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Estudos *in vitro* e *in vivo* de compostos furoxânicos, benzofuroxânicos e quinoxalinas com potencial aplicação para o tratamento da tuberculose

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Tese apresentada ao Programa de Pós-Graduação em Biociências e Biotecnologia Aplicadas à Farmácia, área de concentração: Microbiologia, da Faculdade de Ciências Farmacêuticas, UNESP, como parte dos requisitos para obtenção do Título de Doutor em Biociências e Biotecnologia aplicadas à Farmácia.

Orientador: Prof. Dr. Fernando Rogério Pavan

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"Elegância de comportamento não é algo que se tem, é algo que se é! A verdadeira elegância é a do caráter, porque procede da essência do ser."

Autor desconhecido

RESUMO

A busca por novos fármacos faz-se necessária sob dois principais pontos: a cura/tratamento do paciente pela melhora de qualidade de vida e a importância de trata-lo a fim de eliminar focos de propagação das doenças infecto-contagiosas. A tuberculose (TB) se insere neste panorama, visto que é uma doença infecciosa que tem como principal patógeno o *Mycobacterium tuberculosis* (*Mtb*). A taxa de mortalidade da TB caiu 45% desde 1990 e o mundo estaria a caminho de atingir a meta global de redução de 50% até o ano de 2015. A última pesquisa conduzida no ano de 2015 mostrou 6,3 milhões de casos de TB notificados pelos programas nacionais da doença, dos quais 55% correspondem a casos de co-infecção TB-HIV. Uma recente problemática repercute: a resistência. Fato comprovado pela estimativa de 580.000 novos casos de TB-MDR no ano de 2015 comparados aos 480.000 casos registrados em 2014; e o relato de 9,7% desses casos serem na verdade TB-XDR. Baseado nesses dados epidemiológicos observamos a necessidade da busca de novos fármacos a fim de melhorar a terapia e consequentemente a qualidade de vida e cura dos pacientes. Nesse sentido, essa tese buscou investigar o potencial biológico de vinte e dois novos compostos n-óxidos: derivados furoxânicos, benzofuroxânicos e derivados quinoxalínicos; *in vitro* e *in vivo*. Investigamos também a segurança e dados preliminares ao mecanismo de ação. Os compostos demonstraram atividade (CIM_{90}) entre 0,40 – 62 µM. Dentre todos os compostos n-óxidos estudados, o derivado benzofuroxânico **8** demonstrou-se mais promissor, com atividade frente ao *Mtb* com valores de CIM_{90} de 1,10 e 6,62 µM frente a bactéria no estado ativo e latente, respectivamente. A citotoxicidade frente à linhagem celular MRC-5 (IC_{50}) foi obtida na concentração de 519,2 µM. Esse composto também apresentou atividade frente às cepas monorresistentes a importantes fármacos da terapia (isoniazida, rifampicina, moxifloxacina, bedaquilina, cicloserina e estreptomicina); assim como demostrou uma atividade intramacrofágica similar ao fármaco da terapia rifampicina – aproximadamente 90% de inibição para todas as concentrações testadas. Para os estudos *in vivo* (biodisponibilidade oral, tolerabilidade, infecção e tratamento) os compostos foram incorporados em microemulssão devