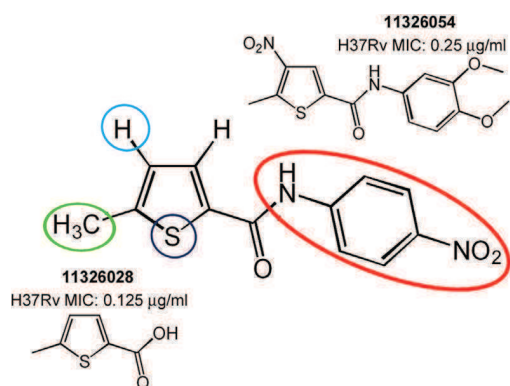
(B) Surface “cut-through” of the synthetase active site, with UTP- and ATP-binding pockets, and “cut section” that shows a possible NH<sub>4</sub><sup>+</sup> channel connecting the glutaminase to the synthetase site. The electrostatic surface potential has been calculated and rendered by PyMol (Schroedinger, <http://www.pymol.org>).

(C) Surface view of ATP-binding pocket, occupied by AMP-PCP, to show the location of Val186 (orange) mutated in Gly in the *M. tuberculosis*-resistant strains. Side chains of hydrophobic residues surrounding Val186 are depicted as sticks.

See also Table S3; Figures S4 and S5.

for ATP and UTP due to nucleotide-driven tetramerization. Indeed, another structure of the enzyme in the apo form at lower resolution (3.5 Å; Table S3), revealed a homodimeric protein, each homodimer representing half of the functional tetramer and showing a dimerization surface of  $\sim 1,350 \text{ \AA}^2$  per monomer (Figure 4A), all in good agreement with previous structural studies (Endrizzi et al., 2004, 2005; Goto et al., 2004; Lauritsen et al., 2011). Also, consistent with the oligomeric assembly as a dimer of dimers being triggered by ATP/UTP, the nucleotide-binding pockets were delimited by residues from two (ATP) or three (UTP) different subunits (Figures S4A and S4B). Surprisingly, in the highest-resolution structure available (Table S3), UTP was found lying in both pockets, a likely artifact due to the high concentration of the nucleotide (5 mM) in the co-crystallization conditions (Figure S4C). It should be noted that the UTP orientation in the substrate-binding pocket is unproductive for the course of the reaction, as the pyrimidine ring points away from ATP. Moreover, this UTP orientation coincides with the CTP orientation observed in *E. coli* PyrG in complex with CTP and ADP (PDB: 2ad5; Figure S4C), suggesting that this structure likely represents an inhibited enzyme (Endrizzi et al., 2005).

In contrast, in the independent crystal form, grown in the presence of AMP-PCP as well as UTP and the glutamine analog 6-diazo-5-oxo-L-norleucine (L-DON), AMP-PCP lies in the ATP-binding site, as expected, with UTP maintaining the same orientation as above (Figures 4B and S4B). In addition, a covalent adduct was observed between the Cys393 sulfur and oxonorleucine, as expected from the reaction with L-DON (Hart and Powers-Lee, 2008), therefore confirming the role of Cys393 as the catalytic nucleophile within a Gln-hydrolyzing triad that includes His524 and Glu526 (Figures S4D and S4E). In agreement with similar observations made on *E. coli* PyrG (Endrizzi et al., 2004), a putative ammonia diffusion channel was visualized connecting the glutaminase active site in the C-terminal domain to the synthetase site at the N-terminal domain (Figure 4B). However, the tunnel is not continuous, but appears to be blocked by the side chains of residues Pro55, Pro60, and Val66, all located on the long  $\beta 2$  to  $\beta 3$  linker that includes the short  $\alpha 2$  (Figures S4D and S4E), forming a constriction in the channel (Figure 4B). The residue Val186 mutated to Gly in the *M. tuberculosis*-resistant mutants lies on the  $\beta 7$  strand behind the conserved P loop (Gly16 to Gly25) that contributes



**Figure 5. SAR Optimization Strategy of 7947882 Compound**

Modifications made on the thiophene ring (positions 4, 5 and the sulfur atom) and the 4-nitroaniline moiety led to two more active compounds (11326028 and 11326054).

See also Tables S4 and S5.

to bind the ATP phosphates, its side chain being at closest  $\sim 7$  Å from the AMP-PCP  $\beta$ -phosphate (Figure 4C). Despite being located in the proximity of the ATP-binding pocket, the Val186Gly substitution does not provide any obvious explanation for the resistance profile to an ATP competitive inhibitor. Moreover, this mutation should have a destabilizing effect on the P loop and on its proper positioning within the ATP-binding cleft, leading to a decreased affinity for ATP. This hypothesis, suggested by the observation that the Val186 side chain is situated in a hydrophobic pocket delimited by Val14, Leu22 (belonging to the P loop), Leu184, Leu188, and Ile221 (Figure 4C), is further supported by the steady-state kinetics analysis of the V186G mutant enzyme reported above.

The availability of the *M. tuberculosis* PyrG structure is useful for both structure-activity relationship (SAR) studies and in silico docking approaches to find new PyrG inhibitors that do not require EthA activation.

### SAR Study of 7947882

To improve the antitubercular activity of 7947882, and to understand the substituent requirements needed to achieve activity against *M. tuberculosis*, SAR studies were performed (Figure 5). 105 derivatives of compound 7947882 were synthesized and tested for their activity against *M. tuberculosis* H37Rv. The substitutions concerned mainly the thiophene ring and the 4-nitroaniline moiety.

Substitution of the thiophene ring (with furan, pyrazole, or methylthiazole) led to inactive compounds (Table S4A). The substitution of the 4-nitroaniline led, in general, to decreased potency of the compounds (Table S4B). The presence of the *p*-nitro group was associated with the best MIC, but was not strictly required, since its substitution with halogen atoms or a methyl group caused only a small increase in MIC. Moreover, the addition of further substituents to the other positions of the phenyl ring did not improve efficacy. For instance, introduction of bulkier substituents, such as *S*-methyl or sulfonamide, or the substitution of the aniline with formimidamide derivatives, was even detrimental for activity, as these compounds all showed a higher MIC (Table S4B).

Furthermore, no improvement arose from modification of the substituents in the thiophene moiety. Lack of the methyl group in position 5 of the thiophene led to an increase in the MIC (Table S4C), as did introduction of a methyl or nitro group in position 4, with the exception of compound 11326054 which showed a lower MIC value (0.25  $\mu\text{g/ml}$ ) (Figure 5; Tables S4D and S4E). Curiously, analogs of this compound lacking the nitro group (11326008) did not show lower MIC (2  $\mu\text{g/ml}$ ). Moreover, all compounds with the thiophene moiety substituted with a nitro group showed lower potency than those lacking this group. Such high activity of compound 11326054 is conceivably connected with the antimycobacterial properties of nitrothiophenes (Hartkoorn et al., 2014), and in parallel with the amide moiety discussed in this paper.

Finally, substitution of the aniline with a hydroxy group, to give the 5-methylthiophene-2-carboxylic acid (11326028), led to the most potent compound (MIC of 0.128  $\mu\text{g/ml}$ , Figure 5). The carboxylic group of compound 11326028 was fundamental for its antimycobacterial activity, since the carboxamide derivative was less active and, likewise for compound 7947882, modification of the thiophene substituents led to less active derivatives (Table S4F).

The *M. tuberculosis* *pyrG* mutant 88.7 showed levels of resistance to both 11326028 and 11326054 derivatives that were significantly higher (MIC values  $> 8$   $\mu\text{g/ml}$ ) compared with that of the wild-type strain, thus confirming that they still target PyrG. Similarly, the 81.10 mutant (mutated in *ethA* gene) was resistant to both compounds, indicating that they still need to be activated by EthA. This result was confirmed by the fact that the compounds are substrates of the enzyme ( $k_{\text{cat}}$  values of  $1.33 \pm 0.02 \text{ min}^{-1}$  and  $0.98 \pm 0.03 \text{ min}^{-1}$  for 11326028 and 11326054, respectively).

Finally, five derivatives of the active EthA metabolite 11426026 were synthesized. These compounds were all active toward wild-type PyrG, but not against the V186G mutant. However, none of these compounds showed an improved MIC compared with the active metabolite of 7947882 (Table S5).

### Docking of the 11426026 Active Metabolite and PyrG Inhibitors

To acquire insight into the binding between the active metabolite of 11426026 and PyrG, a careful computational analysis of the possible poses of the compound was performed. Docking the 11426026 compound demonstrated that it would only successfully dock in the PyrG ATP site (Figures S5A, S5B and S5C). The superimposition with the UTP molecule shows a partial overlap. The phenyl ring is suggested to pi-stack with Arg223 while the nitro group is proposed to interact with Ala253 and Asp252.

Similarity searching based on the 4-nitroacetanilide portion of the molecule resulted in 12 similar compounds present in the Collaborative Drug Discovery (CDD) database (Ekins and Bunin, 2013; Ananthan et al., 2009; Ekins et al., 2014; Maddry et al., 2009; Reynolds et al., 2012). Four of these compounds were tested in vitro against PyrG enzymatic activity. One compound, CDD-823953 (LibDock score 106.7), was a weak PyrG inhibitor ( $K_i = 88.9 \mu\text{M}$ ). Figures S5D and S5E show how this compound may bind less optimally in the ATP-binding site with the 4-nitroacetanilide portion in a different position to that seen with 11426026. Docking of compounds may be instructive for SAR

until the co-crystal structure with a ligand is obtained. For example, the 11326054 sulfone was also docked in the PyrG structure and was shown to be in an orientation similar to that of the 11426026 active metabolite (Figures S5F and S5G).

## SIGNIFICANCE

**New leads and new targets are required for tuberculosis drug development. Using phenotypic screening of a chemical library, two thiophenecarboxamide derivatives were identified that inhibited *M. tuberculosis* under replicating, non-replicating, and intracellular growth conditions. Both compounds were activated by the EthA monooxygenase, and the main metabolite of one of them (7947882), identified by mass spectrometry, was shown to target the CTP synthetase PyrG. The active metabolite was synthesized and shown to behave as a competitive inhibitor toward the ATP-binding site of PyrG, thus validating this enzyme as a new antitubercular drug target. Further validation was obtained genetically using conditional knockdown of *pyrG* to prove its essentiality in all the physiological states. A metabolomic approach demonstrated that the thiophenecarboxamide derivatives caused general deregulation of nucleotide metabolism, consistent with the inhibition of CTP synthetase. The combined evidence strongly indicates that PyrG is potentially a clinically relevant drug target. To overcome the requirement for EthA activation, we obtained high-resolution crystal structures of PyrG to underpin structure-based drug design. This approach has already generated additional lead compounds that inhibit this new drug target under all conditions tested.**

## EXPERIMENTAL PROCEDURES

### NIAID Library Screening

CB2, a library of 594 compounds selected from an HTS screen on *M. tuberculosis* H37Rv (Ananthan et al., 2009; Goldman and Laughon, 2009; Maddy et al., 2009), was kindly provided by NIAID (Prof. R.C. Goldman). These compounds were initially screened at 10  $\mu$ g/ml in duplicate for activity against H37Rv and ss18b in 96-well format, using the resazurin reduction microtiter assay. Compounds with a percentage of inhibition of H37Rv growth of more than 80% were subsequently analyzed for their MIC, intracellular activity against H37Rv, and cytotoxicity against the human hepatocellular carcinoma cell line HepG2 and Huh7, the human lung epithelial cell line A549, and the murine macrophage cell line RAW 264.7 (see Supplemental Experimental Procedures).

### Compounds Used and Synthesis of Their Derivatives

7904688 and 7947882 were purchased from ChemBridge Corp (<http://www.chembridge.com/index.php>). Synthetic routes of thiophene derivatives, experimental details, and compound characterization data are provided in the Supplemental Experimental Procedures.

### Isolation and Characterization of *M. tuberculosis* Mutants Resistant to 7947882 and 7904688

The isolation of *M. tuberculosis* mutants was performed by plating  $\sim 10^{10}$  cells from an exponential growth phase wild-type culture onto 7H11 medium containing different concentrations of 7947882 and 7904688, ranging from 5- to 20-fold the MIC of the wild-type strain.

Genomic DNA of *M. tuberculosis*-resistant mutants and wild-type strain was isolated and sequenced by using Illumina HiSeq2000 technology at IGA Technology Services (Udine, Italy). For the bioinformatic analysis of Illumina data, repetitive PE and PPE gene families were discarded as well as SNPs and indels

with less than 50% probability. The mutations found in *ethA* (Rv3854c) and *pyrG* (Rv1699) (<http://tuberculist.epfl.ch/>) were confirmed by Sanger sequencing (Eurofins MWG Operon), after PCR amplification using the oligonucleotides presented in the Supplemental Information. PCR products were purified using the Wizard SV Gel and PCR Clean-Up system (Promega).

### Overexpression of *ethA* in *M. tuberculosis* H37Rv

*M. tuberculosis ethA* was cloned into pSODIT-2, a shuttle expression vector containing the hygromycin resistance determinant, after PCR amplification using primers indicated in the Supplemental Information, *Pfu* DNA Polymerase (Promega), and genomic DNA as template. PCR fragments were digested with *Bam*HI and *Hind*III and ligated to the pSODIT-2 generating pSODIT/*ethA*. *M. tuberculosis* H37Rv competent cells were transformed with pSODIT-2 or pSODIT/*ethA*, and plated onto complete Middlebrook 7H11 agar plates supplemented with 20  $\mu$ g/ml hygromycin and different concentrations of 7947882 or 7904688, ranging from 0.125 to 20  $\mu$ g/ml.

### Enzyme Production and Characterization

*M. tuberculosis* PyrG and EthA were obtained in recombinant forms in *E. coli* and purified by standard methods. Enzymatic assays were performed according to the published methods (Fraaije et al., 2004; Lunn et al., 2008). See also Supplemental Experimental Procedures.

### Construction of a *M. tuberculosis pyrG* Knockdown Mutant

The first 714 bp of *pyrG* coding sequence was amplified using RP1609 and RP1610 primers and cloned in the suicide plasmid pFRA170 downstream of a *P<sub>ptr</sub>*-derived promoter. To replace *pyrG* promoter with *P<sub>ptr</sub>*, 10  $\mu$ g of this plasmid was used to transform TB38, an H37Rv derivative harboring the TetR-PipOFF system in its genome at the L5 *attB* site (Boldrin et al., 2010). Selection of recombinants was achieved using 7H10 agar plates containing hygromycin (50  $\mu$ g/ml). Integration of the suicide plasmid was confirmed by PCR. Since *pyrG* might be co-transcribed with its downstream genes (*rv1700-rv1701*), the latter genes were provided in *trans* on a pMV261-derived plasmid. In this way the final *pyrG* conditional knockdown (cKD) strain was obtained. This cKD strain was used for *pyrG* essentiality evaluation in both in vitro and ex vivo experiments (see Supplemental Experimental Procedures).

### PyrG Crystallization, Data Collection, and Structure Determination

Crystallization screenings of PyrG in the presence of various ligands were carried out at 18°C by sitting drop in 96-well format (200 + 200 nl drops) with a Mosquito dispensing robot (TTP Labtech). Crystals were identified in several conditions. PyrG in apo form: 10% PEG8000, 200 mM Ca acetate, 100 mM HEPES (pH 7.5); complex with UTP: 17% PEG20000, 100 mM MgCl<sub>2</sub>, 100 mM Tris-HCl (pH 8.5); complex with AMP-PCP, UTP, and L-DON: 30% PEG2000 MME, 100 mM NaCl, 100 mM bicine (pH 9.0). All data sets were collected on the Proxima-1 beamline at the Soleil synchrotron (Gif-sur-Yvette, France) from single crystals at 100 K, processed with XDS (Kabsch, 2010) and merged with Aimless from the CCP4 suite (Winn et al., 2011). The structure was first solved by molecular replacement with the program MOLREP (Murshudov et al., 1997) on a data set collected from a PyrG-UTP crystal, using the structure of *Thermus thermophilus* CTP synthetase in complex with sulfate (PDB: 1vcn; Goto et al., 2004) as the search model. Refinement was carried out with Refmac5 (Murshudov et al., 2011) or autoBUSTER (Bricogne et al., 2011). The other data sets were solved by molecular replacement with MOLREP and the coordinates of a partially refined *M. tuberculosis* PyrG structure as the search model. Final models were validated through the Molprobit server (Chen et al., 2010). Docking of PyrG inhibitors was performed as described in the Supplemental Experimental Procedures.

### In Vitro EthA Metabolite Production and Identification

For in vitro EthA metabolite production, 30 mg of 7947882 was incubated with 10 mg of EthA in 50 mM potassium phosphate (pH 8.0), 500  $\mu$ M NADPH, 10  $\mu$ M BSA, at 37°C for 5 hr under agitation. To produce the PyrG-EthA metabolite complex, the EthA reaction was performed in the presence of 45  $\mu$ M PyrG. Reaction products were purified and analyzed as described in Supplemental Experimental Procedures.

### Metabolomic Experiments

Experimental Procedures have been described elsewhere (Larrouy-Maumus et al., 2013; Brauer et al., 2006). In brief, *M. tuberculosis* H37Rv was grown initially in 7H9 (with 0.5 g/l BSA, 0.05% tyloxapol, 0.2% glycerol, 0.2% glucose, and 0.085% NaCl) until late logarithmic phase ( $OD_{600} = 1.0$ ) and 1 ml was layered onto 22-mm nitrocellulose filters (0.22  $\mu$ m) under vacuum filtration. *M. tuberculosis*-laden filters were placed atop 7H10 (supplemented as 7H9) and incubated at 37°C for 5 days, after which the filters were transferred to 7H10 containing 7947882 (5 $\times$  MIC = 2.5  $\mu$ g/ml) or the control. After 24 hr, the bacteria were metabolically quenched by plunging *M. tuberculosis*-laden filters into acetonitrile/methanol/H<sub>2</sub>O (2:2:1) pre-cooled to -40°C. The metabolites were extracted by mechanical lysing of the *M. tuberculosis*-containing solution with 0.1-mm zirconia beads. Lysates were clarified by centrifugation, filtered, and metabolites analyzed by liquid chromatography-mass spectrometry as described in Supplemental Experimental Procedures.

For metabolic labeling of *M. tuberculosis* H37Ra with [<sup>14</sup>C]uracil, 10 ml of the GAS medium (Takayama et al., 1975) was inoculated in the ratio 1:100 with an *M. tuberculosis* H37Ra pre-culture grown in Sauton medium. After 7 days of static growth at 37°C, the culture was split into two aliquots, and 11426026 (final concentration of 16  $\mu$ g/ml) was added to one and DMSO to the other as a control. After 1 hr, [<sup>14</sup>C]uracil (American Radiolabeled Chemicals, specific activity 53 mCi/mmol) was added to a final concentration 1  $\mu$ Ci/ml. Radiolabeling was carried out for 3 hr, then two 2-ml batches were removed from each culture. The bacteria were harvested by centrifugation, washed twice with a cold physiological solution, and immediately extracted with 110  $\mu$ l of ice-cold 9% (v/v) formic acid for 30 min. The formic acid extract was recovered by centrifugation and its radioactivity was quantified by scintillation spectrometry in 5 ml of EcoLite scintillation liquid (MP Biomedicals). The nucleotide extract was stored at -20°C and was typically analyzed by TLC or HPLC within 24 hr (see Supplemental Experimental Procedures). Alternatively, radiolabeling was performed as above with *M. tuberculosis* H37Ra culture grown to  $OD_{600} = 0.375$  in 7H9 medium at 37°C.

### ACCESSION NUMBERS

The PDB accession numbers for coordinates and structure factors of the crystal structures described here are PDB: 4ZDI (PyrG in apo form); PDB: 4ZDJ (PyrG in complex with two molecules of UTP/Mg<sup>2+</sup>); and PDB: 4ZDK (PyrG in complex with UTP, AMP-PCP, Mg<sup>2+</sup>, and 5-oxo-L-norleucine).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, list of oligonucleotide primers, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.chembiol.2015.05.016>.

### AUTHOR CONTRIBUTIONS

R.C.H. performed the screening of NIAID library procured by S.T.C.; G.M., A.L.dJ.L.R., and M.R.P. isolated and characterized the resistant mutants; M.F. performed bioinformatics analysis of Illumina data; G.M., L.R.C., M.E., and N.B. performed cloning, and protein expression and purification; L.R.C. and M.E. performed enzymatic assays; L.R.C., M.E., and A.P. performed metabolite isolation and identification; V.M., E.K., and A.L. performed derivative synthesis; G.D. and F.B. performed studies on *pyrG* essentiality; L.B.M., I.C., Z.S., and J.B. performed metabolomic studies; M.B. performed crystallographic studies; S.E. and M.B. performed docking experiments; R.F., G.Z., V.M., R.M., L.P.S.C., A.R.B., K.M., P.M.A., G.R., S.T.C., and M.R.P. supervised and directed the work; L.R.C., M.B., S.E., R.F., G.Z., V.M., R.M., L.P.S.C., A.R.B., K.M., G.R., S.T.C., and M.R.P. wrote the paper. All authors discussed the results and commented on the manuscript.

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## Antitubercular activity of Ru (II) isoniazid complexes



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### ABSTRACT

Despite the resistance developed by the *Mycobacterium tuberculosis* (MTb) strains, isoniazid (INH) has been recognized as one of the best drug for treatment of Tuberculosis (Tb). The coordination of INH to ruthenium metal centers was investigated as a strategy to enhance the activity of this drug against the sensitive and resistant strains of MTb. The complexes *trans*-[Ru(NH<sub>3</sub>)<sub>4</sub>(L)(INH)]<sup>2+</sup> (L = SO<sub>2</sub> or NH<sub>3</sub>) were isolated and their chemical and antituberculosis properties studied. The minimal inhibitory concentration (MIC) data show that [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> was active in both resistant and sensitive strains, whereas free INH (non-coordinated) showed to be active only against the sensitive strain. The coordination of INH to the metal center in both [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> and *trans*-[Ru(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)]<sup>2+</sup> complexes led to a shift in the INH oxidation potential to less positive values compared to free INH. Despite, the ease of oxidation of INH did not lead to an increase in the *in vitro* INH activity against MTb, it might have provided sensitivity toward resistant strains. Furthermore, ruthenium complexes with chemical structures analogous to those described above were synthesized using the oxidation products of INH as ligands (namely, isonicotinic acid and isonicotinamide). These last compounds were not active against any strains of MTb. Moreover, according to DFT calculations the formation of the acyl radical, a proposed intermediate in the INH oxidation, is favored in the [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> complex by 50.7 kcal mol<sup>-1</sup> with respect to the free INH. This result suggests that the stabilization of the acyl radical promoted by the metal center would be a more important feature than the oxidation potential of the INH for the antituberculosis activity against resistant strains.

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### 1. Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis* (MTb), is the second leading cause of death from an infectious disease and it is surpassed by the human immunodeficiency virus (HIV) (WHO, 2012). The chemotherapy of tuberculosis is in general based on the administration of Isoniazid, Rifampicin, Pyrazinamide and Ethambutol (WHO, 2009). Among these drugs, the prodrug isoniazid (isonicotinic acid hydrazide, INH), first synthesized in 1912 (Meyer and Mally, 1912), is the most prescribed (Riccardi et al., 2009; Rozwarski et al., 1998). INH action involves the inhibition of the mycolic acid biosynthesis, which is one of the most important cell wall components of MTb (Winder and Collins, 1970). In fact, the most accepted action mechanism of this prodrug requires a conversion of the INH into an acyl radical promoted by the KatG enzyme (Lei et al., 2000). This radical is able to link with NAD<sup>+</sup> and

form a covalent adduct (Vilcheze et al., 2006) potentially capable of inhibiting the FASII enoyl-ACP reductase InhA (Lei et al., 2000; Nguyen et al., 2002; Rawat et al., 2003; Vilcheze et al., 2006). Despite this, the mechanism of action of INH has been related to occurs by other pathways, as the inhibition of nucleic acids (Gangadharam et al., 1963), phospholipids (Brennan et al., 1970) synthesis and NAD<sup>+</sup> metabolism (Bekierkunst, 1966; Zatman et al., 1954).

Unfortunately, MTb have become resistant to INH due to their high ability to change DNA under selective pressure (Barry et al., 1998). The most common alterations related to INH resistance occur in the genes: *katG*, *inhA*, *ahpC*, *kasA* and *ndh* (Schroeder et al., 2002). The *katG* gene encodes the KatG protein (catalase-peroxidase-peroxynitritase T), responsible for the INH activation and also for mutations in the gene. This is the most accepted mechanism for INH resistance (Kapetanaki et al., 2005). Alterations in the *inhA* gene can occur in two different ways: through mutations in the gene promoter region and through mutations in the InhA domain responsible for the binding with the NAD-INH adduct. In

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general, resistant strains which have a mutated KatG show a loss of catalase-peroxidase activity, which is a protective mechanism (Pym et al., 2002). In an attempt to compensate this loss and adapt the protective fitness, these bacteria overexpress the AhpC protein, an alkyl hydroperoxide reductase, through mutations in the *ahpC* promoter region. The mutation in the -46 position is one of the most found mutation (Hazbón et al., 2006; Hoshide et al., 2013; Jnawali et al., 2013).

Mutations in *katG* and *inhA* do not cover the totality of resistant clinical isolates (Mdluli et al., 1998). Mdluli and co-authors (Mdluli et al., 1998) conducted a study aiming at a new target to INH. They found that a treatment with a dose of  $1 \mu\text{g mL}^{-1}$  of INH could generate a saturated hexacosanoic acid accumulation in a 12 kDa protein known as AcpM. The same study (Mdluli et al., 1998) showed an upregulation of an 80 kDa-protein of the same amino terminal of AcpM under a treatment with INH. Actually, such an 80 kDa was a complex covalently formed by AcpM, INH and  $\beta$ -ketoacyl ACP synthase, called KasA.

The discovery of new anti-TB drugs is essential for the overcoming the major problems related to tuberculosis: (1) nonadherence of the patients due to the duration and complexity of the treatment; (2) treatment side effects; (3) emergence of MDR (Multidrug Resistant) and XDR (Extensive Drug Resistant) strains; (4) drug interactions between antiretroviral and available anti-TB drugs and (5) need for a drug capable of acting in latent bacteria (van den Boogaard et al., 2009). The treatment duration is one of the main problems of the disease, since it results in nonadherence by the patient. The attempts to shorten the therapy include studies with Sutezolid (phase II of clinical development) (Williams et al., 2009), rifamycins (Rosenthal et al., 2007), nitroimidazoles (Lin et al., 2012) and fluoroquinolones (present in OfloTub, ReMox and Rifaquin trials) (Zumla et al., 2013).

One of the strategies to overcome the problems related to INH therapy is the coordination of this prodrug to metal complexes (Basso et al., 2010; Oliveira et al., 2006, 2004; Rodrigues et al., 2012; Sousa et al., 2012; Vasconcelos et al., 2008), as previously performed with  $[\text{Fe}(\text{CN})_5(\text{INH})]^{3-}$  and  $[\text{Ru}(\text{CN})_5(\text{INH})]^{3-}$ . These compounds have been tested *in vitro* and *in vivo* against tuberculosis.  $[\text{Fe}(\text{CN})_5(\text{INH})]^{3-}$  exhibited good activity ( $0.2 \mu\text{g mL}^{-1}$ ) as opposed to  $[\text{Ru}(\text{CN})_5(\text{INH})]^{3-}$ , which was not active (Sousa et al., 2012). Indeed, there has been growing concern about the medicinal applications of coordination compounds of iron and ruthenium against cancer, parasitosis and tuberculosis (Pavan et al., 2011, 2010; Santos et al., 2013). The association of bioactive molecules with metal complexes may result in a potentialization of drug activity and, in some cases, improvement on other physical chemical properties, like an increase in water solubility of these molecules regarding the free drug. For example, the coordination of benzimidazole (Bz), the mostly used drug in therapy of Chagas' disease (Silva et al., 2008), to the tetraammineruthenium complex  $\text{trans-}[\text{Ru}(\text{Bz})(\text{NH}_3)_4(\text{SO}_2)](\text{CF}_3\text{SO}_3)_2$  resulted in a ninefold increase in water solubility and a thousand-fold smaller dose (*in vivo* assays) regarding the optimal dose of Bz (Silva et al., 2008). After the coordination of Bz to the ruthenium(II) complex, the reduction potential of the nitro group (in Bz) was shifted to more positive values ( $\Delta E = 0.10 \text{ V}$ ). The highest activity observed for  $\text{trans-}[\text{Ru}(\text{Bz})(\text{NH}_3)_4(\text{SO}_2)](\text{CF}_3\text{SO}_3)_2$  regarding the non-coordinated Bz was attributed to this feature.

A similar strategy was used in the present study, i. e.  $[\text{Ru}(\text{NH}_3)_5(\text{INH})](\text{BF}_4)_2$  and  $\text{trans-}[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})](\text{BF}_4)_2$  complexes (Fig. 1) were synthesized and their antituberculosis activity investigated. Analogous ruthenium(II) complexes with isn (isonicotinamide) and ina (isonicotinic acid) ligands, known as two of the isoniazid oxidation products (Johnsson et al., 1995), were also synthesized (Fig. 1). The antituberculosis activity of these compounds was also investigated. DFT calculations were carried out so as to

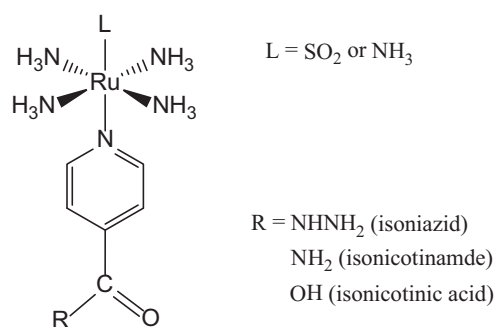


Fig. 1. Chemical structure of the ruthenium(II) complexes.

verify the relative stability of the possible intermediates generated during the INH oxidation.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals and reagents were of analytical grade (Aldrich, Merck or Panreac) and used without further purification.

### 2.2. Synthesis of the complexes

The precursors  $\text{trans-}[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)\text{Cl}](\text{BF}_4)_2$  (Vogt et al., 1965) and  $[\text{Ru}(\text{NH}_3)_5\text{Cl}]\text{Cl}_2$  (Kuehn and Taube, 1976), and the complexes  $\text{K}_3[\text{Ru}(\text{CN})_5(\text{INH})]$  (Johnson and Shepherd, 1983),  $\text{Na}_3[\text{Fe}(\text{CN})_5(\text{INH})]$  (Sousa et al., 2012),  $[\text{Ru}(\text{NH}_3)_5(\text{isn})](\text{BF}_4)_2$ ,  $[\text{Ru}(\text{NH}_3)_5(\text{INH})](\text{BF}_4)_2$  (Gaunders and Taube, 1970),  $\text{trans-}[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)(\text{isn})](\text{BF}_4)_2$  (Isied and Taube, 1974),  $[\text{Ru}(\text{NH}_3)_5(\text{ina})](\text{BF}_4)_2$  (Chou et al., 1992) were synthesized as previously reported. The well known complexes  $\text{trans-}[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{NO}^+)(\text{L})]^{3+}$ , where L is isonicotinamide (isn), pyridine (py), 4-picoline (pic), nicotinamide (nic), isonicotinic acid (ina) or imidazol (imN) were synthesized by following earlier described procedures (Roveda et al., 2014; Tfouni et al., 2003).

#### 2.2.1. $\text{trans-}[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)(\text{ina})](\text{BF}_4)_2$

0.40 g of isonicotinic acid were added to 10 mL of water and solid NaOH was added until the total dissolution of the ligand. The final pH was adjusted to 9.0 (using trifluoroacetic acid) and the solution was deaerated by argon flux for 30 min.  $\text{trans-}[\text{Ru}(\text{NH}_3)_4(\text{Cl})(\text{SO}_2)]\text{Cl}$  (0.15 g, 0.5 mmol) was dissolved in 9.0 mL of previously deaerated water (under argon) and sodium carbonate was added until reach pH 9.0. The isonicotinic acid solution was added to this pale green-yellow solution through Teflon tubing. The color changed to orange and a suspension was formed. The reaction was kept under stirring for 15 min and then 3.0 mL of 6.0 M  $\text{HBF}_4$  solution were added. The product was precipitated with an excess of acetone, filtered and dried under vacuum. Anal. calcd.: C, 13.60; H, 3.23; N, 13.21. Found: C, 12.93; H, 3.15; N, 13.22.  $^1\text{H NMR}$  (400 MHz):  $\delta$  8.99 ppm (d, 2H),  $\delta$  8.56 ppm (d, 2H).

#### 2.2.2. $\text{trans-}[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})](\text{BF}_4)_2$

$\text{trans-}[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)\text{Cl}](\text{Cl})_2$  (0.10 g, 0.49 mmol) was dissolved in 9.0 mL of deaerated water and sodium carbonate was added until reach pH 9.0. Isoniazid was added to this solution (0.68 g, 4.98 mmol) and the mixture was left to react for 30 min at room temperature. 3.0 mL of  $\text{HBF}_4$  6.0 M were then added. The orange precipitate was collected by filtration, washed with cold 6.0 M  $\text{HBF}_4$  and dried under vacuum. The solid was suspended in  $\text{HBF}_4$  and filtrated. Yield: 75%. Anal. Calc. C, 13.25; H, 3.52; N, 18.02.

Found: C,12.69; H, 3.56; N,17.29.  $^1\text{H}$  NMR (400 MHz):  $\delta$  8.81 ppm (d, 2H),  $\delta$  7.99 ppm (d, 2H).

### 2.3. Equipment

The electronic spectra were recorded on a Multiskan Go model Thermo Scientific UV–vis spectrophotometer using a 1.0 cm path length quartz cell in argon-degassed solutions at 25 °C. The ionic strength ( $\mu = 0.10 \text{ mol L}^{-1}$ ) was maintained by using trifluoroacetic acid/sodium trifluoroacetate ( $\text{CF}_3\text{CO}_2\text{H}/\text{CF}_3\text{CO}_2\text{Na}$ ) solution as background electrolyte. The infrared measurements were performed using KBr pellets on a Bomem MB-102 series spectrometer in the 4000–350  $\text{cm}^{-1}$  region. Cyclic voltammograms were carried out at room temperature ( $25 \pm 1$  °C) using EG&G PAR model 264A equipment. A conventional three-electrode system was used. The reference electrode was a saturated calomel electrode (SCE), the auxiliary electrode was a platinum plate and the working electrode was a boron doped diamond (BDD). All the potential values were reported vs. SCE. During this experiment, the solutions ( $\mu = 0.10 \text{ mol L}^{-1}$   $\text{CF}_3\text{CO}_2\text{H}/\text{CF}_3\text{CO}_2\text{Na}$ ) were degassed with argon and maintained under inert atmosphere during the measurements. The  $^1\text{H}$  NMR spectra were obtained on a Bruker DRX 400 MHz. Deuterated trifluoroacetic acid was used as the solvent and the chemical shifts were reported in ppm ( $\delta$ ) relative to the internal standard 3-(Trimethylsilyl)propionic-2,2,3,3- $\text{d}_4$  acid sodium salt. Elemental analysis was conducted on a CHNS 2400 Perkin-Elmer.

### 2.4. DFT studies

DFT calculations (Kohn and Sham, 1965) were performed with Gaussian 03 package (Frisch et al., 2004). The optimized molecular geometries were calculated using the hybrid B3LYP (Becke, 1993; Lee et al., 1998) exchange–correlation functional. The Density Gauss Double-Zeta Valence Polarized basis set (DGDZVP) (Hay and Wadt, 1985; Hay, 1985; Wadt and Hay, 1985) and effective core potential (LANL2DZ) were used for all atoms. The ground-state geometries of the molecules were optimized in a cavity surrounded by a continuum, to mimic the solvent effect. The solvent water was included in the calculations by the IEFPCM (Integral Equation Formalism for the Polarizable Continuum Model) (Cances et al., 1997; Reed et al., 1988; Reed and Weinhold, 1995) method and the electronic analysis was obtained through TD-DFT calculations (20 excited states).

The enthalpies and Gibbs free energies for each step of the mechanism of action, using thermodynamic correction terms (at 298.15 K), were obtained through the calculation of vibrational modes for all structures, ensuring that imaginary frequencies were not generated in the minimum structures. The INH oxidation reactions were carried out theoretically using the potential energy surface scanning (Foresman and Frisch, 1996) and the transition states were obtained by the QST3 (Hratchian and Schlegel, 2005) method. In these calculations, the explicit solvation by water molecules was employed besides of the IEFPCM. The bond dissociation enthalpies (BDE) were used for calculation of relative energy of the first species involved in the oxidation pathway (Nenadis et al., 2011). Since the oxidation of the metal center occurs in a potential less positive than the INH oxidation (please see voltammograms in Figs. 4), for all BDE and theoretical kinetics calculations the oxidation state of the ruthenium metal center was considered as being (III). For the *trans*- $[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})]^{2+}$  complex, the  $\text{SO}_2$  was considered as  $\text{SO}_3^{2-}$  as a function of the physiologic medium (pH 7.4), as described for similar systems (Isied and Taube, 1974). The simulations were carried out considering both the singlet and the triplet form of the coordinated acyl radical. Owing to the higher energies of the triplet form, only the results for the more reliable singlet form were considered.

### 2.5. Anti-*M. tuberculosis* activity assay

The anti-*M. tuberculosis* activity of the complexes was determined by the Resazurin Microtiter Assay (REMA). Stock solutions of the compounds were prepared in  $\text{H}_2\text{O}$  and diluted in Middlebrook 7H9 broth (Difco), supplemented with oleic acid, albumin, dextrose and catalase (OADC enrichment BBL/Becton Dickinson, Sparks, MD, USA) so that final drug concentration ranges from 0.15 to 250  $\mu\text{g mL}^{-1}$  could be obtained. Serial dilutions were performed in a Precision XS Microplate Sample Processor (Biotek™). Isoniazid was dissolved in distilled water, according to the manufacturers recommendations (Difco laboratories, Detroit, MI, USA) and used as a standard drug. Culture aliquots of MTb H<sub>37</sub>Rv were thawed and added to appropriate solutions of the test compounds in microplate wells, yielding a final testing volume of 200  $\mu\text{L}$  with  $2 \times 10^4$  CFU/mL. Each test was set up in triplicate. Microplates were incubated for 7 days at 37 °C, after which Resazurin was added for the reading and incubated for 24 h. The fluorescence was read (530 nm excitation filter and 590 nm emission filter) in a SPECTRAfluor Plus (Tecan) microfluorimeter.

The minimum inhibitory concentration (MIC) of isoniazid was determined on each microplate. The acceptable range of isoniazid MIC is 0.015 to 0.050  $\mu\text{g mL}^{-1}$  (Palomino et al., 2002). Each test was set up in triplicate.

### 2.6. Cytotoxicity assay and selectivity index

*In vitro* cytotoxicity assays ( $\text{IC}_{50}$ ) were performed first on VERO epithelial cells (ATCC CCL81). Low-cytotoxicity compounds were investigated on the J774A.1 (ATCC TIB-67) mouse cell line. Both studies were recommended by Pavan et al. (2010). The cells were routinely maintained in a complete medium (DMEM for VERO and RPMI-1640 (VitroCell®) for J774A.1) supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus gentamicin (50  $\text{mg L}^{-1}$ ) and anfotericin B (2  $\text{mg/L}$ ), at 37 °C, in a humidified 5%  $\text{CO}_2$  atmosphere. After reaching confluence, the cells were detached and counted. For the cytotoxicity assay,  $1 \times 10^5 \text{ mL}^{-1}$  cells were seeded in 200  $\mu\text{L}$  of a complete medium on 96-well plates (NUNC™). The plates were incubated under the same conditions for 24 h, for a cell adhesion prior to drug testing. The compounds were dissolved in water and subjected to twofold serial dilution from 1250 to 3.9  $\mu\text{g mL}^{-1}$ . The cells were exposed to the compounds at various concentrations for 24 h. A resazurin solution was then added to the cell cultures and incubated for 6 h. The cell respiration, as an indicator of cell viability, was detected by the reduction of resazurin to Resorufin, whose pink color and fluorescence indicate cell viability. A persistent blue color of resazurin is a sign of the cell death. Fluorescence measurements (530 nm excitation filter and 590 nm emission filter) were performed in a SPECTRAfluor Plus (Tecan) microfluorimeter. The  $\text{IC}_{50}$  value was defined as the highest drug concentration at which 50% of the cells are viable in comparison to the control. Each test was set up in triplicate.

The selectivity index (SI) was calculated by dividing  $\text{IC}_{50}$  by the MIC for the pathogen, if the SI is > 10, the compound is then evaluated further.

## 3. Results and discussion

### 3.1. Theoretical and spectroscopic properties

All our attempts to grow diffraction-quality crystals of the synthesized complexes were unsuccessful so, to determine the structure of the Ru compounds, DFT calculations were carried out. Through the optimized structure is possible to note that the bond

length Ru-N(INH) does not change considerably when the ligand *trans* to INH is NH<sub>3</sub> or SO<sub>2</sub> (2.08 and 2.09 Å, respectively). Comparable bond lengths were reported for tetra- and pentaammineruthenium(II) complexes containing aromatic N-heterocycles (Borges et al., 1998; Bottomley, 1974; Gress et al., 1981). Similar distortion is observed in the octahedral structure for both complexes, in the complex *trans*-[Ru(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)]<sup>2+</sup> the angles between the amine groups N<sub>2</sub>-Ru-N<sub>3</sub> and N<sub>3</sub>-Ru-N<sub>4</sub> are 87° and 92°, respectively (Fig. S1), whereas the angle N(INH)-Ru-S(SO<sub>2</sub>) is 180° (Gress et al., 1981). For the pentaamine complex similar angles (N<sub>2</sub>-Ru-N<sub>3</sub> and N<sub>3</sub>-Ru-N<sub>4</sub> are 88° and 91°, respectively) were observed (Fig. S1).

The infrared spectra data for [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup>, *trans*-[Ru(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)]<sup>2+</sup> and INH are described in Table S1. The ligand INH exhibit strong bands at 3300–3000 cm<sup>-1</sup> (ν<sub>NH</sub>), 3100–2900 cm<sup>-1</sup> (ν<sub>CHar</sub>), 1674 cm<sup>-1</sup> (ν<sub>CO</sub>), and 1545 cm<sup>-1</sup> (ν<sub>CN</sub>) (Gunasekaran et al., 2009). Upon coordination to Ru(II) center, these bands are slightly shifted (Table S1, Fig. S2). The complex *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)]<sup>2+</sup> exhibited bands at 1285 and 1108 cm<sup>-1</sup> which are related to the coordination of the SO<sub>2</sub> by the sulfur atom, as reported for the complex *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(Bz)]<sup>2+</sup> (Bz = benzimidazole) (Silva et al., 2008).

The electronic spectra of free INH and coordinated to Ru(II) complexes are shown in Fig. 2. Depending on the pH, the protonation of free isoniazid at different sites may be favored (pK<sub>a</sub> 1.8 – hydrazine nitrogen, 3.5 – pyridine nitrogen and 10.8 – acidic group) (Ofoefule et al., 2002). Thus, all the spectra were recorded at pH 2.0. In this pH, the free ligand exhibits two intense transitions at 224 and 266 nm, attributed to  $\pi \rightarrow \pi^*$  and  $n \rightarrow \pi^*$ , respectively (Fig. 2) (Manoussa et al., 1973). The *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)](BF<sub>4</sub>)<sub>2</sub> complex exhibit those bands shifted to high energy, 217 and 253 nm, whereas in the [Ru<sup>II</sup>(NH<sub>3</sub>)<sub>5</sub>(INH)](BF<sub>4</sub>)<sub>2</sub> the  $\pi \rightarrow \pi^*$  and  $n \rightarrow \pi^*$  resemble those of the free ligand.

The bands observed at 486 nm (log  $\bar{\epsilon}_{\max}$  = 3.73) for complex [Ru<sup>II</sup>(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> and at 328 nm (log  $\bar{\epsilon}_{\max}$  = 2.42) for *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(INH)SO<sub>2</sub>](BF<sub>4</sub>)<sub>2</sub> were assigned as metal-to-ligand charge-transfer (MLCT, (Ru<sup>II</sup>)4d  $\rightarrow$  p\*(L)) (Fig. 2, Tables S2 and S3). Theoretical computational studies (TD-DFT) have addressed the electronic absorption spectra of INH and ruthenium complexes and the predicted (TD-DFT) MLCT (Ru<sup>II</sup>) 4d  $\rightarrow$  (INH)p\* (442 nm, HOMO  $\rightarrow$  LUMO) of the [Ru<sup>II</sup>(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> complex exhibits lower energy in comparison to the MLCT (Ru<sup>II</sup>)4d  $\rightarrow$  (SO<sub>2</sub>)p\* (376 nm, HOMO-4  $\rightarrow$  LUMO) of the *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)]<sup>2+</sup> complex, in agreement with the experimental data. Analogous assignments

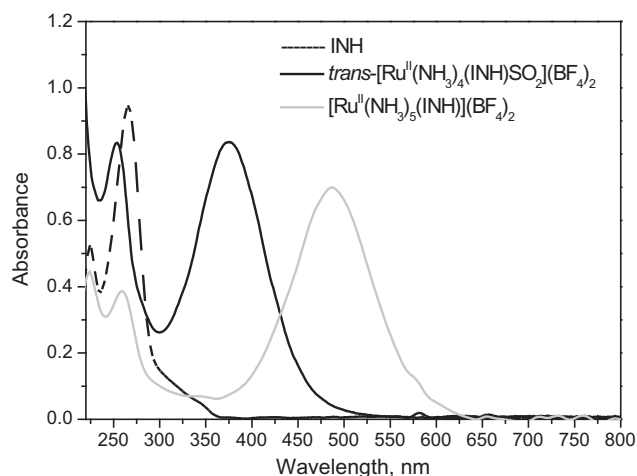


Fig. 2. UV-vis spectra of INH (black line), *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)](BF<sub>4</sub>)<sub>2</sub> (red line) and [Ru<sup>II</sup>(NH<sub>3</sub>)<sub>5</sub>(INH)](BF<sub>4</sub>)<sub>2</sub> (blue line). Conditions: aqueous solution pH 2.0 ( $\mu$  = 0.10 M CF<sub>3</sub>CO<sub>2</sub>H/CF<sub>3</sub>CO<sub>2</sub>Na).

were also reported for similar complexes (Doro et al., 2007; Ford et al., 1968; Tfouni, 2000).

### 3.2. Electrochemical data

Hydrazides can be oxidized either chemically by a large number of reagents (Takale and Telvekar, 2010; Varale and Hilage, 2009) or electrochemically, yielding mainly acids, aldehydes and esters as oxidation products (Yalgudre and Gokavi, 2012).

Cyclic voltammetry experiments were performed for comparing the oxidation potentials of free and coordinated INH (Figs. 3 and 4). The formal potentials of the INH oxidation and the metal redox couple are listed in Table 1. A comparison between the voltammogram of the non-coordinated INH using glassy carbon (GC) and boron doped diamond (BDD) electrodes were performed (Fig. 3). As can be seen in the experiment with GC, the current begins to increase when the potential reaches 1.0 V for the solution containing 1.0 mM of INH (blue line in Fig. 3), however the oxidation process is not well defined. The increase in the current is not observed in the voltammogram of the electrolyte solution alone (gray line in Fig. 3), suggesting that the increase in the current is related to the process of INH oxidation. However, in the voltammograms of the solution containing INH using BDD electrodes a well-defined oxidation process is observed in potentials close to 1.2 V (pH 2.0), Fig. 4A. Furthermore the observed peak current, is at least three times greater than the value observed in the voltammogram obtained with the GC electrode. Although several electrochemical experiments of INH in presence of a mediator has been reported (Gao et al., 2006; Karimi et al., 2010; Tong et al., 1997), herein the BDD electrode by itself is capable of activating the INH oxidation. The existence of an empty *p* orbital in the boron atom, provides in the electrode a site which would promote an interaction between the electrode surface and the neighbors INH particles, and thus making easier the electron transfer process (McCreery, 2008). The BDD electrode has some advantages regarding to others electrodes. The main ones are high thermal conductivity, high hardness and chemical inertness (Alfaro et al., 2006; Salazar-Banda et al., 2013). CV in different scan rates demonstrates that the oxidation of INH with BDD electrode is a diffusion controlled electrochemical process, Fig. S3.

The coordination of INH to the complex stabilizes the metal center regarding oxidation. At pH 2.0, the cyclic voltammogram for the [Ru<sup>II</sup>(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> complex consists of a reversible process assigned to the Ru<sup>III</sup>/Ru<sup>II</sup> couple with  $E_{1/2}$  = 0.16 V, which is shifted by 290 mV regarding to the synthetic precursor aquopentaammineruthenium (II or III) species ( $E_{1/2}$  = -0.13 V) (Kuehn and Taube,

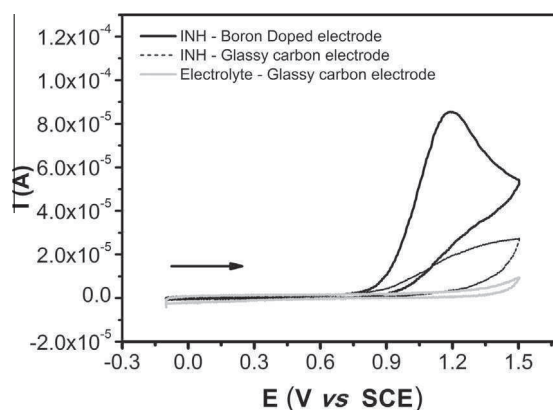
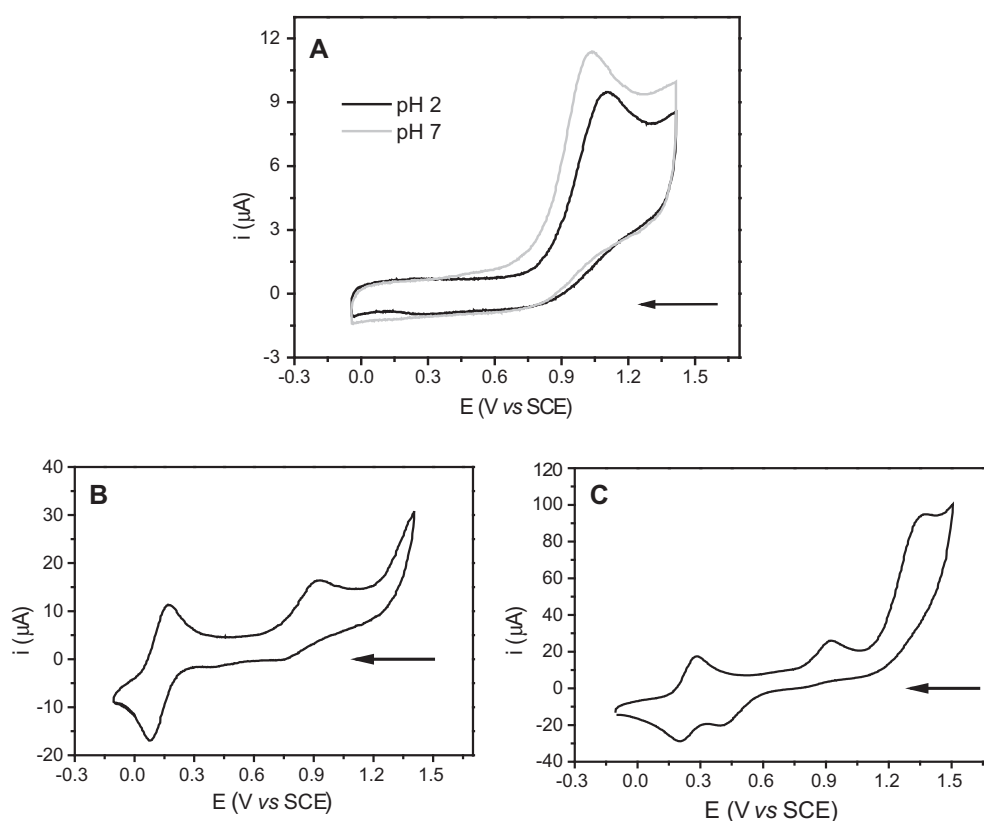


Fig. 3. Cyclic Voltammograms of INH performed with boron doped diamond and glassy carbon electrodes. Conditions: aqueous solution pH 2.0,  $\mu$  = 0.10 mol L<sup>-1</sup> CF<sub>3</sub>COOH/CF<sub>3</sub>COONa; C<sub>INH</sub> = 1.0 mM.



**Fig. 4.** Cyclic voltammograms of (A) free INH ( $C = 1.0 \times 10^{-4} \text{ mol L}^{-1}$ ); (B)  $[\text{Ru}^{\text{II}}(\text{NH}_3)_5(\text{INH})]^{2+}$  ( $C = 7.5 \times 10^{-4} \text{ mol L}^{-1}$ ); (C)  $\text{trans-}[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})]^{2+}$  ( $C = 2.0 \times 10^{-3} \text{ mol L}^{-1}$ ). Conditions:  $\text{CF}_3\text{CO}_2\text{H}/\text{CF}_3\text{CO}_2\text{Na}$  aqueous solution, pH 2.0,  $\mu = 0.10 \text{ mol L}^{-1}$ , scan rate =  $200 \text{ mV s}^{-1}$ ,  $25^\circ\text{C}$ .

**Table 1**

Half-wave potentials ( $E_{1/2}$ ) of the  $\text{Ru}^{\text{III/II}}$  and  $\text{Fe}^{\text{III/II}}$  redox couple, anodic peak potential ( $E_{p_a}$ ) of the INH, and variation of free energy ( $\Delta G$ ,  $\text{kcal mol}^{-1}$ ) for the INH oxidation,  $T = 25^\circ\text{C}$ , V vs SCE,  $\mu = 0.10 \text{ mol L}^{-1}$ .

Complex	$E_{1/2} \text{ M}^{\text{III/II}}$ (V) <sup>*</sup>	$E_{p_a}$ INH (V)		$\Delta G^{**}$	
		pH 2.0	pH 7.0	pH 2.0	pH 7.0
$[\text{Ru}^{\text{II}}(\text{NH}_3)_5(\text{INH})]^{2+}$	0.16	1.06	0.84	-0.90	-4.38
$\text{trans-}[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{INH})(\text{SO}_2)]^{2+}$	0.29	1.03	0.82	-1.50	-4.84
$[\text{Fe}^{\text{II}}(\text{CN})_5\text{INH}]^{3-}$	0.30	0.97	0.83	-3.00	-4.61
$[\text{Ru}^{\text{II}}(\text{NH}_3)_5(\text{H}_2\text{O})]^{2+}$	-0.13 <sup>a</sup>	-	-	-	-
$\text{trans-}[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{Cl})(\text{SO}_2)]^{2+}$	0.28	-	-	-	-
Isoniazid (INH)	-	1.10	1.03	-	-

<sup>\*</sup> pH 2.0.

<sup>\*\*</sup> Values calculated with respect to the oxidation potential of non-coordinated INH.

<sup>a</sup> Reference Metzker et al. (2014).

1976; Metzker et al., 2014). For the complex  $[\text{Ru}^{\text{II}}(\text{NH}_3)_5(\text{INH})]^{2+}$ , the oxidative process of INH is negatively shifted ( $E = 40 \text{ mV}$  for pH 2.0 and  $E = 190 \text{ mV}$  for pH 7.0) in comparison to the non-coordinated INH (Table 1). This feature implies that in terms of free energy ( $\Delta G$ ) the INH oxidation after coordination to the complex  $[\text{Ru}^{\text{II}}(\text{NH}_3)_5(\text{INH})]^{2+}$  is facilitated by  $0.90 \text{ kcal mol}^{-1}$  (pH 2.0) and  $4.38 \text{ kcal mol}^{-1}$  (pH 7.0).

Analogously, the  $\text{Ru}^{\text{III/II}}$  couple for the compound  $\text{trans-}[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})]^{2+}$  is observed at  $E_{1/2} = 0.29 \text{ V}$  (pH 2.0). Moreover, the irreversible process attributed to the one-electron oxidation of INH in this complex, occurs at potentials  $70 \text{ mV}$  (pH 2.0) and  $210 \text{ mV}$  (pH 7.0) less positive than that observed for free INH (Table 1). Based on these data, the INH oxidation for the complex  $\text{trans-}[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})]^{2+}$  was facilitated by  $1.5 \text{ kcal mol}^{-1}$  at pH 2.0 and  $4.84 \text{ kcal mol}^{-1}$  at pH 7.0. By analogy to a similar system (Isied and Taube, 1974), the third electrochemical process at  $+1.43 \text{ V}$  (Fig. 4) was attributed to the oxidation of the ligand  $\text{SO}_2$ .

Similar electrochemical behavior was observed for the  $\text{Na}_3[\text{Fe}(\text{CN})_5(\text{INH})]$  complex. The irreversible process of INH oxidation was observed at  $+0.97 \text{ V}$  (pH 2.0) and  $+0.83 \text{ V}$  (pH 7.0) and the potentials are shifted by  $130 \text{ mV}$  (pH 2.0) and  $200 \text{ mV}$  (pH 7.0) to less positive values in comparison to the non-coordinated INH.

### 3.3. Anti-M. tuberculosis activity and cytotoxicity assay

Owing to our experience on the use of ruthenium nitrosyls complexes (NO-donors) in treatment of infectious diseases (e.g. Chagas' Disease, Leishmaniasis) (Tfouni et al., 2010, 2012), some of these complexes, with general formula  $\text{trans-}[\text{Ru}^{\text{II}}(\text{NH}_3)_4(\text{NO}^+)(\text{L})]^{3+}$ , where L is isonicotinamide (isn), pyridine (py), 4-picoline (pic), nicotinamide (nic), isonicotinic acid (ina) or imidazol (imN) were selected and tested in the treatment of tuberculosis. The results of anti-tuberculosis activity are shown in Table 2. As can be noticed, ruthenium nitrosyl complexes (complexes 10–16 in

**Table 2**  
Data of MIC, IC<sub>50</sub> (J774A.1) and Ep<sub>a</sub>(INH) for the Ru(II) compounds and free INH against the H<sub>37</sub>Rv and CF45 and CF74 resistant strains.

Compounds	MIC (μg mL <sup>-1</sup> )						IC <sub>50</sub> (μg/mL)	Selectivity Index (SI)	Ep <sub>a</sub> INH <sup>+</sup> (V)
	H <sub>37</sub> Rv	SD (±)	CF45	SD (±)	CF74	SD (±)			
1 Isoniazid (INH)	0.07	0.02	>25	*	>25	*	500	7142.85	1.03
2 Isonicotinamide (isn)	>25	*	>25	*	>25	*	*	*	*
3 Isonicotinic acid (ina)	>25	*	>25	*	>25	*	*	*	*
4 [Ru(NH <sub>3</sub> ) <sub>5</sub> (INH)] <sup>2+</sup>	0.60	0.17	22.55	1.06	24.26	0.54	250	416.66	0.84
5 [Ru(NH <sub>3</sub> ) <sub>5</sub> (isn)] <sup>2+</sup>	>25	*	>25	*	>25	*	*	*	*
6 [Ru(NH <sub>3</sub> ) <sub>5</sub> (ina)] <sup>2+</sup>	>25	*	>25	*	>25	*	*	*	*
7 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (SO <sub>2</sub> )(INH)] <sup>2+</sup>	0.88	0.50	>25	*	>25	*	500	568.18	0.82
8 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (SO <sub>2</sub> )(isn)] <sup>2+</sup>	>25	*	>25	*	>25	*	*	*	*
9 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (SO <sub>2</sub> )(ina)] <sup>2+</sup>	>25	*	>25	*	>25	*	*	*	*
10 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (NO)(imN)] <sup>3+</sup>	5.9	*	*	*	*	*	>815	>138.13	*
11 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (NO)(py)] <sup>3+</sup>	>25	*	*	*	*	*	*	*	*
12 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (NO)(pic)] <sup>3+</sup>	>25	*	*	*	*	*	*	*	*
13 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (NO)(nic)] <sup>3+</sup>	>25	*	*	*	*	*	*	*	*
14 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (NO)(isn)] <sup>3+</sup>	>25	*	*	*	*	*	*	*	*
15 <i>trans</i> -[Ru(NH <sub>3</sub> ) <sub>4</sub> (NO)(ina)] <sup>3+</sup>	>25	*	*	*	*	*	*	*	*

SD = Standard deviation.

\* Ep<sub>a</sub> vs. SCE: pH 7.0, μ = 0.10 mol L<sup>-1</sup>.

Table 2) exhibited low or none activity against the MTB H<sub>37</sub>Rv strain. Attempts to synthesize the complex *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(NO<sup>+</sup>)(L)]<sup>3+</sup> with L = INH failed owing to necessary step of oxidation used in the sulfite/sulfate (Tfouni et al., 2003) which could oxidize the ligand INH. If this synthesis was successful, a synergic effect between nitric oxide and INH would be expected in the treatment of tuberculosis (Silva et al., 2008). Despite this, the complexes [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> and *trans*-[Ru(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)]<sup>2+</sup>, which have INH in the coordination sphere, exhibited activity against sensitive and resistant strains of MTb.

The changes in the oxidation potential of coordinated INH by coordination to the metal complexes could be an interesting approach to overcome the problems in the treatment of resistant strains of *M. tuberculosis*, in which isoniazid has little or no activity (Vasconcelos et al., 2008). Again, it is interesting to recall that the INH lack of activity in most of the resistant strains is attributed to the absence of isoniazid-activating enzyme KatG, which is the main responsible agent for the isoniazid activation (Vasconcelos et al., 2008). Thus, as discussed above about the electrochemical data, the coordination of INH makes less positive the oxidation potential of this ligand. This approach might lead to the isoniazid oxidation by other compounds, such as Mn<sup>2+</sup> instead of KatG pathway (Magliozzo and Marcinkeviciene, 1997). Manganese alone can induce the aerobic oxidation of INH so as to yield the same products observed for the KatG activation (Magliozzo and Marcinkeviciene, 1997). The oxidation of the INH occurs after the peroxidation of Mn(II) into Mn(III) (Magliozzo and Marcinkeviciene, 1997). Despite some literature considers this mechanism, the possibility of the manganese acting as an oxidant is not fully accepted (Wei et al., 2003). Recently, an alternative pathway cropped up from interesting results of the acyl radical formation from the oxidation of the INH pentacyanoferrate complex by hydrogen peroxide and superoxide have been shown. (Sousa et al., 2014). According to the authors the results reveals that hydrogen peroxide and superoxide are capable to promote a very fast and efficient oxidation of the coordinated INH (Sousa et al., 2014). This proposal seems reasonable since Mtb resides mainly inside the macrophage, one of the most oxidizing environments found in living cells (Sousa et al., 2014).

Additionally, based on previous discussions about isoniazid-like metal complexes (Sousa et al., 2012), the electrochemical potential for Ru(III/II) would also play an important role in the antituberculosis activity. Sousa et al. (2012) compared the ability of [Ru(CN)<sub>5</sub>(INH)]<sup>3-</sup> and [Fe(CN)<sub>5</sub>(INH)]<sup>3-</sup> complexes to inhibit

the InhA enzyme. Despite the similarity on the coordination sphere of these complexes, only the iron complex was able to inhibit InhA (Sousa et al., 2012). According to the authors (Sousa et al., 2012), the non-activity of the ruthenium complex regarding to the iron complex would be related to the difference in the electrochemical potential of the metal center (E<sub>1/2</sub>), which was E<sub>1/2</sub> ~1000 mV vs. Ag/AgCl for the couple Ru(III/II) in the complex [Ru(CN)<sub>5</sub>(INH)]<sup>3-</sup> and E<sub>1/2</sub> = 578 mV vs. Ag/AgCl for the couple Fe(III/II) in [Fe(CN)<sub>5</sub>(INH)]<sup>3-</sup>.

The molecules isn and ina are known to be products of isoniazid oxidation and have demonstrated no activity against MTb (Johnsson et al., 1995). Ruthenium complexes containing these ligands in the coordination sphere (*trans*-[Ru(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(L)](BF<sub>4</sub>)<sub>2</sub> and [Ru(NH<sub>3</sub>)<sub>5</sub>(L)](BF<sub>4</sub>)<sub>2</sub>, where L = isn or ina) were synthesized and tested *in vitro* against MTb, and no activity was observed (Table 2). The collected data suggests that in the absence of isoniazid as ligand, these two inorganic moieties ([Ru(NH<sub>3</sub>)<sub>5</sub>] and [Ru(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)] have no activity against MTb.

The most promising results in the present study are related to the antituberculosis activity against resistant strains of MTb obtained with the complex [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)](BF<sub>4</sub>)<sub>2</sub>. According to the MIC and SI data of Table 2, the performance of this ruthenium complex was comparable to or better than the first- and second-line drugs of the current therapeutic treatment against sensitive strains, Rifampicin (0.4 μg/ml), Streptomycin (1.0 μg/ml), Ofloxacin (0.5 μg/ml) and Ethambutol (0.5 μg/ml) (Handbook of anti-tuberculosis agents, 2008). Since none of the compounds showed cytotoxic against VERO epithelial cells in the concentrations tested, the cytotoxicity (IC<sub>50</sub>) of the compounds against J774A.1 macrophage cells (Table 2) was investigated. As macrophages are the major phagocytic cells of the immune system, they are the first cells involved in the immune response and this cell is a host for Mtb. Again, the compounds showed relative low cytotoxicity against J774A.1, with IC<sub>50</sub> higher than 250 μg/mL. As the SI is used to estimate the therapeutic window of a drug and to identify drug candidates for further studies, the complex [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> (MIC = 0.6 μg/mL, IC<sub>50</sub> = 250 μg/mL and SI = 416.66) is very promising as a new tuberculosis candidate, and the index is comparable to the SI of Rifampicin (MIC = 0.2 μg/mL, IC<sub>50</sub> = 156.3 μg/mL: SI = 781) (Collins and Franzblau, 1997), which is used together with isoniazid to treat tuberculosis.

The *in vitro* data obtained with the ruthenium complexes *trans*-[Ru<sup>II</sup>(NH<sub>3</sub>)<sub>4</sub>(SO<sub>2</sub>)(INH)]<sup>2+</sup> and [Ru(NH<sub>3</sub>)<sub>5</sub>(INH)]<sup>2+</sup> in treatments against *M. tuberculosis* were promising especially regarding to the

$[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{2+}$  which showed some degree of activity in strains resistant to Isoniazid and Rifampicin. This feature is important and deserves further attention taking into account the worrying emergence to combat these strains (Handbook of anti-tuberculosis agents, 2008).

One of the isoniazid-resistant clinical isolates ( $\text{MIC} > 25.00 \mu\text{g}/\text{mL}$ ) used in the activity tests showed a single mutation, i.e. a change in position 315 of the *katG* gene with the replacement of serine for threonine (Ser315Thr). This mutation can reduce 6-fold the catalase activity of the protein, as well as 2-fold its peroxidase activity by decreasing the conversion efficiency of isoniazid to isonicotinic acid (Middlebrook, 1954). Although the values of activity obtained by the  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{2+}$  complex in resistant strains are still not ideal for the development of a new drug against the disease, such structures can be a guide of great value in tailoring new species to circumvent the resistance mechanisms of the *bacillus* using coordination complexes (Pavan et al., 2011). Considering the other clinical isolate, in which the only found mutation is silent (in the 6th codon of the *ahpC* gene), the activity of the compound was similar to the first one. Regarding the fact that a 90% inhibition in growth in concentrations lower than  $25.00 \mu\text{g}/\text{mL}$  was confirmed for the compound  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{2+}$ , the perspectives would be very promising. Isoniazid, one of the standard drugs for TB treatment, in the same test, did not show any inhibition even in the concentration of  $25.00 \mu\text{g}/\text{mL}$ . In this context, selectivity indexes for the compounds are considered acceptable for a drug discovery as suggested by the cut-off value of  $>10.00$  (Secrist et al., 2001).

### 3.4. DFT studies

In order to obtain more insight about the INH activation through the coordination to metal complexes, DFT studies were carried out for verifying the stability of the possible intermediates generated during the INH oxidation in the  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{2+}$  and

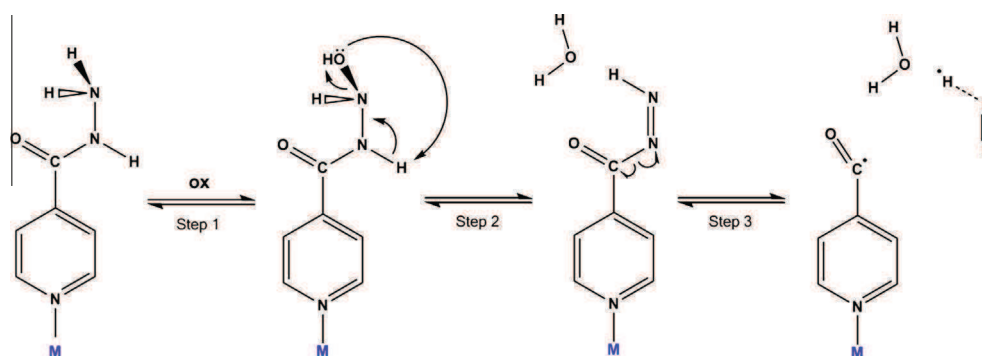
$\text{trans}-[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})]^{2+}$  species. The kinetic and thermodynamic parameters involved in INH oxidation were calculated to assist in understanding the determining step for the activity of the  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{2+}$  and  $\text{trans}-[\text{Ru}(\text{NH}_3)_4(\text{SO}_2)(\text{INH})]^{2+}$  complexes against the resistant strains. As described in Section 2.4, for the theoretical investigations the oxidation state of the ruthenium metal center was considered as being (III), since the oxidation of the metal center occurs in a potential more favorable than the INH oxidation (please see voltammograms in Fig. 4). Also, the ligand  $\text{SO}_2$  was considered as  $\text{SO}_3^{2-}$  as a function of the physiologic medium (pH 7.4) (Isied and Taube, 1974). Thus, the chemical formula of the complexes considered in DFT studies was  $\text{trans}-[\text{Ru}(\text{NH}_3)_4(\text{SO}_3)(\text{INH})]^+$  and  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{3+}$ .

As proposed by Metushi et al. (2011), the oxidation of INH occurs through a 3 step pathways (Fig. 5). The last step is responsible for the acyl radical formation, which will react with  $\text{NAD}^+$  to form the NAD-INH adduct, inhibiting the FASII enoyl-ACP reductase InhA. By adopting this mechanism as a guide to DFT investigations is possible to compare the calculated activation barrier for the oxidation of non-coordinated INH and for the complexes  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{3+}$  and  $\text{trans}-[\text{Ru}(\text{NH}_3)_4(\text{SO}_3)(\text{INH})]^+$ .

In the first step (step 1), the energies were obtained by BDE for the interaction between INH and oxygen. In the subsequent steps a potential energy surface mappings were carried out to obtain the saddle points for the minimum conformational. Water molecules were added in the matrix, in order to explicitly simulate the effect of the solvent in addition to the IEFPCM (Amorati et al., 2013; Silva et al., 2014), which considerably lowers the activation parameters for the oxidation, Table 3.

The activation barrier have similar values for the coordinated and non-coordinated INH (Table 3), which suggests that in the oxidation process, the coordination of the metal center does not affect the energy barriers.

Conversely to the observed for the free INH and  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{3+}$ , our calculations suggest that the oxidation of INH in the



**Fig. 5.** Proposed mechanism for the oxidation of non-coordinated INH and  $[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{3+}$ . For complex  $\text{trans}-[\text{Ru}(\text{NH}_3)_4(\text{SO}_3)(\text{INH})]^+$  the step 2 is not observed (Metushi et al., 2011).

**Table 3**

Kinetic ( $\Delta G^\ddagger$ ) and thermodynamic (BDE and  $\Delta G$ ) parameters ( $\text{kcal mol}^{-1}$ ), according to mechanism proposed in Fig. 5.

		IEFPCM		Explicit solvent effect	
		INH	INH	$[\text{Ru}(\text{NH}_3)_5(\text{INH})]^{3+}$	$\text{trans}-[\text{Ru}(\text{NH}_3)_4(\text{SO}_3)(\text{INH})]^+$
BDE <sub>step1</sub>	( $\text{kcal mol}^{-1}$ )	-50.2	-58.1	-55.8	-58.5
$\Delta G_{\text{step2}}^\ddagger$		46.4	12.5	13.1	12.9
$\Delta G_{\text{step2}}$		-7.4	3.4	6.4	-18.0
$\Delta G_{\text{step3}}^\ddagger$		37.4	12.2	2.8	-
$\Delta G_{\text{step2}}$		35.4	4.2	-46.5	-

complex  $trans-[Ru(NH_3)_4(SO_3)(INH)]^+$  occurs in 2 steps, since the destabilization of the intermediate formed in step 2 automatically degrades it, generating the acyl radical, Fig. 5.

According to Table 3, the values of BDE found for free INH,  $[Ru(NH_3)_5(INH)]^{3+}$  and  $trans-[Ru(NH_3)_4(SO_3)(INH)]^+$  compounds were very similar (55–58.8 kcal mol<sup>-1</sup>), which strongly suggests that the first step of oxidation is not the relevant step for the activity. However, the formation of the acyl radical (Fig. 5 – step 3) was favored in the  $[Ru(NH_3)_5(INH)]^{3+}$  complex by 50.7 kcal mol<sup>-1</sup> regarding to the non-coordinated INH molecule (Table 3), whereas for the  $trans-[Ru(NH_3)_4(SO_3)(INH)]^+$ , the acyl radical formation was unfavorable by 28.5 kcal mol<sup>-1</sup> with respect to  $[Ru(NH_3)_5(INH)]^{3+}$ . The differences in the relative enthalpies are coherent with the MIC values experimentally obtained (see Table 2). Thus, the experimental and calculated data suggest that the more favorable the formation of the acyl radical the more efficient would be the compound against resistant MTb strains.

#### 4. Conclusions

In this work the synthesis and characterization of new isoniazid-like Ru(II) complexes and the *in vitro* activity of these complexes against tuberculosis were reported.

The ruthenium complex  $[Ru(NH_3)_5(INH)]^{2+}$  exhibited antitubercular activity against sensitive and resistant strains, and this result becomes more significant, since in general, the first-line drugs have no activity against resistant strains. Reactions pathways for the oxidation of free and coordinated INH were studied using DFT calculations. These studies suggested that the coordination of INH to the complex  $[Ru(NH_3)_5(INH)]^{2+}$  stabilized the acyl radical formed after INH oxidation, which is likely a key intermediate species in the drug action mechanism.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejps.2015.01.008>.

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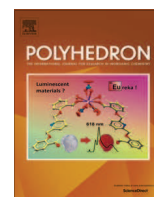
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## Pyrazolyl Pd(II) complexes containing triphenylphosphine: Synthesis and antimycobacterial activity



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### ABSTRACT

Complexes of the type *trans*-[PdCl<sub>2</sub>(HL)(PPh<sub>3</sub>)], where HL = pyrazole (**1**); 3,5-dimethylpyrazole (**2**); 4-nitropyrazole (**3**); 4-iodopyrazole (**4**) and PPh<sub>3</sub> = triphenylphosphine, were synthesized and characterized by elemental analyses, infrared and <sup>1</sup>H NMR spectroscopies. Single-crystal X-ray diffraction determination on **3**·0.9 CHCl<sub>3</sub> and **4** showed that the coordination geometry around Pd(II) is nearly square-planar, with the chloro ligands in a *trans* configuration. *In vitro* antimycobacterial evaluation demonstrated that compound **4** displayed a minimum inhibitory concentration (MIC) of 7.61 ± 2.18 μM, being superior to the values observed for some commonly used antituberculosis drugs and other metal-based complexes.

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### 1. Introduction

Tuberculosis (TB), an airborne infectious disease caused by *Mycobacterium tuberculosis* (MTB) and other mycobacteria, is still a major worldwide health threat [1]. The urgent need to discover new anti-TB agents is justified by many important reasons, mainly: (i) the outbreak of multidrug resistant (MDR) and extensively drug-resistant (XDR-TB) TB strains of *M. tuberculosis*; (ii) the spreading of the human immunodeficiency virus (HIV) and its deadly synergy with TB and nontubercular mycobacterial infections; (iii) the poor compliance with the complexity and toxicity of the current chemotherapeutic regimens [1–3].

In this sense, pyrazole-type heterocycles have emerged as a class of potential antimycobacterial agents [4–7]. Horrocks et al. [7] have synthesized some 3-(4-chlorophenyl)-4-substituted pyrazole derivatives which displayed not only an interesting activity against the tested strain of *M. tuberculosis* H<sub>37</sub>Rv, but also exhibited remarkable antifungal activity against four pathogenic strains. Velaparthy et al. [8] have described a series of 5-tert-butyl-N-pyrazol-4-yl-4,5,6,7-tetrahydrobenzo[d]isoxazole-3-carboxamide

derivatives as novel potent *M. tuberculosis* pantothenate synthetase inhibitors showing cytotoxicity activity (IC<sub>50</sub>) ranging from 90 nM to 7.13 μM.

The importance of pyrazole derivatives has also been accompanied by an increasing interest focused on the coordination chemistry of these heterocycles [9–11]. Particularly, metal-based compounds of pyrazoles containing phosphines as co-ligands have also received attention due to their possible use as antitumor and antimicrobial drugs [12,13]. Nomiya et al. [13] synthesized the complex [Au(HPz)(PPh<sub>3</sub>)] (HPz = pyrazole; PPh<sub>3</sub> = triphenylphosphine) which showed activities against Gram-positive bacteria (*Staphylococcus aureus*) and one yeast (*Candida albicans*). Nevertheless, studies on the antimycobacterial activity towards TB involving pyrazolyl complexes bearing phosphine ligands remain scarce in literature.

For many years, our research has been focused on the synthesis of metal compounds containing N-, P- or S-based ligands and the evaluation of their activity against tumour cell lines and *M. tuberculosis* [14–19]. Ferreira et al. have obtained the organometallic compound [Pd(C-bzan)(SCN)(dppp)] {bzan = N-benzylideneaniline, dppp = 1,3-bis(diphenylphosphino)propane} which displayed significant antimycobacterial activity (MIC<sub>90</sub> = 5.15 μM) [20]. Recently, we have described the synthesis of binuclear compounds of the type [Pd(μ-L)(N<sub>3</sub>)(PPh<sub>3</sub>)<sub>2</sub>] {L = pyrazolate (Pz); 3,5-dimethylpyrazolate (dmPz); 4-iodopyrazolate (IPz)} [21]. *In vitro* antimycobacterial

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assays demonstrated that compound  $[\text{Pd}(\mu\text{-Pz})(\text{N}_3)(\text{PPh}_3)_2]$  exhibited a MIC of 8.16  $\mu\text{M}$ . This findings have prompted us to evaluate the anti-TB activity of other Pd(II) compounds bearing phosphine ligands. Inspired by the promising biological results obtained for mononuclear compounds of the type  $[\text{Au}(\text{pyrazoles})(\text{PPh}_3)]$  [12,13] and as a part of our ongoing studies on coordination chemistry of pyrazolyl ligands [22–24], we prepared analogous Pd(II) derivatives with general formulae  $\text{trans-}[\text{PdCl}_2(\text{HL})(\text{PPh}_3)]$ , where  $\text{PPh}_3$  = triphenylphosphine and HL are ligands of type pyrazole (HPz); 3,5-dimethylpyrazole (HdmPz); 4-nitropyrazole ( $\text{HNO}_2\text{Pz}$ ) and 4-iodopyrazole (HIPz), and investigated their antimycobacterial activity against *M. tuberculosis*. The crystallographic structures of the compounds **3**·0.9  $\text{CHCl}_3$  and **4** are also described in this work.

## 2. Material and methods

### 2.1. General methods

Synthesis were performed at ambient temperature. The precursor  $[\text{PdCl}_2(\text{MeCN})_2]$  was prepared as previously described [25]. Pyrazolyl ligands and triphenylphosphine were purchased from Sigma Aldrich or Merck. Reagents and solvents were employed without further purification.

### 2.2. Synthesis

Compounds  $[\text{PdCl}_2(\text{HPz})(\text{PPh}_3)]$  (**1**),  $[\text{PdCl}_2(\text{HdmPz})(\text{PPh}_3)]$  (**2**),  $[\text{PdCl}_2(\text{HNO}_2\text{Pz})(\text{PPh}_3)]$  (**3**) and  $[\text{PdCl}_2(\text{HIPz})(\text{PPh}_3)]$  (**4**) were prepared by adding a mixture containing 1.17 mmol of the appropriated pyrazolyl ligand {79.6 mg of HPz (**1**), 112 mg of HdmPz (**2**), 132 mg of  $\text{HNO}_2\text{Pz}$  (**3**) or 227 mg of HIPz (**4**)} and triphenylphosphine (307 mg; 1.17 mmol) in 5 mL of  $\text{CHCl}_3$  to an orange solution of  $[\text{PdCl}_2(\text{CH}_3\text{CN})_2]$  (301 mg; 1.16 mmol) in 15 mL of  $\text{CHCl}_3$ . The mixtures were stirred magnetically for 2 h, and then the solutions were concentrated to 2 mL under reduced pressure. The addition of pentane (30 mL) resulted in the precipitation of the products **1–4**, which were filtered and dried under vacuum. Yield 79–90%.

### 2.3. Physical measurements

C, H and N analyses were performed on a Perkin Elmer 2400. Conductivities were measured with a Digimed-DM-31 conductometer using  $1 \times 10^{-3}$  mol  $\text{L}^{-1}$  DMSO solutions. Infrared spectra were recorded as KBr pellets on a Nicolet FTIR-Impact 400 spectrophotometer in the spectral range 4000–400  $\text{cm}^{-1}$  with resolution of 2  $\text{cm}^{-1}$ . The  $^1\text{H}$  NMR spectra were obtained using  $\text{CDCl}_3$  solutions, on a Varian INOVA 500 spectrometer.

### 2.4. Single-crystal X-ray diffraction studies

Single crystals for X-ray crystallography of **3** and **4** were obtained by slow diffusion of pentane into a solution of the complexes in chloroform. X-ray diffraction data for **3**·0.9  $\text{CHCl}_3$  and **4** were collected on a Bruker X8 Kappa APEX II with a charge-coupled device (CCD) area-detector diffractometer (Mo  $\text{K}\alpha$  graphite-monochromated radiation,  $\lambda = 0.71073$  Å) controlled by the APEX2 software package [26], and equipped with an Oxford Cryosystems Series 700 cryostream monitored remotely using the software interface Cryoapd [27]. Images were processed using the software package SAINT+ [28], and data were corrected for absorption by the multi-scan semi-empirical method implemented in SADABS [29]. The crystal structures of **3**·0.9  $\text{CHCl}_3$  and **4** were solved using the Patterson synthesis algorithm implemented in SHELXS-97 [30], which allowed the immediate location of the crystallographically independent  $\text{Pd}^{2+}$  centers and the most of

the heaviest atoms. All remaining non-hydrogen atoms were located from difference Fourier maps calculated from successive full-matrix least squares refinement cycles on  $F^2$  using SHELXL-2014 [31,32], and refined using anisotropic displacement parameters. Refinement of solvent occupancy of the chloroform molecule in **3** converged at 0.892(2), however this was fixed at 0.9.

Hydrogen atoms bound to carbon were placed at their idealized positions using appropriate HFIX instructions in SHELXL: 13 for the –CH from chloroform and 43 for the CH groups of the aromatic rings. These atoms were included in subsequent refinement cycles in riding motion approximation with isotropic thermal displacements parameters ( $U_{\text{iso}}$ ) fixed at  $1.2 \times U_{\text{eq}}$  of the parent carbon atoms. In opposition, H atoms bonded to nitrogen were located from difference Fourier maps and the N–H distance and isotropic thermal displacements parameters ( $U_{\text{iso}}$ ) fixed at 0.88 Å and  $1.5 \times U_{\text{eq}}$  of the nitrogen atoms, respectively.

For compound **3**·0.9  $\text{CHCl}_3$ , the last difference Fourier map synthesis showed the highest peak ( $0.89 \text{ e}\text{\AA}^{-3}$ ) located at 1.02 Å from Cl4, and the deepest hole ( $-1.12 \text{ e}\text{\AA}^{-3}$ ) at 0.76 Å from Pd1. For compound **4**, the last difference Fourier map synthesis showed the highest peak ( $2.59 \text{ e}\text{\AA}^{-3}$ ), and the deepest hole ( $-3.22 \text{ e}\text{\AA}^{-3}$ ), located at 0.44 and 1.06 Å from I1, respectively. Details of the crystal data and structure refinement parameters for **3**·0.9  $\text{CHCl}_3$  and **4** are summarized in Table 1.

### 2.5. Antimycobacterial assays

The anti-*M. tuberculosis* activity was determined by the Resazurin Microtiter Assay (REMA) [33,34]. Stock solutions of the test compounds were prepared in DMSO and diluted in

**Table 1**  
Crystal and structure refinement data for  $[\text{PdCl}_2(\text{HNO}_2\text{Pz})(\text{PPh}_3)] \cdot 0.9 \text{ CHCl}_3$  (**3**·0.9  $\text{CHCl}_3$ ) and  $[\text{PdCl}_2(\text{HIPz})(\text{PPh}_3)]$  (**4**).

	( <b>3</b> ·0.9 $\text{CHCl}_3$ )	( <b>4</b> )
Formula	$\text{C}_{21}\text{H}_{18}\text{Cl}_2\text{N}_3\text{O}_2\text{PPd} \cdot 0.9(\text{CHCl}_3)$	$\text{C}_{21}\text{H}_{18}\text{Cl}_2\text{I}_2\text{N}_2\text{PPd}$
Formula weight	660.09	633.54
Crystal system	monoclinic	triclinic
Space group	$P2_1/n$	$P\bar{1}$
T (K)	150	150
a (Å)	15.0018(8)	10.2246(5)
b (Å)	9.4988(5)	10.5312(5)
c (Å)	18.6528(10)	12.0140(5)
$\alpha$ (°)	90	102.004(2)
$\beta$ (°)	103.784(3)	96.708(2)
$\gamma$ (°)	90	116.699(2)
V (Å <sup>3</sup> )	2581.5(2)	1097.22(19)
Z	4	2
$D_{\text{calc}}$ ( $\text{g cm}^{-3}$ )	1.698	1.918
$\theta$ range (°)	3.6–33.1	2.3–25.4
$\mu$ (Mo $\text{K}\alpha$ ) ( $\text{mm}^{-1}$ )	1.29	2.58
Crystal type	Yellow block	Orange block
Crystal size (mm)	0.15 × 0.12 × 0.08	0.15 × 0.12 × 0.08
Index ranges	$-23 \leq h \leq 17$ , $-14 \leq k \leq 14$ , $-27 \leq l \leq 28$	$-12 \leq h \leq 12$ , $-12 \leq k \leq 12$ , $-14 \leq l \leq 14$
Reflections collected	32371	52973
Independent reflections	9819 [ $R_{\text{int}} = 0.062$ ]	4014 [ $R_{\text{int}} = 0.0648$ ]
Completeness	99.7% (to $\theta = 30.0$ )	100% (to $\theta = 25.4$ )
Final R indices	$R_1 = 0.047$ , $wR_2 = 0.112$	$R_1 = 0.040$ , $wR_2 = 0.089$
Weighting scheme <sup>c</sup>	$m = 0.0446$ $n = 0$	$m = 0.183$ $n = 6.1607$
Largest diff. peak and hole ( $\text{e}\text{\AA}^{-3}$ )	0.89 and $-1.12$	2.59 and $-3.22$

<sup>a</sup>  $R_1 = \sum ||F_o| - |F_c|| / \sum |F_o|$ .

<sup>b</sup>  $wR_2 = \sqrt{\sum [w(F_o^2 - F_c^2)]^2 / \sum [w(F_o^2)]^2}$ .

<sup>c</sup>  $w = 1 / [\sigma^2(F_o^2) + (mP)^2 + nP]$  where  $P = (F_o^2 + 2F_c^2) / 3$ .

Middlebrook 7H9 broth (Difco), supplemented with oleic acid, albumin, dextrose and catalase (OADC enrichment – BBL/Becton Dickinson, Sparks, MD, USA), to obtain final compound concentration ranging from 0.15 to 250  $\mu\text{g mL}^{-1}$ . The serial dilutions were performed in a Precision XS Microplate Sample Processor (Biotek™). The isoniazid was dissolved in distilled water, according to the manufacturers' recommendations (Difco laboratories, Detroit, MI, USA), and used as a standard drug. *M. tuberculosis* H<sub>37</sub>Rv ATCC 27294 was grown for 7–10 days in Middlebrook 7H9 broth supplemented with OADC, plus 0.05% Tween 80 to avoid clumps. Suspensions were prepared and their turbidities matched to the optical density of the McFarland No. 1 standard. After a further dilution of 1:25 in Middlebrook 7H9 broth supplemented with OADC, 100  $\mu\text{L}$  of the culture were transferred to each well of a 96-well microtiter plate (NUNC), together with the test compounds. Each test was set up in triplicate. Microplates were incubated for 7 days at 37 °C, after which resazurin was added for the reading. Wells that turned from blue to pink, with the development of fluorescence, indicated growth of bacterial cells; maintenance of the blue colour indicated bacterial inhibition. The fluorescence was read (530 nm excitation filter and 590 nm emission filter) in a SPECTRA fluor Plus (Tecan) microfluorimeter. The MIC was defined as the lowest concentration resulting in 90% inhibition of growth of *M. tuberculosis*. As a standard test, the MIC of isoniazid was determined on each microplate. The acceptable range of isoniazid MIC is from 0.015 to 0.05  $\mu\text{g mL}^{-1}$  [34,35].

### 3. Results and discussion

Compound  $[\text{PdCl}_2(\text{MeCN})_2]$  reacts with the appropriate pyrazolyl ligand (HL) and triphenylphosphine ( $\text{PPh}_3$ ), in a 1:1:1 molar ratio, respectively, to yield mononuclear compounds of the type  $[\text{PdCl}_2(\text{HL})(\text{PPh}_3)]$ . A general scheme which represents the strategy employed for the synthesis of the complexes is given in Scheme 1.

Complexes 1–4 are air and light stable solids soluble in DMSO and chloroform and exhibit colour that varies from light yellow to orange. The elemental analysis and molar conductivity results are given in Table 2.

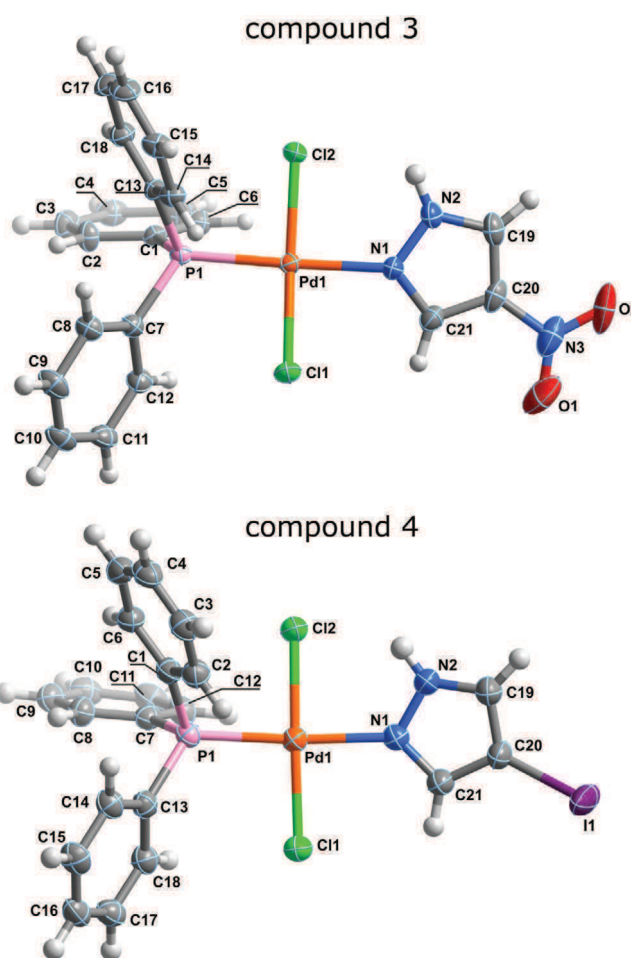
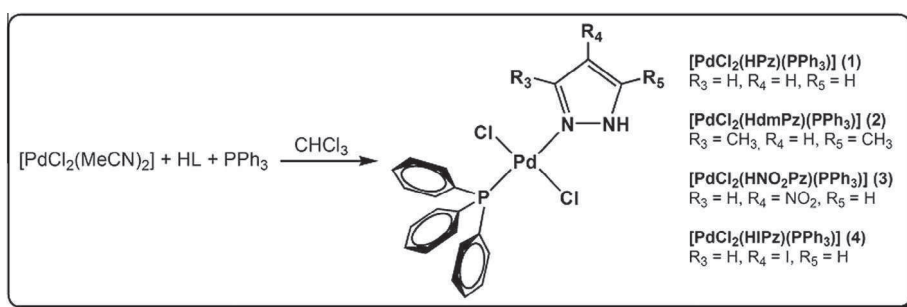


Fig. 1.  $[\text{PdCl}_2(\text{HNO}_2\text{Pz})(\text{PPh}_3)] \cdot 0.9\text{CHCl}_3$  (**3**·0.9CHCl<sub>3</sub>) and  $[\text{PdCl}_2(\text{HIPz})(\text{PPh}_3)]$  (**4**) coordination complexes showing non-hydrogen atoms represented as displacement ellipsoids drawn at 50% probability level and hydrogen atoms as spheres with arbitrary radii. Labels are provided for all non-hydrogen atoms.



Scheme 1. A synthesis scheme for the compounds of the type  $[\text{PdCl}_2(\text{HL})(\text{PPh}_3)]$ , where HL = pyrazole (**1**); 3,5-dimethylpyrazole (**2**); 4-nitropyrazole (**3**); 4-iodopyrazole (**4**) and  $\text{PPh}_3$  = triphenylphosphine.

Table 2

Analytical and physico-chemical data on complexes  $[\text{PdCl}_2(\text{HPz})(\text{PPh}_3)]$  (**1**),  $[\text{PdCl}_2(\text{HdmPz})(\text{PPh}_3)]$  (**2**),  $[\text{PdCl}_2(\text{HNO}_2\text{Pz})(\text{PPh}_3)]$  (**3**) and  $[\text{PdCl}_2(\text{HIPz})(\text{PPh}_3)]$  (**4**).

Complex	$\Lambda_M$ ( $\Omega^{-1} \text{cm}^2 \text{mol}^{-1}$ )	Colour	Carbon		Nitrogen		Hydrogen	
			Found (%)	Calc. (%)	Found (%)	Calc. (%)	Found (%)	Calc. (%)
$\text{C}_{21}\text{H}_{19}\text{Cl}_2\text{N}_2\text{PPd}$ ( <b>1</b> )	1.64	Yellow	49.80	49.86	5.50	5.52	3.82	3.77
$\text{C}_{23}\text{H}_{23}\text{Cl}_2\text{N}_2\text{PPd}$ ( <b>2</b> )	2.24	Yellow orange	52.00	51.56	5.40	5.23	4.53	5.10
$\text{C}_{21}\text{H}_{18}\text{Cl}_2\text{N}_3\text{O}_2\text{PPd}$ ( <b>3</b> )	4.59	Orange	45.28	45.64	7.70	7.60	3.15	3.28
$\text{C}_{21}\text{H}_{18}\text{Cl}_2\text{IN}_2\text{PPd}$ ( <b>4</b> )	4.59	Light yellow	39.79	39.81	4.44	4.42	2.80	2.86

Analytical results are in agreement with the proposed formulae. The molar conductivities of **1–4** in DMSO are between 1.64 and  $4.59 \Omega^{-1} \text{cm}^2 \text{mol}^{-1}$ , in agreement with their nonelectrolytic nature [36]. These values were significantly lower than those observed for Pd(II) and Pt(II) complexes with 1:1 electrolyte behaviour ( $\Lambda_M = 20\text{--}30 \Omega^{-1} \text{cm}^2 \text{mol}^{-1}$ ) [37,38]. In addition, the molar conductivity of **1–4** shows little change after storage at room temperature for 24 h (see Table S1 – Supporting Information), indicating that these species are relatively stable in DMSO solution over a period of 24 h.

### 3.1. Single-crystal X-ray diffraction studies

The monomeric nature and important structural features of **3** and **4** were investigated using X-ray diffraction studies. The molecular structures of compounds **3**·0.9 CHCl<sub>3</sub> and **4** are depicted in Fig. 1. Selected bond lengths, bond angles and supramolecular interactions are given in Table 3.

Compound **3** crystallizes in the monoclinic space group  $P2_1/c$ , with the asymmetric unit being composed by a whole complex molecule and one fraction (0.9) of a chloroform molecule. The metal centre is coordinated to two chloride anions, one molecule of HNO<sub>2</sub>Pz and one PPh<sub>3</sub> in a *trans* configuration, formulated as *trans*-[PdCl<sub>2</sub>(HNO<sub>2</sub>Pz)(PPh<sub>3</sub>)] (Fig. 1). The geometry around Pd(II) is slightly distorted square planar, in which the *trans* angles, Cl1–Pd–Cl2 and N1–Pd–P1, are 176.15(3)° and 175.32(7)°, respectively; *cis* angles vary in the interval 87.44(3)–94.19(3)°. The Pd–Cl2 bond [2.3004(7) Å] is slightly longer than Pd–Cl1 of [2.2794(7) Å] and the Pd–N1 bond [2.115(2) Å] is higher than those found in regular coordination polyhedron such as [Pd(SCN)<sub>2</sub>(HPz)<sub>2</sub>], [Pd(SCN)<sub>2</sub>(phmPz)<sub>2</sub>] (phmPz = 1-phenyl-3-methylpyrazole) [39], [PdCl<sub>2</sub>(phmPz)<sub>2</sub>] and [Pd(N<sub>3</sub>)<sub>2</sub>(phmPz)<sub>2</sub>] [40] where Pd–N bond was found in the range 2.011–2.018 Å. This difference can presumably be ascribed to the *trans* effect of the phosphine ligand. The plane of the pyrazole ring and the planar coordination environment around the metal centre are almost co-planar with an interplanar angle of 18.38(13)°.

Compound **4** crystallises in the triclinic space group  $P\bar{1}$ , comprising an unsolvated complex *trans*-[PdCl<sub>2</sub>(HIPz)(PPh<sub>3</sub>)] with coordination environment around the metal centre similar to that of **3**·0.9 CHCl<sub>3</sub>, with the ligand HIPz present instead of HNO<sub>2</sub>Pz. Conversely, complex **4** has a quadratic planar geometry slightly distorted like **3** with *trans* angles, Cl1–Pd–Cl2 and N1–Pd–P1 of 177.49(5)° and 174.58(11)°, respectively; the *cis* angles are found in the range 87.57(5)–94.02(5)°. The Pd–Cl1/Cl2 and Pd–N1 bonds show the same behaviour as those of complex **3** with bonds distances 2.2792(13)/2.3119(12) Å and 2.120(4) Å, respectively. The interplanar angle between the plane of the pyrazole ring and the planar coordination environment around the metal centre is 20.0(2)°.

Hydrogen bonding interactions share in the two structures similar features. Both structures show a ring with graph set motif  $R_2^2(4)$  (Fig. 2) formed by the hydrogen bonds interactions between a pair of donors (N2) and a pair of acceptors (Cl2) [32]. This feature is also present in the complex [PdCl(HPz)(C<sub>6</sub>H<sub>5</sub>)(PPh<sub>3</sub>)], HPz = pyrazole [41]. While in **3** the bifurcated N2–H2Z···(Cl2)<sub>2</sub> interaction shows similar D···A distances and D–H···A angles, in **4** the corresponding geometrical parameters are highly unsymmetrical, with a relatively strong intramolecular interaction and a very weak intermolecular interaction (details in Table 2 and Fig. 2). Additionally, the two structures exhibit weak interactions of the type C–H···π. Weak forces of the type C–Cl···π and Cl···I are observed in the crystal structures of **3**·0.9 CHCl<sub>3</sub> and **4**, respectively (details in Table 3).

### 3.2. IR spectroscopy

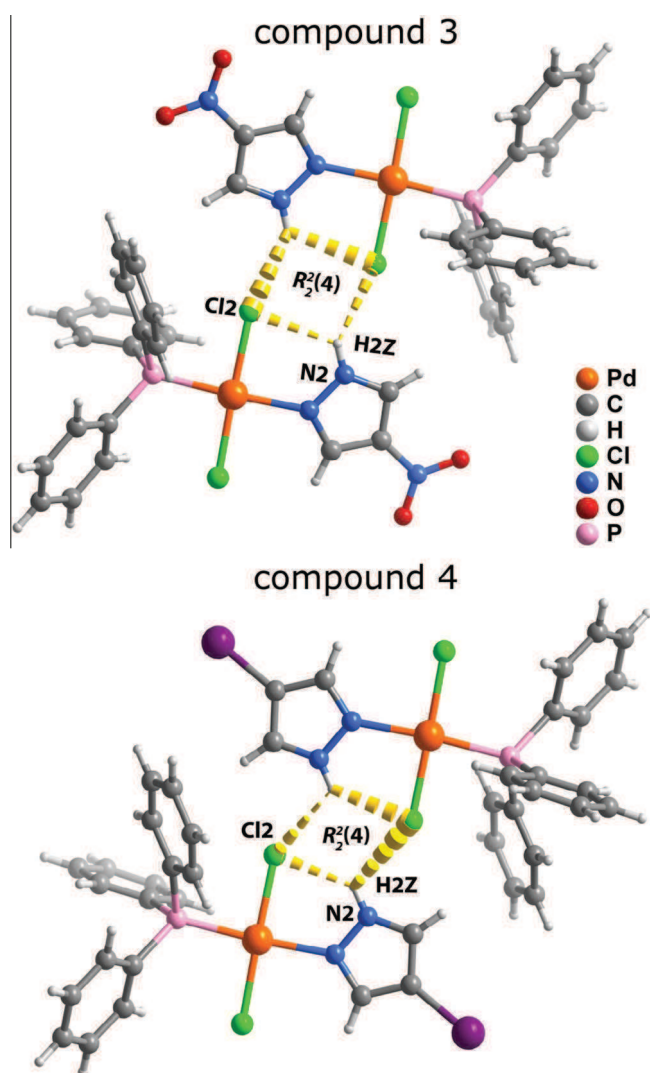
The FT-IR spectra of **1–4** showed the typical absorptions of coordinated triphenylphosphine at  $\sim 748 \text{cm}^{-1}$  ( $\nu_{\text{CH}}$ ),  $1100\text{--}1091 \text{cm}^{-1}$  (q),  $\sim 1480 \text{cm}^{-1}$  ( $\nu_{\text{ring}}$ ) and  $\sim 690 \text{cm}^{-1}$  ( $\gamma_{\text{ring}}$ ) [42]. The vibrational modes of  $\nu_{\text{NH}}$  appear at  $3324\text{--}3175 \text{cm}^{-1}$  and the displacement of the  $\nu_{\text{ring}}$  band at  $1600\text{--}1530 \text{cm}^{-1}$  to  $1583\text{--}1510 \text{cm}^{-1}$  suggests that the pyrazoles are coordinated as neutral and monodentate ligands [23,39,43]. The most important IR frequencies of the complexes **1–4** along with their assignments are presented in Table 4.

**Table 3**

Selected geometric parameters (Å, °) for [PdCl<sub>2</sub>(HNO<sub>2</sub>Pz)(PPh<sub>3</sub>)] (**3**·0.9 CHCl<sub>3</sub>) and [PdCl<sub>2</sub>(HIPz)(PPh<sub>3</sub>)] (**4**).

	<b>3·0.9CHCl<sub>3</sub></b>		<b>4</b>	
Pd1–N1	2.115(2)		2.120(4)	
Pd1–P1	2.2354(8)		2.2473(13)	
Pd1–Cl1	2.2794(7)		2.2792(13)	
Pd1–Cl2	2.3004(7)		2.3119(12)	
N1–Pd1–P1	175.32(7)		174.58(11)	
N1–Pd1–Cl1	88.90(7)		89.81(11)	
P1–Pd1–Cl1	94.19(3)		94.02(5)	
N1–Pd1–Cl2	89.69(7)		88.74(11)	
P1–Pd1–Cl2	87.44(3)		87.57(5)	
Cl1–Pd1–Cl2	176.15(3)		177.49(5)	
<i>Hydrogen bonding interactions</i>				
D···A	D···A	<D–H···A	D···A	<D–H···A
N2–H2Z···Cl2	3.082(3)	122	3.010(4)	135
N2–H2Z···Cl2 <sup>i</sup>	3.195(3)	129		
N2–H2Z···Cl2 <sup>ii</sup>			3.272(5)	109
<i>C–H···π contacts</i>				
	C16–H16···Cg2 <sup>iii</sup>	C···Cg	C2–H2···Cg4 <sup>v</sup>	C···Cg
		3.682(3)	C15–H15···Cg1 <sup>v</sup>	3.742(7)
			C17–H17···Cg3 <sup>vi</sup>	3.759(6)
			C19–H19···Cg2 <sup>vii</sup>	3.637(6)
				3.708(6)
<i>Other contacts</i>				
	Cl3···Cg1 <sup>iv</sup>	3.3015(16)	Cl2···I <sup>viii</sup>	3.7157(16)

Notes: The two crystal structures have common nomenclature for equivalent features. Cg1: Centroid of {N1, N2, C19–C21}; Cg2: Centroid of {C1–C6}; Cg3: Centroid of {C7–C12}; Cg4: Centroid of {C13–C18}. Symmetry operations: (i)  $-x + 1, -y + 2, -z$ ; (ii)  $-x, -y, -z + 1$ ; (iii)  $x, 1 + y, z$ ; (iv)  $1 - x, 1 - y, -z$ ; (v)  $-x, -y, -z$ ; (vi)  $-x, 1 - y, -z$ ; (vii)  $-x, -y, 1 - z$ ; (viii)  $1 + x, y, z$ .



**Fig. 2.** Supramolecular dimers present in the crystal structures of *trans*-[PdCl<sub>2</sub>(HNO<sub>2</sub>Pz)(PPh<sub>3</sub>)]·0.9 CHCl<sub>3</sub> (**3**·0.9 CHCl<sub>3</sub>) and *trans*-[PdCl<sub>2</sub>(HIPz)(PPh<sub>3</sub>)] (**4**). Hydrogen bonds are represented by yellow dashed lines. For details, see Table 3. (Colour online.)

Compounds **2** and **3** presented specific vibrational modes related to the pyrazoles substituents. The FT-IR spectra showed the expected  $\nu_{\text{CH}_3}$  bands at 2930–2850 cm<sup>-1</sup> for the complex **2** and the nitro group at 1550–1520 cm<sup>-1</sup> for **3**.

### 3.3. <sup>1</sup>H NMR spectroscopy

The <sup>1</sup>H NMR results are given in Table 5. As expected the <sup>1</sup>H NMR spectra of compounds **1–4** showed a multiplet attributable

to the phenyl groups of the phosphine ligand over the spectral range of 7.89–7.22 ppm. With exception of **2**, <sup>1</sup>H NMR spectra of the complexes exhibited only one set of the expected signals from coordinated pyrazolyl-type ligands, indicating the existence of a single species in solution.

The presence of neutral monodentate pyrazole in **1** was substantiated by appearance of the characteristic AMX pattern for the H<sub>3</sub>, H<sub>4</sub> and H<sub>5</sub> atoms of the pyrazole nucleus (see Scheme 1) whereas the monodentate coordination of 4-substituted pyrazoles in **3** and **4** was evidenced by the appearance of separate singlet resonances for the H<sub>3</sub> and H<sub>5</sub> atoms [39].

The pyrrolic hydrogen at N<sub>1</sub> was detected in all cases by the presence of a broadened singlet at ~12 ppm. The <sup>1</sup>H NMR spectrum of **2** at room temperature clearly indicated the existence of two conformational isomers in solution, in an intensity ratio of approximately 5:2, by the appearance of two sets of signals for each proton of the coordinated HdmPz ligand. Such solution behaviour has already been observed in other Pd(II) compounds bearing 3,5-dimethylpyrazole [44,45].

### 3.4. Antimycobacterial assays

*In vitro* antimycobacterial activities of the ligands, [PdCl<sub>2</sub>(MeCN)<sub>2</sub>] and the compounds **1–4** were evaluated against the strains of the *M. tuberculosis* H<sub>37</sub>Rv ATCC 27294 [46]. The microplate Alamar Blue assay (MABA) was used to measure the minimal inhibitory concentration (MIC) for the tested compounds (minimum concentration necessary to inhibit 90% growth of *M. tuberculosis* H<sub>37</sub>Rv ATCC 27294) [46]. The minimum inhibitory concentration (MIC) values are depicted in Table 6.

According to Table 6, the organic ligands and [PdCl<sub>2</sub>(MeCN)<sub>2</sub>] precursor displayed no response at compound concentrations <125 μg mL<sup>-1</sup> against MTB, and thus have to be considered inactive. However, in most of the cases, simultaneous coordination of pyrazoles and triphenylphosphine on palladium centre resulted in compounds with MIC values ranging from 5.43 to 20.3 μM. The most active complex was **4**, with a MIC of 7.61 ± 2.18 μM. On the other hand, the compound **2** did not inhibit the growth of *M. tuberculosis*, indicating that none of the conformational isomers are active.

From the inspection of MIC values found for complexes **1**, **3** and **4**, it was noticed a progressive increase on their antimycobacterial activity according to the type of coordinated pyrazolyl ligand (HL), following the order HNO<sub>2</sub>Pz < HPz ≈ HIPz. This finding may suggest that the antimycobacterial effectiveness is dependent, at least in part, on substituent hydrophobicity. Taking into account that –NO<sub>2</sub> group has a lower contribution to molecular hydrophobicity than –I and –H [47], one could roughly speculate that the metal-based derivative containing 4-nitropyrazole (**3**) possesses decreased lipophilicity. This is evident from the improved activity of **1** and **4**, compared with the complex **3**, presumably because of their relative easiness in crossing the highly impermeable lipid

**Table 4**  
Selected vibrational data (4000–400 cm<sup>-1</sup>) for the complexes [PdCl<sub>2</sub>(HPz)(PPh<sub>3</sub>)] (**1**), [PdCl<sub>2</sub>(HdmPz)(PPh<sub>3</sub>)] (**2**), [PdCl<sub>2</sub>(HNO<sub>2</sub>Pz)(PPh<sub>3</sub>)] (**3**) and [PdCl<sub>2</sub>(HIPz)(PPh<sub>3</sub>)] (**4**).

Vibrational frequency (cm <sup>-1</sup> )				Assignments
<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	
3324 s	3175s	3132 m	3297 s	$\nu_{\text{NH}}$
1515 w	1579 m	1583 w	1510 w	$\nu_{\text{ring}}$
1436 m, 1479 w	1478 m, 1435 s	1480 m	1481 m, 1457 m, 1431 m	$\nu_{\text{anel}}(\text{PPh}_3)$
1095 m	1096 s	1100 m	1091 s	q (PPh <sub>3</sub> )
766 m, 748 m	748 s	749 s	747 m	$\gamma_{\text{CH}}(\text{PPh}_3)$
690 s	692s	689 s	691 s	$\gamma_{\text{anel}}(\text{PPh}_3)$

$\nu$  = stretching;  $\phi$  = breathing ring;  $\gamma$  = out-of-plane bending; q = sensitive mode; s = strong, m = medium, w = weak.

**Table 5**<sup>1</sup>H NMR data (ppm) and assignment for complexes **1–4** at 298 K, in CDCl<sub>3</sub>, given as δ (<sup>1</sup>H), multiplicity [integration].

		<sup>1</sup> H NMR			
		<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
Pyrazole ring	NH	11.89 s [1H]	11.70 s [1H]; 10.39 s [1H]	12.47 s [1H]	12.02 s [1H]
	R <sub>3</sub>	8.26 br [1H]	2.67 s [3H]; 2.52 s [3H]	8.87 s [1H]	8.30 s [1H]
	R <sub>4</sub>	6.41 m [1H]	5.70 s [1H]; 5.87 s [1H]		
	R <sub>5</sub>	7.59 br [1H]	1.96 s [3H]; 2.22 s [3H]	8.28 s [1Hs]	7.66 s [1H]
Phenyl ring	H	7.83–7.28 m	7.88–7.22 m	7.70–7.25 m	7.89–7.22 m

s = singlet; br = broadened; m = multiplet.

**Table 6**MIC values of the ligands, [PdCl<sub>2</sub>(CH<sub>3</sub>CN)<sub>2</sub>] and compounds **1–4** against *M. tuberculosis* H<sub>37</sub>Rv.

Compound	MIC	
	μg mL <sup>-1</sup>	μM
Pyrazole (HPz)	NHIC	NHIC
3,5-Dimethylpyrazole (HdmPz)	>250	
4-Nitropyrazole (HNO <sub>2</sub> Pz)	>250	
4-Iodopyrazole (HIPz)	>250	
Triphenylphosphine (PPh <sub>3</sub> )	>250	
[PdCl <sub>2</sub> (CH <sub>3</sub> CN) <sub>2</sub> ]	125	481
[PdCl <sub>2</sub> (HPz)(PPh <sub>3</sub> )] ( <b>1</b> )	6.07 ± 0.82	11.95 ± 1.62
[PdCl <sub>2</sub> (HdmPz)(PPh <sub>3</sub> )] ( <b>2</b> )	>250	
[PdCl <sub>2</sub> (HNO <sub>2</sub> Pz)(PPh <sub>3</sub> )] ( <b>3</b> )	10.30 ± 0.94	18.63 ± 1.70
[PdCl <sub>2</sub> (HIPz)(PPh <sub>3</sub> )] ( <b>4</b> )	4.82 ± 1.38	7.61 ± 2.18

membrane cell wall of TB. Nevertheless, such structure-activity relationship is only preliminary taking into account that they were based on a limited number of Pd(II) compounds. Further experiments on this class of complexes are required in order to rationalize the obtained MIC values in terms of structure-activity relationship as well as to correlate the observed activity with the molecular hydrophobicity of the complexes.

In addition, the incorporation of phosphine-based ligands into the molecular structure of Pd(II) complexes demonstrated to be an interesting strategy to obtain compounds with *in vitro* antiproliferative activity against *M. tuberculosis*. The complex **4** (MIC = 7.61 ± 2.18 μM) exhibited a MIC value comparable to those found for [Pd(2-acetylpyridine-N(4)-phenyl-thiosemicarbazone)(PPh<sub>3</sub>)](NO<sub>3</sub>)·H<sub>2</sub>O, 8.7 μM [48] and [Pd(C-benzylideneaniline)(SCN)(1,3-bis(diphenylphosphino)propane)], 5.15 μM [20]. The *in vitro* activity of **4** is also comparable to those observed for some commonly used anti-TB agents (gentamicin, 4.19–8.38 μM; tobramycin, 8.56–17.11 μM; clarithromycin, 10.70–21.40 μM) [49]. It is important to emphasize that the antimycobacterial studies described here is only the first in a long series of assays that would have to be employed to establish safety and efficacy.

#### 4. Conclusions

In conclusion, the new compounds **1–4** were successfully prepared and characterized in this work. The results obtained from *in vitro* antimycobacterial assays indicated that the enhancement of the hydrophobic character of the substituents in the 4-position on the pyrazole ring results in an increase of the antiproliferative activity (**3** < **1** ≈ **4**) among the pyrazolyl-based Pd(II) complexes containing triphenylphosphine as a co-ligand. However, it must be pointed out that the activity data reported here should not be rigorously interpreted to mean that the structural integrity of complexes is maintained intact during the experiments and the MIC values reproduce precisely the effects of either the Pd(II) complexes or their products formed from the interaction with the components of the biological media.

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#### Appendix A. Supplementary data

CCDC 1044493 (compound **3**·0.9 CHCl<sub>3</sub>) and 1044494 (compound **4**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via <http://www.ccdc.cam.ac.uk/conts/retrieving.html>, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: [deposit@ccdc.cam.ac.uk](mailto:deposit@ccdc.cam.ac.uk). Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.poly.2015.07.009>.

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## Original article

# Bioactivity of pyridine-2-thiolato-1-oxide metal complexes: Bi(III), Fe(III) and Ga(III) complexes as potent anti-*Mycobacterium tuberculosis* prospective agents



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## ABSTRACT

In the search for new therapeutic tools against tuberculosis and to further address the therapeutic potential of pyridine-2-thiol 1-oxide (Hmpo) metal complexes, two new octahedral  $[M^{III}(\text{mpo})_3]$  complexes, with  $M = \text{Ga}$  or  $\text{Bi}$ , were synthesized and characterized in the solid state and in solution. Attempts to crystallize  $[\text{Ga}^{III}(\text{mpo})_3]$  in  $\text{CH}_2\text{Cl}_2$  led to single crystals of the reaction product  $[\text{GaCl}(\text{mpo})_2]$ , where the gallium(III) ion is in a square basis pyramidal environment, *trans*-coordinated at the basis to two pyridine-2-thiolato 1-oxide anions acting as bidentate ligands through their oxygen and sulfur atoms.

The biological activity of the new  $[M^{III}(\text{mpo})_3]$  complexes together with that of the previously reported Fe(III) analogous compound and the pyridine-2-thiol 1-oxide sodium salt ( $\text{Na mpo}$ ) was evaluated on *Mycobacterium tuberculosis*. The compounds showed excellent activity, both in the standard strain H<sub>37</sub>Rv ATCC 27294 (pan-susceptible) and in five clinical isolates that are resistant to the standard first-line anti-tuberculosis drugs isoniazid and rifampicin. These pyridine-2-thiol 1-oxide derivatives are promising compounds for the treatment of resistant tuberculosis.

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## 1. Introduction

Tuberculosis (TB) is an airborne and highly contagious infectious disease caused by *Mycobacterium tuberculosis* (MTB). Although TB is curable and preventable it still remains the second killing disease worldwide due to a single infectious agent after HIV/AIDS. In 2012 about 95% of the deaths occurred in low- and middle-income countries. About one-third of the world's population is infected but is not yet ill and cannot transmit the disease (latent TB). People bearing latent TB work as reservoir of the disease and those with compromised immune systems (HIV, malnutrition or diabetes) have a higher risk of getting ill [1,2]. Moreover, the increasing emergence of multi-drug resistant (MDR)

and extensively drug resistant (XDR) MTB strains has also contributed to the alarming high morbidity and mortality of the disease [3,4]. Only few drugs are active against the *Mycobacterium* bacilli and MDR and XDR MTB organisms are virtually untreatable by the current drugs in immune compromised patients [5,6]. Although research on anti-TB drug development has substantially increased during the past decade, no new drug has already reached the pharmaceutical market [6]. Bedaquiline (Sirturo™) was approved in 2012 by the Food and Drug Administration but only for the treatment of resistant infections, mainly due to its side effects. New strategies need to evolve for the discovery of new drugs that could reduce the duration of the treatment, be active against resistant strains, do not interfere with antiretroviral drugs and be active against latent bacilli [7–9].

Inorganic Medicinal Chemistry could be a source of metal-based anti-TB drugs. In particular, we have successfully developed novel iron complexes with bioactive 3-aminoquinoxaline-2-carbonitrile

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$N^1, N^4$ -dioxide derivatives as ligands that showed promising anti-MTB properties and improved activity as compared with the organic ligands [10–12]. At that stage iron (III) was selected as metal center taking into account the well known relationship between iron, mycobacteria and tuberculosis. In particular, it has been described that the treatment of mycobacteria with PAS (*p*-aminosalicylic acid), one of the oldest drugs against TB, resulted more effective when the cells were grown in media with adequate iron levels [13]. In addition, iron is needed by the bacteria and different iron complexes of bioactive ligands have shown anti-mycobacterial activity [10,12,14].

During the last years some of us have been studying the effect of metal coordination on the bioactivity of another aromatic amine *N*-oxide, namely pyridine-2-thiol 1-oxide (Fig. 1, Hmpo) [15–17]. Square planar complexes  $[M^{II}(\text{mpo})_2]$ , where  $M(\text{II}) = \text{Pd}(\text{II})$  or  $\text{Pt}(\text{II})$ , and a Au(I) homodinuclear mixed-ligand complex,  $[\text{Au}^I_2(\text{mpo})_2(\text{PPh}_3)_2]$ , showed high activity on *Trypanosoma cruzi*, the protozoan parasite responsible of Chagas disease. Although they may have other intraparasite targets, the free ligand and the metal complexes inhibit the parasite specific enzyme NADH-fumarate reductase. This enzyme is responsible of the conversion of fumarate to succinate as energy source [15–18]. Generation of succinate through a similar enzymatic reductive process has been described in latent TB lung granulomas where the oxygen amount is severely limited. The utilization of the generated succinate enables MTB to maintain basic physiologic functions although it is not enough to allow growth [19].

Taking into account these antecedents and that we had previously assessed the high activity of mpo sodium salt on MTB, we decided to explore the effect of metal complexation on the anti-mycobacterial activity of structurally related octahedral mpo complexes by changing the metal center (Fe(III), Ga(III) or Bi(III)).

Fe(III) was the first choice metal center due to the arguments given above. Fe(III) and Ga(III) similarities have lead to potential biological competition between both metal ions *in vivo* [20–22]. Therefore, Ga(III) could be interesting for the development of drugs against MTB. On the other hand, Bi(III) shows an interesting bio-inorganic chemistry, interacting with proteins in the iron binding sites, and its compounds have also shown medicinal applications, mainly as antimicrobial agents, but its anti-TB potentiality has not been explored yet [23–26].

In this work we further address the therapeutic potential of pyridine-2-thiolato 1-oxide metal complexes by synthesizing two new octahedral  $[M^{III}(\text{mpo})_3]$  complexes, with  $M = \text{Ga}$  or  $\text{Bi}$ . The complexes were characterized in the solid state and in solution by different techniques. Their biological activity together with that of the previously reported Fe(III) analogous compound and mpo sodium salt was evaluated on the *M. tuberculosis* standard strain H<sub>37</sub>Rv ATCC 27294 (pan-susceptible) as well as on clinical isolates showing resistance to the standard drugs isoniazid and rifampicin.

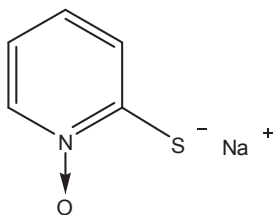


Fig. 1. Pyridine-2-thiol 1-oxide sodium salt (Na mpo).

## 2. Results and discussion

Two novel  $[M^{III}(\text{mpo})_3]$  complexes of the bioactive ligand pyridine-2-thiolato 1-oxide were synthesized with high purity and good yield by the reaction of the selected  $M(\text{III})$  salt and pyridine-2-thiol 1-oxide sodium salt (Na mpo) in methanol at reflux. Unfortunately, it was impossible to obtain adequate single crystals of the complexes for structural crystallographic studies. Both were characterized by using elemental analyses, conductometric measurements, and FTIR and  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy.  $[\text{Fe}^{III}(\text{mpo})_3]$  was synthesized with high purity and good yield by a different route to that previously reported and was characterized to assure its identity according to the literature [27].

Elemental analyses agree with the proposed formula. The molar conductivity value obtained for the complexes in DMSO solution shows that they are non-electrolytes, a fact that demonstrates the non-charged nature of the complexes [28]. No conductivity changes were observed during at least 7 days, hence suggesting that the complexes are stable in DMSO solution.

### 2.1. Characterization of the $[M^{III}(\text{mpo})_3]$ complexes in the solid state and in solution

Characterization of the three complexes by FTIR and of Bi and Ga complexes by NMR spectroscopy has been performed and compared with the corresponding results for Na mpo and the previously reported Pd(II), Pt(II) and Au(I) complexes [15,16].

#### 2.1.1. IR spectroscopic studies of the $[M^{III}(\text{mpo})_3]$ complexes, 1–3

Relevant IR absorption bands were tentatively assigned to molecular vibration modes (Table 1). The assignment of the typical bands due to  $\nu(\text{N}-\text{O})$ ,  $\delta(\text{N}-\text{O})$  and  $\nu(\text{C}-\text{S})$  modes is in agreement with those previously reported for Na mpo and other mpo metal complexes [15,16,29]. The frequency shift of these bands upon coordination to the metal is in agreement with the bidentate coordination of the ligand through the oxygen of the  $\text{N}-\text{O}$  group and the sulfur. Bands corresponding to metal-to-ligand stretching modes were also tentatively identified in the low wave number region ( $500\text{--}300\text{ cm}^{-1}$ ) and assigned to  $\nu(\text{M}-\text{O})$  and  $\nu(\text{M}-\text{S})$  stretches.

#### 2.1.2. NMR characterization of the $[M^{III}(\text{mpo})_3]$ complexes, 2–3

Stability of the complexes in DMSO during at least 24 h was confirmed by NMR spectroscopy. Neither free ligand nor coordinated DMSO was detected. Table 2 shows the  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts ( $\delta$ ) of Na mpo and the complexes and the chemical shift differences between them (expressed as  $\Delta\delta$ ). The table also includes a figure showing the numbering scheme.  $^1\text{H}$  NMR integrations and signal multiplicities were in agreement with the proposed formula and structure. HMQC and HMBC experiments allowed the assignment of all the signals of mpo and the investigated complexes. A deshielding effect of all the protons is observed due to electron withdrawing caused by coordination to the  $M(\text{III})$  center. Upon coordination, the most distinguishing feature of the  $^{13}\text{C}$  NMR spectra was the change in the chemical shift of carbon 1 which drops approximately from 168 to 156–159 ppm (Table 2). This result is in agreement with the previous report for Pd(II) and Pt(II)  $[M^{II}(\text{mpo})_2]$  complexes [16].

### 2.2. Crystal structure of $[\text{GaCl}(\text{mpo})_2]$

Although  $[\text{GaCl}(\text{mpo})_2]$  complex is not of direct interest for the current work, the obtained  $[\text{GaCl}(\text{mpo})_2]$  single crystal affords a complete structural X-ray study to bring further support to the coordination mode of mpo to Ga(III) center and therefore its solid state molecular architecture is reported here. The partial

**Table 1**

Tentative assignment of the main characteristic IR bands of mpo sodium salt and the Fe, Ga and Bi [M<sup>III</sup>(mpo)<sub>3</sub>] complexes. Assignments for previously reported [Pd<sup>II</sup>(mpo)<sub>2</sub>] and [Pt<sup>II</sup>(mpo)<sub>2</sub>] complexes are included for comparison (wave numbers in cm<sup>-1</sup>) [16].

Assignment	Na mpo	[Fe <sup>III</sup> (mpo) <sub>3</sub> ]	[Ga <sup>III</sup> (mpo) <sub>3</sub> ]	[Bi <sup>III</sup> (mpo) <sub>3</sub> ]	[Pd(mpo) <sub>2</sub> ]	[Pt(mpo) <sub>2</sub> ]
ν(CH)	3068 m	3099 m	3103 m	3094 m	3095 m	3098 m
	3043 m	3067 m	3067 m	3072 m	3067 m	3085 m
ν(C=C)	1541 s	1539 vs	1545 vs	1540 vs	1547 vs	1551 vs
	1206 s	1227 s	1226 s	1190 s	1247 s	1250 s
δ(N–O)	834 s	829 s	829 s	825 s	821 s	814 m
ν(C–S)	702 s	712 s	712 s	698 s	708 s/695 sh	711 s/686 sh
ν(M–O)	–	440 w	440 w	417 w	442 w	434 w
ν(M–S)	–	351 w	372 w	340 w	392 w	386 w
		342 w	353 w	318 w	313 w	300 w

v: stretching; δ: bending; s: strong, m: medium; w: weak.

generation (few single crystals) of [GaCl(mpo)<sub>2</sub>] from [Ga(mpo)<sub>3</sub>] only when the crystallization is performed in CH<sub>2</sub>Cl<sub>2</sub> could be probably explained considering that aliphatic halides like CH<sub>2</sub>Cl<sub>2</sub> are likely to be contaminated with halogen acids from which they have been prepared [30].

Fig. 2 shows an ORTEP [31] drawing of the gallium complex. Intra-molecular bond distances and angles are given in Table 3. The Ga(III) ion is in a distorted square basis pyramidal coordination. It is *trans*-coordinated at the pyramid basis to two pyridine-2-thiolato 1-oxide anions acting as bidentate ligands through their O-atoms [Ga–O distances equal to 1.993(4) Å] and S-atoms [Ga–S distances of 2.2835(8) and 2.2763(9) Å]. The pyramid apex is occupied by a chloride ion [d(Ga–Cl) = 2.2248(9) Å]. As expected for an extended π-bonding delocalization, the ligands are planar [*rms* deviation of atoms from the best least-squares plane less than 0.016 Å]. The ligands slightly depart from mutual co-planarity [angled at 19.04(4)° from each other].

The observed inter-atomic distances and angles in the heterocycles are consistent with the description of their molecular structure in terms of resonant bonds. C–C bond distances are in the range from 1.351(4) to 1.396(4) Å and N–C lengths are in the 1.344(4)–1.374(4) Å interval. Single bonds N–O distances are 1.347(3) and 1.338(3) Å and C–S lengths are 1.717(3) and 1.710(3) Å.

### 2.3. Biological results

The analogous Ga(III), Fe(III) and Bi(III) compounds and the mpo sodium salt were evaluated on *M. tuberculosis*, both on the standard strain H<sub>37</sub>Rv ATCC 27294 (pan-susceptible) and on five clinical isolates with resistance already observed to the standard drugs isoniazid (INH) and rifampicin (RIF) (Table 4). All of them showed excellent activity either on H<sub>37</sub>Rv ATCC 27294 strain or on the INH and RIF resistant isolates (Tables 5 and 6).

In the field of drug discovery based on phenotypic screening, the first point to be observed is the activity of the compounds against the MTB H<sub>37</sub>Rv. The MIC (Minimal Inhibitory Concentration) results for the four *in vitro* evaluated compounds range from 1.06 μM to 3.29 μM (Table 5). The compounds show an equal or greater activity against the bacillus than drugs already used in therapy shown in the table. In a scheme of basic treatment recommended by the WHO (World Health Organization) the drugs used in a first phase are: isoniazid (MIC = 0.18 μM), rifampicin (MIC value around 0.49 μM), pyrazinamide (MIC value varying in the range of 48.74–406.14 μM at pH 5.5) and ethambutol (MIC = 2.45 μM) [32]. When we compare our results with the second line drugs or some new molecules in clinical trials, the expectation is even better (Table 5).

The Ga(III) complex was the most active among the compounds studied, being able to eliminate 90% of the bacteria in the *in vitro*

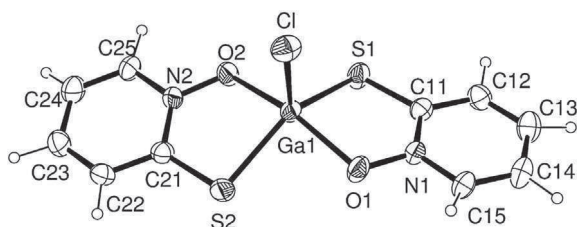
assay at a concentration of 1.06 μM. This value is similar to those of molecules in Phase II of clinical development as SQ109, and is lower than those of molecules already in clinical use as streptomycin, ethambutol and moxifloxacin [32,33]. The Bi(III) complex showed the lower activity with a MIC value of 3.29 μM, but still qualifies as an excellent candidate for new anti-TB molecule as it presents a higher activity than pyrazinamide (at least 48.74 μM) and kanamycin (3.43 μM) [32]. Consequently, these compounds meet the first requirement of a new anti-TB molecule, showing significantly higher activity than the drugs already used in the treatment. They could be of great value (after further study) in an attempt to shorten the long drug therapy. Fe(III) and Ga(III) coordination of mpo led to an increase in activity.

Looking for satisfying the second anti-TB drug development criteria, the high activity of these compounds against the clinical isolates resistant to INH and RIF was also verified. The results were extremely promising (Table 6). A very striking fact is the similarity of the MIC values against resistant and sensitive strains for these compounds, which might indicate a different mechanism of action or target than those of INH and RIF. The Ga(III) and Fe(III) compounds were the most active complexes in the resistant strains, varying their strength according to the type of isolate. Their MIC values are extremely low in these strains that have been previously characterized by Miyata et al. [34] as highly resistant to the two main first-line anti-TB drugs. These results classify these compounds as candidates for drug discovery, meeting the second requirement needed for new anti-TB molecules. These results point at the tested pyridine-2-thiol 1-oxide compounds as promising new candidates against the major concern in the context of the disease: the emergence of resistance.

In order to get further insight into the potentiality of both most promising metal compounds, [Ga(mpo)<sub>3</sub>] and [Fe(mpo)<sub>3</sub>], their cytotoxicities on VERO epithelial cells (ATCC CCL81) was *in vitro* assayed. The IC<sub>50</sub> values on these cells together with the selectivity indexes (SI = IC<sub>50</sub> VERO cells (μM)/MIC *M. tuberculosis* H<sub>37</sub>Rv (μM)) where determined: IC<sub>50</sub> Vero cells [Ga(mpo)<sub>3</sub>] 10.04 μM, [Fe(mpo)<sub>3</sub>] 4.61 μM; SI [Ga(mpo)<sub>3</sub>] 9.37, [Fe(mpo)<sub>3</sub>] 3.03. Usually a compound is considered promising for further testing if a SI close or higher than 10 is observed [35]. Accordingly, [Ga(mpo)<sub>3</sub>] shows to be the most promising compound for further drug development. Mpo sodium salt showed a higher selectivity index than the metal complexes when considering Vero cells as mammalian cells model (SI = 80) but not if considering previous results on other mammalian cells like J774 macrophages (SI < 1) [16]. Nevertheless, the metal complexes showed much higher activities than Na mpo on pan-susceptible and resistant *M. tuberculosis* deserving therefore to perform further biological studies in order to establish their real therapeutic potentiality.

**Table 2**<sup>1</sup>H and <sup>13</sup>C NMR chemical shift values ( $\delta$ ) in ppm, of the ligand and the complexes, in DMSO-*d*<sub>6</sub>:D<sub>2</sub>O (9:1) at 30 °C.

Proton	$\delta_H$ (multiplicity)					Carbon	$\delta_C$				
	Ligand	[Ga <sup>III</sup> (mpo) <sub>3</sub> ]	$\Delta\delta^a$	[Bi <sup>III</sup> (mpo) <sub>3</sub> ]	$\Delta\delta^a$		Ligand	[Ga <sup>III</sup> (mpo) <sub>3</sub> ]	$\Delta\delta^a$	[Bi <sup>III</sup> (mpo) <sub>3</sub> ]	$\Delta\delta^a$
–	–	–	–	–	–	<b>1</b>	167.91	158.93	–8.98	155.90	–12.01
<b>2</b>	7.32 (d)	7.60 (d)	0.28	7.42 (d)	0.10	<b>2</b>	132.32	128.96	–3.36	131.26	–1.06
<b>3</b>	6.74 (t)	7.42 (t)	0.68	7.26 (t)	0.52	<b>3</b>	124.02	131.61	7.59	129.09	5.07
<b>4</b>	6.55 (t)	7.10 (t)	0.55	6.95 (t)	0.40	<b>4</b>	114.66	118.94	4.28	119.32	4.66
<b>5</b>	7.97 (d)	8.47 (d)	0.50	8.09 (d)	0.12	<b>5</b>	139.04	137.15	–1.89	140.15	1.11

<sup>a</sup>  $\Delta\delta = (\delta_{\text{Complex}} - \delta_{\text{Ligand}})$ . Multiplicity: d: doublet, t: triplet.**Fig. 2.** Drawing of pyridine-2-thiolato 1-oxide gallium(III) complex, [GaCl(mpo)<sub>2</sub>], showing the labeling of the non-H atoms and their displacement ellipsoids at the 30% probability level.**Table 3**Bond lengths [Å] and angles [°] for [GaCl(mpo)<sub>2</sub>].

C(11)–N(1)	1.365(4)	N(1)–C(15)–C(14)	120.6(3)
C(11)–C(12)	1.387(4)	N(2)–C(21)–C(22)	116.4(3)
C(11)–S(1)	1.717(3)	N(2)–C(21)–S(2)	119.2(2)
C(12)–C(13)	1.363(5)	C(22)–C(21)–S(2)	124.4(3)
C(13)–C(14)	1.388(5)	C(23)–C(22)–C(21)	121.3(3)
C(14)–C(15)	1.352(5)	C(22)–C(23)–C(24)	119.9(4)
C(15)–N(1)	1.345(4)	C(25)–C(24)–C(23)	119.1(3)
C(21)–N(2)	1.374(4)	N(2)–C(25)–C(24)	120.9(4)
C(21)–C(22)	1.396(4)	N(1)–O(1)–Ga(1)	115.81(17)
C(21)–S(2)	1.710(3)	N(2)–O(2)–Ga(1)	116.31(17)
C(22)–C(23)	1.358(5)	O(2)–Ga(1)–O(1)	158.66(11)
C(23)–C(24)	1.381(5)	O(2)–Ga(1)–Cl	100.89(8)
C(24)–C(25)	1.351(4)	O(1)–Ga(1)–Cl	100.44(9)
C(25)–N(2)	1.344(4)	O(2)–Ga(1)–S(2)	85.57(6)
O(1)–N(1)	1.347(3)	O(1)–Ga(1)–S(2)	88.32(7)
O(1)–Ga(1)	1.993(2)	Cl–Ga(1)–S(2)	108.22(4)
O(2)–N(2)	1.338(3)	O(2)–Ga(1)–S(1)	86.82(7)
O(2)–Ga(1)	1.993(2)	O(1)–Ga(1)–S(1)	85.02(7)
Ga(1)–Cl	2.2248(9)	Cl–Ga(1)–S(1)	111.13(4)
Ga(1)–S(2)	2.2763(9)	S(2)–Ga(1)–S(1)	140.65(4)
Ga(1)–S(1)	2.2835(8)	C(11)–S(1)–Ga(1)	96.14(11)
		C(21)–S(2)–Ga(1)	96.30(11)
N(1)–C(11)–C(12)	116.5(3)	O(1)–N(1)–C(15)	116.4(3)
N(1)–C(11)–S(1)	118.8(2)	O(1)–N(1)–C(11)	120.6(2)
C(12)–C(11)–S(1)	124.7(3)	C(15)–N(1)–C(11)	122.9(3)
C(13)–C(12)–C(11)	121.4(4)	C(25)–N(2)–O(2)	117.2(3)
C(12)–C(13)–C(14)	119.7(4)	C(25)–N(2)–C(21)	122.3(3)
C(15)–C(14)–C(13)	118.9(3)	O(2)–N(2)–C(21)	120.5(2)

Although we cannot hypothesize about the mechanism of action of the metal complexes and the real role of metal coordination of this bioactive ligand, it is well known that coordination modifies electronic and physicochemical properties of bioactive organic ligands. These changes could lead to an improvement of the

bioavailability of the organic drug. Moreover, metal complexes could act as drug transporters into the cell. In addition, coordination of a bioactive organic ligand could improve its activity through metal – drug synergism [36,37].

### 3. Conclusions

Two new [M<sup>III</sup>(mpo)<sub>3</sub>] complexes, where M = Ga or Bi and mpo = pyridine-2-thiolato 1-oxide, were synthesized and characterized in the solid state and in solution. Both, together with pyridine-2-thiol 1-oxide sodium salt and the analogous [Fe<sup>III</sup>(mpo)<sub>3</sub>] compound, were biologically evaluated on sensitive and resistant strains of *M. tuberculosis*.

In the field of drug discovery against tuberculosis, the pyridine-2-thiol 1-oxide derivatives tested in this work are promising compounds for the treatment of resistant tuberculosis and show high potentiality as hits in the development of new bioactive metal complexes series. Further studies are in progress to assess other relevant biological properties of these compounds.

### 4. Materials and methods

#### 4.1. Materials

All common laboratory chemicals were purchased from commercial sources and used without further purification. Pyridine-2-thiol 1-oxide sodium salt (Na mpo), FeCl<sub>3</sub>, BiCl<sub>3</sub> and Ga(NO<sub>3</sub>)<sub>3</sub>·8.15H<sub>2</sub>O were commercially available.

#### 4.2. Syntheses of pyridine-2-thiolato-1-oxide-κS,κO metal complexes, [M<sup>III</sup>(mpo)<sub>3</sub>], 1–3

Complexes 1–3 were prepared by mixing a solution of Na mpo (43 mg, 0.29 mmol) in 8 mL MeOH with a solution of each metal(III) salt in 2 mL MeOH (0.094 mmol, 37 mg Ga(NO<sub>3</sub>)<sub>3</sub>·8.15H<sub>2</sub>O or 15 mg FeCl<sub>3</sub> or 30.0 mg de BiCl<sub>3</sub>). The reaction mixture was kept for 20 h under reflux. In each case a colored solid was isolated by centrifugation and washed with two portions of MeOH.

**[Fe<sup>III</sup>(mpo)<sub>3</sub>], 1.** Yield: 31 mg, 77%. Black solid. Anal (%) calc. for C<sub>15</sub>H<sub>12</sub>FeN<sub>3</sub>O<sub>3</sub>S<sub>3</sub>: C, 41.5; H, 2.8; N, 9.7; S, 22.1. Found: C, 41.4; H, 2.8, N, 9.7; S, 22.0.  $\Delta_M(\text{DMSO})$ : 0.3 Scm<sup>2</sup> mol<sup>–1</sup>.

**[Ga<sup>III</sup>(mpo)<sub>3</sub>], 2.** Yield: 29 mg, 69%. Light yellow solid. Anal (%) calc. for C<sub>15</sub>H<sub>12</sub>GaN<sub>3</sub>O<sub>3</sub>S<sub>3</sub>: C, 40.2; H, 2.7; N, 9.4; S, 21.4. Found: C, 40.0; H, 2.7, N, 9.3; S, 21.2.  $\Delta_M(\text{DMSO})$ : 2.0 Scm<sup>2</sup> mol<sup>–1</sup>.

**Table 4**

Phenotypic and genotypic resistance profiles related to isoniazid (INH) and rifampicin (RIF) of the clinical isolates used in the REMA assays [34].

Clinical isolate	BACTEC		REMA		Sequencing			
	INH	RIF	MIC ( $\mu\text{g/mL}$ )		Genes related to INH resistance			Gene related to RIF resistance
			INH	RIF	<i>inhA</i>	<i>katG</i>	<i>ahpC</i>	<i>rpoB</i>
1	R	R	>25.00	>25.00	*	Ser315Thr	*	Pro445Leu/Pro526Leu
2	R	R	>25.00	>25.00	*	*	*	C $\rightarrow$ G in the positions 450 and 531
3	R	R	>25.00	>25.00	*	Ser315Thr	*	C $\rightarrow$ T in the positions 450 and 531
4	R	S	>25.00	0.212	*	*	*	*
5	R	R	>25.00	>25.00	C $\rightarrow$ T in the position –15	*	*	C $\rightarrow$ T in the positions 450 and 531

R: resistant; S: sensitive; C: cytosine; T: thymine; G: guanine.

Mutations of N  $\rightarrow$  N type (where N is an arbitrary nitrogenous base) indicate a silent mutation in which there was not an amino acid exchange.

**[Bi<sup>III</sup>(mpo)<sub>3</sub>]<sub>3</sub>**. Yield: 49 mg, 90%. Yellow solid. Anal (%) calc. for C<sub>15</sub>H<sub>12</sub>BiN<sub>3</sub>O<sub>3</sub>S<sub>3</sub>: C, 30.7; H, 2.1; N, 7.2; S, 16.4. Found: C, 30.6; H, 2.0, N, 7.1; S, 16.2.  $\Delta_M(\text{DMSO})$ : 0.5 Scm<sup>2</sup> mol<sup>-1</sup>.

#### 4.3 Physicochemical characterization

C, H, N and S analyses were carried out with a Thermo Scientific Flash 2000 elemental analyzer. Conductometric measurements were done over time (7 days) at 25 °C in 10<sup>-3</sup> M DMSO solutions using a Conductivity Meter 4310 Jenway to determine the type of electrolyte and to assess the stability of the complexes in such medium [28]. The FTIR absorption spectra (4000–300 cm<sup>-1</sup>) of the complexes and the free ligand were measured as KBr or CsI pellets with a Shimadzu IRPrestige-21 instrument. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of Na mpo and the complexes were recorded in DMSO-d<sub>6</sub>:D<sub>2</sub>O (9:1) at 30 °C on a Bruker DPX-400 instrument (at 400 MHz and 100 MHz, respectively). Hetero-nuclear correlation experiments (2D-HETCOR), HSQC (hetero-nuclear single quantum correlation) and HMBC (hetero-nuclear multiple bond correlation), were carried out with the same instrument. Tetramethylsilane was used as the internal standard. Chemical shifts are reported in ppm.

#### 4.4 Crystallographic study of [GaCl(mpo)<sub>2</sub>]

When attempting to obtain [Ga(mpo)<sub>3</sub>] adequate crystals for structural X-ray diffraction studies, re-crystallization in CH<sub>2</sub>Cl<sub>2</sub> rather led to single crystals of the reaction product [GaCl(mpo)<sub>2</sub>]. The X-ray measurements were performed on an Oxford Xcalibur Gemini, Eos CCD diffractometer with graphite-monochromated CuK $\alpha$  ( $\lambda = 1.54184 \text{ \AA}$ ) radiation. X-ray diffraction intensities were collected ( $\omega$  scans with  $\vartheta$  and  $\kappa$ -offsets), integrated and scaled with CrysAlisPro suite of programs [38]. The unit cell parameters were obtained by least-squares refinement (based on the angular settings for all collected reflections with intensities larger than seven times the standard deviation of measurement errors) using CrysAlisPro. Data were corrected empirically for absorption employing the multi-scan method implemented in CrysAlisPro. The structure was solved by direct methods with SHELXS-97 [39] and the corresponding molecular model developed by alternated cycles of Fourier methods and full-matrix least-squares refinement on  $F^2$  with SHELXL-97 [40]. The hydrogen atoms were positioned on stereo-chemical basis and refined with the riding model. Crystal data, data collection procedure, structure determination methods and refinement results are summarized in Table 7. Crystallographic structural data have been deposited at the Cambridge Crystallographic Data Centre (CCDC). Any request to the CCDC for this material should quote the full literature citation and the reference number CCDC 817624.

#### 4.5 Biological activity

##### 4.5.1 Determination of Minimal Inhibitory Concentration (MIC)

The anti-MTB activity of the compounds was determined by the REMA (Resazurin Microtiter Assay) method [41]. Stock solutions of the tested compounds were prepared in DMSO and diluted in Middlebrook 7H9 broth (Difco) supplemented with oleic acid, albumin, dextrose and catalase (OADC enrichment – BBL/Becton–Dickinson), to obtain final drug concentration ranges of 0.09–25  $\mu\text{g/mL}$ . Isoniazid was dissolved in distilled water and rifampicin in DMSO, and both were used as standard drugs. A suspension of the MTB H<sub>37</sub>Rv ATCC 27294 and five clinical isolates resistant to isoniazid and rifampicin were cultured in Middlebrook 7H9 broth supplemented with OADC and 0.05% Tween 80. The cultures were frozen at –80 °C in aliquots. After two days the CFU/mL (colony formation unit/mL) of an aliquot was determined. The concentrations were adjusted by  $5 \times 10^5$  CFU/mL and 100  $\mu\text{L}$  of the inoculum were added to each well of a 96-well micro-plate together with 100  $\mu\text{L}$  of the compounds. Samples were set up in triplicate. The plates were incubated for 7 days at 37 °C. After 24 h, 30  $\mu\text{L}$  of 0.01% resazurin (solubilized in water) was added. The fluorescence of the wells was read after 24 h with a TECAN Spectrafluor<sup>®</sup>. The MIC was defined as the lowest concentration resulting in 90% inhibition of growth of MTB.

**Table 5**MIC values of the mpo compounds against *M. tuberculosis* H<sub>37</sub>Rv compared to MIC values of first- and second-line anti-TB drugs, other drugs also used in the treatment and new proposed molecules [32].

Compound	MIC value against <i>M. tuberculosis</i> H <sub>37</sub> Rv	
	$\mu\text{g mL}^{-1}$	$\mu\text{M}$
Na mpo	0.36 $\pm$ 0.01	2.42
[Ga(mpo) <sub>3</sub> ]	0.48 $\pm$ 0.00	1.06
[Bi(mpo) <sub>3</sub> ]	1.47 $\pm$ 0.03	3.29
[Fe(mpo) <sub>3</sub> ]	0.66 $\pm$ 0.08	1.53
Isoniazid	0.02	0.18
Rifampicin	0.40	0.49
Pyrazinamide	6.00–50.00 <sup>a</sup>	48.74–406.14 <sup>a</sup>
Ethambutol	0.50	2.45
Streptomycin	1.00	1.72
Amikacin	0.50–1.00	0.85–1.70
Capreomycin	2.00	2.61
Kanamycin	2.00	3.43
Gatifloxacin	0.25	0.67
Moxifloxacin	0.50	1.14
Bedaquiline	0.06	0.11
Linezolid	0.25	0.74
SQ109	0.35	1.06
PA-824	0.15–0.30	0.42–0.84
Clofazimine	0.10	0.21
Para-aminosalicylic acid	0.30–1.00	1.96–6.53

<sup>a</sup> At pH 5.5.

**Table 6**  
MIC values of the compounds against the resistant clinical isolates listed above in Table 4. The first value represents the MIC in  $\mu\text{g mL}^{-1}$ , while the second represents the MIC value in  $\mu\text{M}$ .

Compound	MIC values against resistant clinical isolates 1–5 ( $\mu\text{g mL}^{-1}/\mu\text{M}$ )				
	1	2	3	4	5
Na mpo	0.71/4.76 $\pm$ 0.03	0.35/2.38 $\pm$ 0.00	0.73/4.90 $\pm$ 0.03	0.59/3.95 $\pm$ 0.07	0.38/2.58 $\pm$ 0.01
[Ga(mpo) <sub>3</sub> ]	0.74/1.66 $\pm$ 0.00	0.42/0.94 $\pm$ 0.85	0.76/1.70 $\pm$ 0.76	0.91/2.03 $\pm$ 0.30	0.46/1.02 $\pm$ 0.10
[Bi(mpo) <sub>3</sub> ]	1.98/3.37 $\pm$ 0.84	1.46/2.49 $\pm$ 0.84	12.43/21.18 $\pm$ 0.30	2.90/4.94 $\pm$ 0.25	3.22/5.49 $\pm$ 2.05
[Fe(mpo) <sub>3</sub> ]	0.62/1.42 $\pm$ 0.21	0.35/0.80 $\pm$ 0.00	1.07/2.46 $\pm$ 0.22	0.97/2.23 $\pm$ 0.38	0.43/0.99 $\pm$ 0.12

#### 4.5.2. Cytotoxicity assay

*In vitro* cytotoxicity assays were performed on VERO epithelial cells (ATCC CCL81). This lineage is widely used for phenotypic screening of drugs being regarded as a normal cell derived from normal human epithelial tissue [42]. The cells were routinely maintained in DMEM complete medium supplemented with 10% heat inactivated fetal bovine serum (FBS) plus gentamicin (50 mg/L) and amphotericin B (2 mg/L) at 37 °C, in a humidified 5% CO<sub>2</sub> atmosphere. After reaching confluence, the cells were detached and counted. For the cytotoxicity assay,  $1 \times 10^5$  cells/mL were seeded in 200  $\mu\text{L}$  of complete medium in 96-well plates (NUNC™). The plates were incubated under the same conditions for 24 h to allow cell adhesion prior to drug testing. The compounds were dissolved in DMSO (5%) and subjected to two-fold serial dilution from 500 to 3.9  $\mu\text{g/mL}$ . The cells were exposed to the compounds at various concentrations for 24 h. Resazurin solution was then added to the cell cultures and incubated for 6 h. Cell respiration, as an indicator of cell viability, was detected by reduction of resazurin to resorufin, whose pink color and fluorescence indicate cell viability. A persistent blue color of resazurin is a sign of cell death. The fluorescence measurements (530 nm excitation filter and 590 nm emission filter) were performed in a SPECTRAfluor Plus (Tecan) micro-fluorimeter. The IC<sub>50</sub> value was defined as the highest drug concentration at which 50% of the cells are viable relative to the control. Each test was set up in triplicate. The selectivity index (SI)

was calculated by dividing IC<sub>50</sub> for the VERO cells by the MIC for the pathogen.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2014.09.067>.

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**Table 7**  
Crystal data and structure refinement results for chloride bis(pyridine-2-thiolato-1-oxide-κS,κO) gallium(III) complex, [GaCl(mpo)<sub>2</sub>].

Empirical formula	C <sub>10</sub> H <sub>8</sub> Cl Ga N <sub>2</sub> O <sub>2</sub> S <sub>2</sub>
Formula weight	357.47
Temperature	293(2) K
Wavelength	1.54184 Å
Crystal system	Orthorhombic
Space group	<i>Pbca</i>
Unit cell dimensions	<i>a</i> = 13.9373(9) Å <i>b</i> = 12.7865(7) Å <i>c</i> = 14.4427(7) Å
Volume	2573.8(3) Å <sup>3</sup>
Z, density (calculated)	8, 1.845 Mg/m <sup>3</sup>
Absorption coefficient	7.841 mm <sup>-1</sup>
<i>F</i> (000)	1424
Crystal size	0.236 × 0.095 × 0.038 mm <sup>3</sup>
Theta range for data collection	5.61–73.95°
Index ranges	−8 ≤ <i>h</i> ≤ 17, −13 ≤ <i>k</i> ≤ 15, −16 < <i>l</i> ≤ 18
Reflections collected	8233
Independent reflections	2600 [ <i>R</i> (int) = 0.0318]
Observed reflections	1482
Completeness to <i>q</i> = 73.95°	99.8%
Refinement method	Full-matrix least-squares on <i>F</i> <sup>2</sup>
Data/restraints/parameters	2600/0/171
Goodness-of-fit on <i>F</i> <sup>2</sup>	0.862
Final <i>R</i> indices <sup>a</sup> [ <i>I</i> > 2σ( <i>I</i> )]	<i>R</i> <sub>1</sub> = 0.0314, <i>wR</i> <sub>2</sub> = 0.0805
<i>R</i> indices (all data)	<i>R</i> <sub>1</sub> = 0.0591, <i>wR</i> <sub>2</sub> = 0.0900
Largest diff. peak and hole	0.711 and −0.259 e.Å <sup>-3</sup>

<sup>a</sup>  $R_1 = \sum ||F_o| - |F_c|| / \sum |F_o|$ ,  $wR_2 = [\sum w(|F_o|^2 - |F_c|^2)^2 / \sum w(|F_o|^2)^2]^{1/2}$ .

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## Synthesis and antimycobacterial activity of new pyrazolate-bridged dinuclear complexes of the type $[\text{Pd}(\mu\text{-L})(\text{N}_3)(\text{PPh}_3)]_2$ ( $\text{PPh}_3 =$ triphenylphosphine; $\text{L} =$ pyrazolates)



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### ABSTRACT

Binuclear compounds of the type  $[\text{Pd}(\mu\text{-L})(\text{N}_3)(\text{PPh}_3)]_2$  ( $\text{L} =$  pyrazolate (**1**); 3,5-dimethylpyrazolate (**2**); 4-iodopyrazolate (**3**);  $\text{PPh}_3 =$  triphenylphosphine) were synthesized and characterized by elemental analyses, infrared and  $^1\text{H}$  and  $^{31}\text{P}\{^1\text{H}\}$  NMR spectroscopies. The crystal and molecular structures of the complex  $[\text{Pd}(\mu\text{-dmPz})(\text{N}_3)(\text{PPh}_3)]_2$  (**2**) were determined by single-crystal X-ray diffraction techniques. In vitro antimycobacterial evaluation demonstrated that compound  $[\text{Pd}(\mu\text{-Pz})(\text{N}_3)(\text{PPh}_3)]_2$  (**1**) displayed a MIC of 8.16  $\mu\text{M}$ , being more active than some commonly used anti-TB drugs and other Pd(II) complexes.

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Tuberculosis (TB), an airborne infectious disease caused by *Mycobacterium tuberculosis* (MTB) and other mycobacteria, remains a major global health problem. In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (including 320,000 deaths among HIV-positive people) [1]. The alarming rise of tuberculosis is mainly due to its conjunction with HIV infection and to the outbreak of multidrug resistant (MDR) and extensively drug-resistant (XDR-TB) TB strains of *M. tuberculosis*. Additionally, the low compliance with the complexity and toxicity of the current chemotherapeutic regimens demands that a renewed effort need to be made to develop new effective anti-tuberculosis drugs.

Particularly, the interest in exploring the therapeutic potential of palladium(II) compounds as antitumor and antimicrobial agents has significantly increased over the past fifteen years [2–6]. To the best of our knowledge, the first report of the internal administration of a palladium compound for the treatment of tuberculosis was in 1943 [7]. Oral dosages of ca. 65 mg  $\text{PdCl}_2/\text{day}$  produced no apparent adverse effects in tuberculosis patients. Recently, we and others have been investigating the antimycobacterial activity of Pd(II) compounds [8–12]. Among them, complexes bearing phosphine-type ligands have displayed promising antimycobacterial activity at micromolar range [8,9]. In addition, we have also observed that the combination of azide groups ( $\text{N}_3^-$ )

with Pd(II) ions produces synergistic inhibition of MTB growth [12]. These findings have motivated us to prepare new analogous Pd(II) azido derivatives bearing phosphines and investigate their activity against MTB.

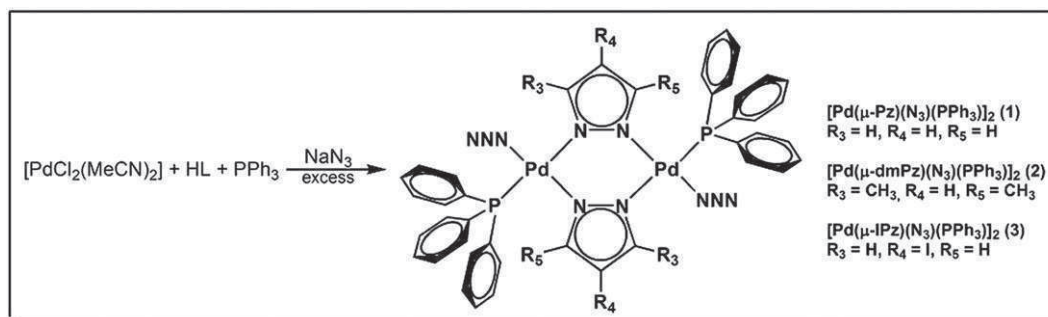
In the framework of our current research on the coordination and biological chemistry of palladium(II) compounds [13–15], we synthesized some palladium(II) compounds of the general formulae  $[\text{Pd}(\mu\text{-L})(\text{N}_3)(\text{PPh}_3)]_2$  ( $\text{L} =$  pyrazolate (**1**); 3,5-dimethylpyrazolate (**2**); 4-iodopyrazolate (**3**);  $\text{PPh}_3 =$  triphenylphosphine) and evaluated their antimycobacterial activity against TB bacillus. The molecular structure of the compound **2** was determined by X-ray crystallography.

The reaction between  $[\text{PdCl}_2(\text{CH}_3\text{CN})_2]$ , neutral pyrazolyl ligand ( $\text{L}$ ) and triphenylphosphine with subsequent addition of sodium azide in 1:1:1:6 molar ratio, respectively, yielded the formation of binuclear pyrazolate bridged complexes of general formulae  $[\text{Pd}(\mu\text{-L})(\text{N}_3)(\text{PPh}_3)]_2$ ,  $\text{L} =$  pyrazolato ( $\text{Pz}$ ), **1**; 3,5-dimethylpyrazolato ( $\text{dmPz}$ ), **2**; 4-iodopyrazolato ( $\text{IPz}$ ), **3**, as depicted in Scheme 1 (see electronic Supporting information).

The IR spectra of **1–3** showed the characteristic absorptions of terminal azido groups at  $\sim 2030\text{ cm}^{-1}$  ( $\nu_{\text{as}}\text{N}_3$ ) and  $\sim 1279\text{ cm}^{-1}$  ( $\nu_{\text{s}}\text{N}_3$ ) [16] as well as the typical bands of the  $\text{PPh}_3$  ligand at  $1097\text{ cm}^{-1}$  ( $\nu_{\text{P-C}}$ ),  $\sim 744\text{ cm}^{-1}$  ( $\gamma_{\text{CH}}$ ), 692 and  $513\text{ cm}^{-1}$  ( $\gamma_{\text{ring}}$ ) [17]. The presence of exobidentate pyrazolato groups was inferred by the absence of an intense and broad  $\nu_{\text{NH}}$  absorption (ca.  $3300\text{ cm}^{-1}$ ) and the appearance of a  $\nu_{\text{ring}}$  band over the spectral range of  $1379\text{–}1348\text{ cm}^{-1}$  [18].

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**Scheme 1.** A schematic representation of the synthesis of compounds  $[\text{Pd}(\mu\text{-L})(\text{N}_3)(\text{PPh}_3)_2]$  {L = pyrazolate (1); 3,5-dimethylpyrazolate (2); 4-iodopyrazolate (3);  $\text{PPh}_3$  = triphenylphosphine}.

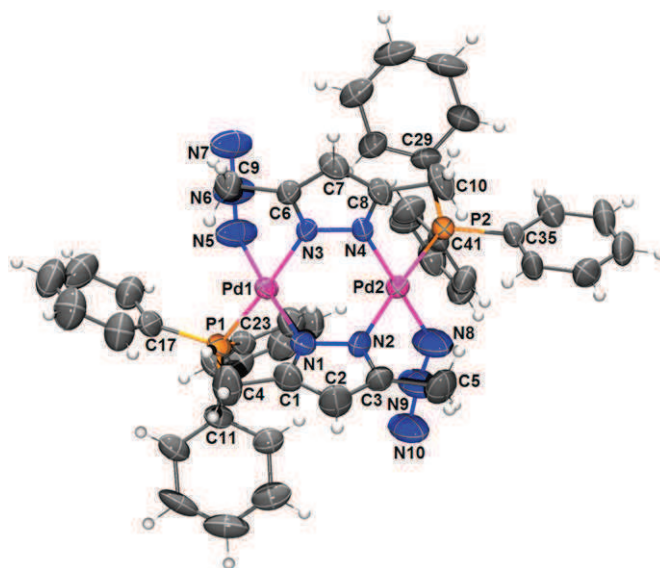
It has been shown that the configuration of bis-(pyrazolato)bridged-complexes of the type  $[\text{M}(\mu\text{-L})(\text{X})(\text{PR}_3)_2]$  (M = Pd, Pt; L = pyrazolates; X = methyl, aryl, chloride;  $\text{PR}_3$  = tertiary phosphines) can be inferred on basis of NMR data [19,20]. According to the  $[\text{Pd}(\mu\text{-L})(\text{N}_3)(\text{PPh}_3)_2]$  formulation, compounds 1–3 could exist as *cis* and *trans* isomers. For the *cis* isomer, two sets of resonances are expected for the substituents at 3, 4 and 5 positions of the ring since two pyrazolate moieties are magnetically nonequivalent. On the other hand, only one signal for the C-4 substituent is expected for the *trans* isomer since both pyrazolate groups are equivalent. The  $^1\text{H}$  NMR spectrum of 1 showed only signals at 5.76 and 6.39 ppm in 1:1 ratio, attributed respectively to the  $\text{H}_4$  and to one of the inequivalent  $\text{H}_3$  or  $\text{H}_5$  of the exobidentate pyrazolate. The  $\text{H}_5$  resonance of the third hydrogen ( $\text{H}_3$  or  $\text{H}_5$ ), is obscured by the complex  $\text{PPh}_3$  envelope at 7.87–7.28 ppm [21]. This magnetic non-equivalence is also detected for the methyl groups at the position 3 and 5 of the dmpz ligand in 2. Besides the presence of one single  $\text{H}_4$  resonance at 5.28 ppm, the methyl groups at the 3 and 5 sites appeared as two equally intense resonances at 1.53 and 2.56 ppm. The same pattern of magnetic equivalence was also observed in the  $^1\text{H}$  NMR spectrum of 3. The  $^{31}\text{P}\{^1\text{H}\}$  NMR spectra of 1–3 display a sharp singlet at 22–27 ppm, suggesting the existence of only one isomeric species in solution.

Suitable single crystals of good quality for X-ray diffraction studies were obtained from slow evaporation of a  $\text{CHCl}_3$  solution of 2 (see electronic Supporting information). The ORTEP representation of compound  $[\text{Pd}(\mu\text{-dmpz})(\text{N}_3)(\text{PPh}_3)_2]$  (2) with the atom scheme is presented in Fig. 1.

In the molecular structure of 2, the individual Pd centers are joined to one another by a pair of bridging exo-bidentate 3,5-dimethylpyrazolate groups defining a  $\text{Pd}_2\text{N}_4$  six-membered metallacycle. This central bridging framework is bent into a boat conformation in which the metal atoms are separated by 3.255(2) Å. The Pd–Pd separation is similar to those observed in related dimers incorporating 3,5-dimethylpyrazolate bridging ligands [22]. The geometric arrangement in complex 2 places each pair of ligands (either  $\text{N}_3$  or  $\text{PPh}_3$ ) mutually *trans* across the metal–metal axis (Fig. 1), resulting in a dissymmetric structure. Within the boat-like  $\text{Pd}_2\text{N}_4$  six-membered ring, the  $\text{N1-Pd1-N3}$  and  $\text{N2-Pd2-N4}$  angles are 85.8(2) and 86.4(2), respectively and the bond distances Pd–N were found to be different. The shorter Pd1–N1 (2.008(5) Å) and Pd2–N4 (2.018(5) Å) display N atoms *cis* to the P atom whereas the longer Pd1–N3 (2.044(5) Å) and Pd2–N2 (2.048(6) Å) exhibit the N atoms approximately *cis* to the  $\text{N}_{\text{azide}}$  donor atoms.

In vitro antimycobacterial activities of sodium azide, triphenylphosphine, pyrazolyl ligands,  $[\text{PdCl}_2(\text{MeCN})_2]$  and the binuclear compounds 1–3 were each evaluated against the strains of the *M. tuberculosis* H37Rv ATCC 27294 (see electronic Supporting information). The microplate alamar blue assay (MABA) was used to measure the minimal inhibitory concentration (MIC) for the tested compounds (minimum concentration necessary to inhibit 90% growth of *M. tuberculosis* H37Rv ATCC 27294) [23]. The minimum inhibitory concentration (MIC) values are depicted in Table 1.

As observed in Table 1, the pyrazolyl ligands, triphenylphosphine and  $[\text{PdCl}_2(\text{MeCN})_2]$  were inactive towards the pathogen. However, the reaction of these ligands with the Pd(II) precursor in the presence of azide ions afforded binuclear derivatives 1–3 with MIC values ranging from 8 to 25  $\mu\text{mol L}^{-1}$ . Keeping in mind the MIC range found for 1–3, our results suggested that the incorporation of the phosphine-type ligands in the molecular backbone of Pd(II) compounds may increase their activity towards *M. tuberculosis*. In fact, a literature survey showed that other phosphine-based Pd(II) compounds exhibited MIC values less than 10  $\mu\text{mol L}^{-1}$ :  $[\text{Pd}(2\text{-acetylpyridine-N}(4)\text{-phenylthiosemicarbazone})(\text{triphenylphosphine})](\text{NO}_3) \cdot \text{H}_2\text{O}$  (MIC = 8.7  $\mu\text{mol L}^{-1}$ ) [8] and  $[\text{Pd}(\text{C}'\text{-N-benzylideneaniline})(\text{SCN})(1,3\text{-bis}(\text{diphenylphosphino})\text{propane})]$  (MIC = 5.15  $\mu\text{mol L}^{-1}$ ) [9]. On the other hand, Pd(II) compounds which do not possess phosphine-like ligands were less active; i.e.  $[\text{Pd}(\text{C}^2\text{-N-dimethylbenzylamine})(\text{thiourea})(\text{X})]$  (X = Cl, Br): MIC range = 58–89  $\mu\text{mol L}^{-1}$  [10] and *trans*- $[\text{PdX}_2(\text{isonicotinamide})_2]$  (X=Cl, NCO, SCN,  $\text{N}_3$ ): MIC range = 36–297  $\mu\text{M}$  [12]. The presence of phosphines in 1–3 and other Pd(II) compounds may increase their lipophilic character, favoring its penetration through the highly impermeable bacterial cell wall of *M. tuberculosis*, which further results in a higher antimycobacterial activity.



**Fig. 1.** ORTEP drawing with labeling scheme for  $[\text{Pd}(\mu\text{-dmpz})(\text{N}_3)(\text{PPh}_3)_2]$  (2). Displacement ellipsoids are drawn at the 50% probability. Selected bond lengths (Å) and bond angles ( $^\circ$ ) are: Pd1–N1 2.008(5), Pd1–N5 2.036(7), Pd1–N3 2.044(5), Pd1–P1 2.262(2), Pd2–N2 2.048(6), Pd2–N4 2.018(5), Pd2–N8 2.021(7), Pd2–P2 2.297(2), N1–Pd1–N3 85.8(2), N2–Pd2–N4 86.4(2), N3–Pd1–N5 92.6(3), N2–Pd2–N8 93.0(0), N3–Pd1–P1 175.4(1), N2–Pd2–P2 176.6(1), N1–Pd1–P1 98.6(2), N4–Pd2–P2 95.8(1), N5–Pd1–P1 83.1(2), N8–Pd2–P2 84.7(2), N1–Pd1–N5 175.2(3), and N4–Pd2–N8 178.7(3).

**Table 1**MIC values of the palladium (II) complexes **1–3** and their ligands against *M. tuberculosis* H<sub>37</sub>Rv.

Compound	MW	MIC (μM)	MIC (μg·mL <sup>-1</sup> )
PPh <sub>3</sub>	262.28	>953.18	>250
NaN <sub>3</sub>	65.01	599.89	38.99
Pz	68.08	*	*
dmPz	96.13	*	*
IPz	193.97	*	*
[PdCl <sub>2</sub> (CH <sub>3</sub> CN) <sub>2</sub> ]	259.43	495.19	125.00
<b>1</b>	955.58	8.16	7.8
<b>2</b>	1011.69	24.71	25.0
<b>3</b>	1207.38	20.70	25.0
Rifampicin	819.40	0.07	0.06
Pyrazinamide	123.10	406.0–812.0	50–100
Isoniazid	137.2	0.22	0.030

\*The compound was inactive at the maximum concentration tested; MW = molecular weight.

In this study, the most active compound was [Pd(μ-Pz)(N<sub>3</sub>)(PPh<sub>3</sub>)<sub>2</sub>] (**1**), being approximately 2.5–3.0 times more active than their analogs **2** and **3**. This finding may suggest that the antimycobacterial activity of these binuclear Pd(II) derivatives is also affected by bridging pyrazolato ligands. The replacement of unsubstituted pyrazolato by 4-iodopyrazolato or 3,5-dimethylpyrazolato decreased the anti-tubercular activity of the [Pd(μ-L)(N<sub>3</sub>)(PPh<sub>3</sub>)<sub>2</sub>] complexes. Nevertheless, such structure–activity relationship is only preliminary taking into account that they were based on only in three Pd(II) compounds.

In addition, the in vitro inhibitory activity of compound **1** is comparable to those found for some commonly drugs used for tuberculosis treatment (gentamicin, 4.19–8.38 μM; tobramycin, 8.56–17.11 μM; clarithromycin, 10.70–21.40 μM; cycloserine, 122.4–489.7 μM) [24]. Nevertheless, further studies are required in order to establish safety and efficacy.

In conclusion, binuclear compounds of the type [Pd(μ-L)(N<sub>3</sub>)(PPh<sub>3</sub>)<sub>2</sub>] {L = pyrazolate (**1**); 3,5-dimethylpyrazolate (**2**); 4-iodopyrazolate (**3**); PPh<sub>3</sub> = triphenylphosphine} have been successfully synthesized and characterized in this work. The results obtained from in vitro antimycobacterial assays demonstrated that the compound **1** displayed an interesting activity against the *M. tuberculosis*. Further investigations on this class of compounds are underway in our laboratories aiming at rationalizing the MIC values in terms of structure–activity relationship as well as to understand the mechanism of action.

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### Appendix A. Supplementary Material

Additional materials, consisting of atomic coordinates and equivalent isotropic displacement parameters for non-hydrogen atoms, H-atom coordinates and isotropic displacement parameters, interatomic bond lengths and bond angles have been deposited with the Cambridge Crystallographic Data Centre, CCDC, no. 990467. Copies free of charge of this information may be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033; email: deposit@ccdc.cam.ac.uk).

Additional descriptions of synthesis, single-crystal X-ray diffraction studies, and antimycobacterial assays are presented in electronic Supporting information file. Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.inoche.2014.09.001>. These data include MOL files and InChIKeys of the most important compounds described in this article.

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Short Communication

## Comparison of resazurin microtiter assay performance and BACTEC MGIT 960 in the susceptibility testing of Brazilian clinical isolates of *Mycobacterium tuberculosis* to four first-line drugs

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### Abstract

We assessed the performance of REMA in comparison with BACTEC MGIT 960 in the susceptibility testing of 80 *Mycobacterium tuberculosis* clinical isolates from Clemente Ferreira Institute against four drugs. REMA proved to be a rapid and accurate method, providing excellent correlation with BACTEC MGIT 960, with the exception of results for the ethambutol drug.

**Key words:** *Mycobacterium tuberculosis*, tuberculosis, drug susceptibility testing.

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Tuberculosis (TB) remains a serious health problem, especially in underdeveloped and developing countries (Sanchoetene *et al.*, 2008). The rising incidence of drug resistant TB gives cause for concern around the world (WHO, 2010). Currently, Brazil occupies the 19th position among the 22 countries with the most cases of TB prevalence (WHO, 2010). The initial treatment for TB involves four drugs (MS/SVS, 2009): isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA) and ethambutol (EMB). Big cities such as São Paulo have high population densities and the high human immunodeficiency virus (HIV) infection rates and large numbers of institutionalized and homeless people contribute to the high prevalence of TB (Ferrari *et al.*, 2010). In this population, other factors contribute to the number of TB cases, such as irregular supply of drugs, unsuitable medical prescriptions and abandonment of treatment (Vareldzis *et al.*, 1994). Low-cost, sustainable and accessible methods (Ferrari *et al.*, 2010) that also exhibit high sensitivity, specificity and accuracy are indispensable in the TB control program, even as to achieve rapid detection of *Mycobacterium tuberculosis* in patients and characterization of drug resistance in the clinical isolates.

BACTEC MGIT 960 TB System is considered a simple and fast *in-vitro* diagnostic instrument, designed and optimized for the fast detection of mycobacteria from clinical specimens (Somoskövi *et al.*, 2000). Resazurin Microtiter Assay (REMA) is a simple and inexpensive method utilizing resazurin, a redox indicator (Franzblau *et al.*, 1998), to test the antimicrobial activity of drugs against clinical isolates of *M. tuberculosis* in low-income countries (Palomino *et al.*, 2002).

We aimed to assess the performance of the REMA method in determining patterns of susceptibility to four first-line drugs in *M. tuberculosis* clinical isolates from Brazil, by comparison with BACTEC MGIT 960, taken as a gold standard employing Minimum Inhibition Concentration (MIC) estimation and cut-off values for each drug.

Eighty *M. tuberculosis* clinical isolates from Clemente Ferreira Institute located in São Paulo city were analyzed by the BACTEC MGIT 960 kit and REMA method to determine their susceptibility patterns to the drugs INH, RMP, streptomycin (STR) and EMB. Multi-drug resistance (MDR) was taken to be resistance to at least INH and RMP. Reference susceptibility patterns were obtained with

BACTEC MGIT 960, performed at the Clemente Ferreira Institute, the TB reference center for São Paulo (SP) state, utilizing commercial kits supplied with fixed concentrations of 0.1 µg/mL, 1.0 µg/mL, 1.0 µg/mL and 5.0 µg/mL, for INH, RMP, STR and EMB, respectively (Siddiqi *et al.*, 2006).

REMA (Palomino *et al.*, 2002) was carried out with a standardized bacterial inoculum, 96-well plate (Nunc Thermo Fisher Scientific, Waltham, MA) and resazurin (Sigma-Aldrich, Steinheim, Germany) as developing agent, to reveal bacterial viability and growth. Each test was carried out in triplicate. MIC was defined as the lowest concentration that inhibited 90% of *M. tuberculosis* growth (Palomino *et al.*, 2002). Comparative analysis between the BACTEC MGIT 960 kit and REMA data allowed the breakpoint concentration to be determined with the MedCalc Software (Mariakerke, Belgium), by means of the Receiver Operating Characteristic (ROC) curve. Accuracy of REMA was evaluated from the area under the curve (AUC): 1 - 0.9 = excellent; 0.9 - 0.8 = good; 0.8 - 0.7 = moderate and < 0.7 weak correlation.

Among the eighty *M. tuberculosis* clinical isolates, BACTEC MGIT 960 detected 57 (71.2%) INH-resistant isolates, 49 (61.2%) RMP-resistant isolates, 29 (36.2%) STR-resistant isolates, 21 (26.2%) EMB-resistant isolates and 48 (60%) MDR isolates (Table 1). REMA determined the MIC values and the MedCalc Software determined cut-off values for each drug, based on the ROC curve concept (Table 1). ROC curve analysis resulted in sensitivity of 100%, 97.2%, 92.6% and 89.5%, specificity of 100%, 100%, 90% and 56.4% and accuracy levels of excellent, excellent, good and reasonable, with cut-off values of 0.0625 µg/mL, 0.125 µg/mL, 0.25 µg/mL and 8 µg/mL for INH, RMP, STR and EMB, respectively. Once the values had been fixed, all clinical isolates were classified as sensitive or resistant, according to their MIC values. REMA results demonstrated that, among the 80 *M. tuberculosis* clinical isolates, 63 (75%) were INH-resistant, 41 (51.2%) were RMP-resistant, 43 (53.7%) were STR-resistant and 47 (58.7%) were EMB-resistant. Simultaneous resistance to INH and RMP was found in 39 (48.7%) isolates, thus considered as MDR (Table 1). Some authors (5, 3, 8) based on these observations have proposed the existence of three categories of susceptibility: susceptible, partially resistant (isolates with MICs close to the cut-off value) and resistant (Mengatto *et al.*, 2006).

Based on a sub-classification (Palomino *et al.*, 2002, Tudó *et al.*, 2010) of resistant isolates by REMA, a division was made between high and low resistance, with cut-off values of 1 µg/mL, 2 µg/mL, 1 µg/mL and 16 µg/mL, for INH, RMP, STR and EMB, respectively. As result of this sub-classification, the resistant isolates were divided into: 53 (84.1%) INH high-resistance and 10 (15.8%) INH low-resistance (Figure 1a); 30 (73.1%) RMP high-resistance and 11 (26.8%) RMP low-resistance (Figure 1b); 19

**Table 1** - Results for 80 *M. tuberculosis* clinical isolates analyzed by BACTEC MGIT 960 and REMA.

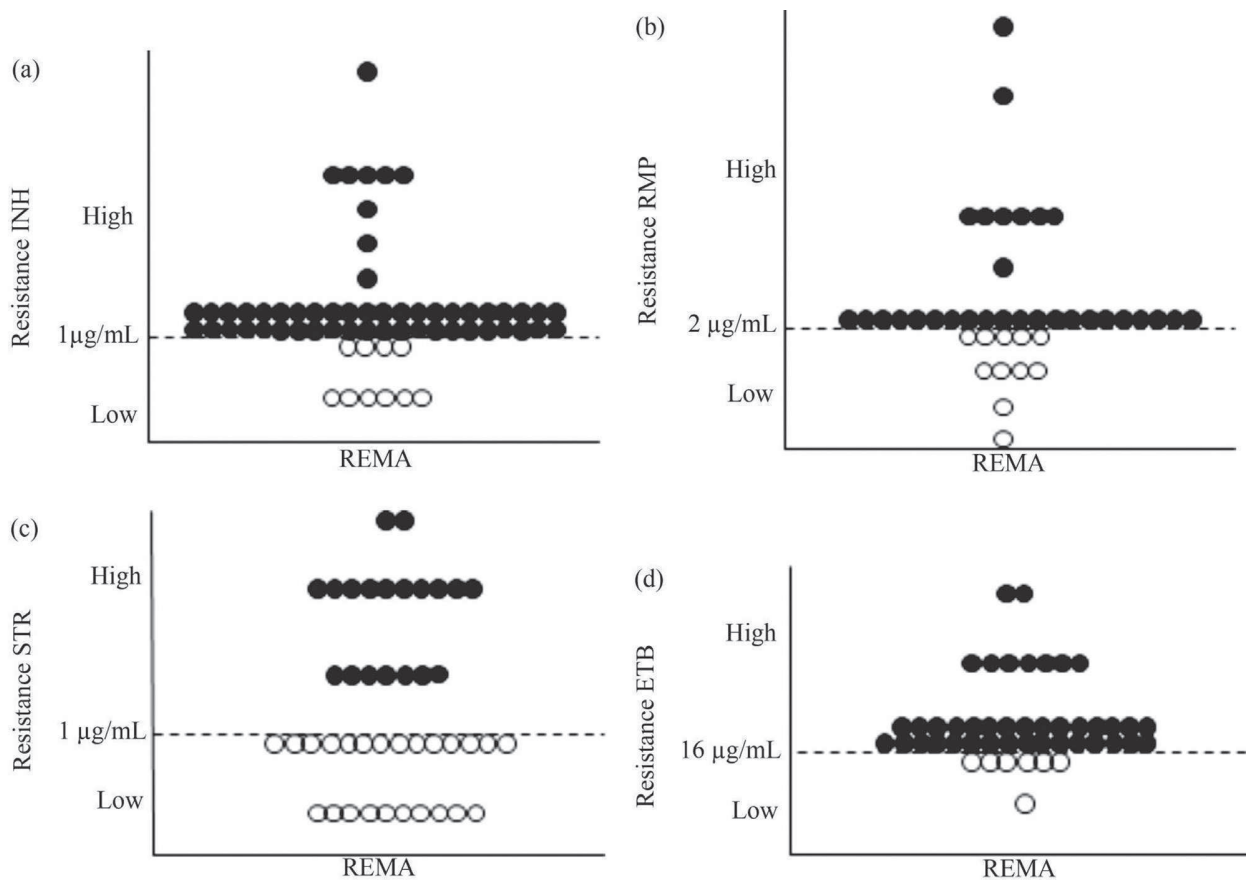
Drug	BACTEC MGIT 960 resistance	REMA resistance	Statistical analysis cut-off value (µg/mL)
INH	57 (71.2%)	63 (75%)	> 0.0625
RMP	49 (61.2%)	41 (51.2%)	> 0.125
STR	29 (36.2%)	43 (53.7%)	> 0.25
EMB	21 (26.2%)	47 (58.7%)	> 8
MDR	48 (60%)	39 (48.7%)	

(44.1%) STR high-resistance and 24 (55.8%) STR low-resistance (Figure 1c) and 40 (85.1%) EMB high-resistance and 7 (14.8%) EMB low-resistance (Figure 1d).

The high percentage of MDR isolates found in this study was probably related to the main objective of the Clemente Ferreira Institute, to assist TB patients with a difficulty history of TB treatment. Studies performed in the period from 1995 to 1998 also revealed a high prevalence of MDR cases (Melo *et al.*, 2003), characterized mainly by treatment failure and abandoned and inappropriate prescriptions (Jardim *et al.*, 2001).

Drug susceptibility patterns of *M. tuberculosis* clinical isolates were tested by REMA because this methodology enables the resistance level to be assessed with more precision in terms of MIC values (Heifets, 1988). The correct evaluation of the MIC is important because if resistant strains are classified as sensitive, the treatment will not reduce the patient's suffering and this resistant strain will spread to other people. Besides that, if sensitive strains are classified as resistant, the patient will be treated with unnecessarily toxic and expensive drugs, resulting in overload of the health system (Ahmad *et al.*, 2007) and patients with collateral effects. Cut-off values found in this paper were lower than those reported by other authors (Montoro *et al.*, 2005, Jadaun *et al.*, 2007). Such differences probably arise bacterial populations coming from different sources, with different susceptibility patterns and proportions of resistant isolates in each study, resulting in different cut-off values for each drug. Despite the discrepancies found, the authors agree that REMA is a fast method for MDR strain detection (Montoro *et al.*, 2005, Jadaun *et al.*, 2007, Rivoire *et al.*, 2007).

For INH, there were no discordant results between REMA and BACTEC MGIT 960 (Table 2) and the sensitivity and specificity of REMA were excellent. Similar results are found in the literature (Palomino *et al.*, 2002, Luna-Herrera *et al.*, 2003, Montoro *et al.*, 2005, Mengatto *et al.*, 2006, Nateche *et al.*, 2006, Rivoire *et al.*, 2007), indicating the viability of REMA in testing patterns of susceptibility to INH, even though cut-off values range between 0.0625 and 0.225 µg/mL and different results for sensitivity and specificity are found in the literature. RMP showed one discrepancy (1 false sensitive) (Table 2), resulting in a



**Figure 1** - Sub-classification within resistant isolates by REMA. (a) INH-resistant isolates. (b) RMP-resistant isolates. (c) STR-resistant isolates. (d) EMB-resistant isolates. Full circles represent high-resistant isolates and empty circles represent low-resistant isolates.

lower sensitivity than that of INH, but with the same specificity and also excellent correlation, with similar results for sensitivity (95%) and specificity (100%) in the literature (Palomino *et al.*, 2002, Luna-Herrera *et al.*, 2003, Montoro *et al.*, 2005, Nateche *et al.*, 2006, Rivoire *et al.*, 2007), as well as excellent correlation (Palomino *et al.*, 2002, Luna-Herrera *et al.*, 2003, Mengatto *et al.*, 2006). For STR, there were 4 discordant results (1 false resistant and 3 false sensitive) (Table 2), resulting in lower values of both sensitivity and specificity and excellent correlation, with similar published results ranging between 88.5 and 100% (Palomino *et al.*, 2002, Luna-Herrera *et al.*, 2003, Montoro *et al.*, 2005, Nateche *et al.*, 2006, Rivoire *et al.*, 2007), as well as an excel-

lent correlation (Luna-Herrera *et al.*, 2003). EMB showed 26 discordant results (3 false resistant and 23 false sensitive) (Table 2), resulting in a lower value of sensitivity, low value of specificity (Montoro *et al.*, 2005) and reasonable correlation; previous sensitivity results ranged between 92 and 98% (6, 8, 13), specificity results between 98 and 100% (Luna-Herrera *et al.*, 2003, Jadaun *et al.*, 2007) and correlation was reasonable (Madison *et al.*, 2002). According to the literature, the INH high-resistance isolates showed similar results to those of Palomino *et al.* (Palomino *et al.*, 2002), RMP and STR high-resistance isolates showed worse results than Palomino *et al.* (2002) and Tudó *et al.* (2010), respectively, and EMB high-resistance isolates showed similar results to those of Siddiqi *et al.* (1985).

**Table 2** - Results from 80 *M. tuberculosis* clinical isolates analyzed by BACTEC MGIT 960 and REMA showing numbers of agreements and disagreements results.

REMA	INH		RMP		STR		EMB	
	R	S	R	S	R	S	R	S
R	41	0	35	0	26	3	13	23
S	0	16	1	21	1	27	3	18
Total	41	16	36	21	27	30	16	41

STR and EMB, but especially EMB, have important factors that may have contributed to the discrepancies in the results: (i) a small proportion of drug-resistant strains in the population studied, (ii) substantial differences in the volume of medium utilized by the two methods, and (iii) different periods of incubation, which may result in different degrees of degradation of the drugs (Mengatto *et al.*, 2006). Partially resistant isolates with borderline MICs were not found in this study, so that MIC values were well defined as susceptible or resistant. Furthermore, some authors have

shown that these “borderline” cultures, more frequently associated with EMB resistance, have classically caused problems in the interpretation of EMB susceptibility testing (Siddiqi *et al.*, 1985), adversely affecting the overall performance of the assay. On the other hand, for STR, a low level of sensitivity was obtained.

Finally, our results demonstrate that REMA is a rapid method for the determination of the susceptibility to drugs of *M. tuberculosis* clinical isolates in reference laboratories such as Clemente Ferreira Institute, which shows a high level of accuracy when compared with BACTEC MGIT 960, except in tests with the drug EMB.

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Monday, 25 January 2016

To Whom It May Concern,

I am delighted to write this recommendation letter to **Mr. Leonardo B. Marino**, supporting his application for a faculty position. His training in metabolomics, mycobacterial research, and antimicrobial pharmacology is outstanding.

I am currently a Head of Laboratory at the Francis Crick Institute, in London, United Kingdom. My lab focuses on the application of liquid chromatography/mass spectrometry metabolomics to enzymology, antibiotic research and mycobacterial physiology.

Leonardo was a member of our laboratory from 10 June 2014 until 16 July 2015, while he was a PhD student at the university. Leonardo worked in a variety of projects during his time in the lab. He explored the mechanism of action of several compounds with antitubercular activity employing cutting-edge methods such as transcriptional profiling and liquid-chromatography coupled to mass spectrometry-based metabolomics. In addition, he worked on the characterization of compounds that affect pyrimidine biosynthesis in *Mycobacterium tuberculosis*. This work has already resulted in a publication in an important journal (*Chem Bio* (2015) 22(7):917-27). In addition to these studies, Leonardo was involved in a few others studies, applying intrabacterial pharmacology techniques to understand how drugs work and which process they inhibit. Two other manuscripts that are partially derived from Leonardo's experiments are currently in review/revision (*J Med Chem*, *in review* and *ACS Infectious Diseases*, *in review*). Finally, Leonardo contributed to the first feasibility, proof-of-concept experiments that allow us to apply for a PhD studentship to study protein lipidation in *M. tuberculosis*. In summary, Leonardo's can-do attitude and commitment make him the most efficient young scientist we ever had in the laboratory.

Finally, I would like to state that Leonardo taught us a lot. Our laboratory is currently much better positioned to carry out antibiotic pharmacology studies than it was before his arrival. It was a pleasure to have him here for this year, and I would be very happy to host other scientists that despite their young age have the calibre and good personality, such as he does.



Regarding personality we were all extremely happy with Leonardo. He is a fantastic colleague, easy to talk to, hard worker, careful and meticulous experimentalist, and has a very good scientific background. People from the laboratory and immediate area of interest were very fond of him.

If I may say, I believe he is one of the most qualified graduate students I ever met, and it would be a mistake to not hire him.

Please, feel free to contact me if you have any further questions.

Yours sincerely,

A handwritten signature in black ink, appearing to read 'L. Pedro' or similar, written in a cursive style.

Luiz Pedro S. de Carvalho, Ph.D.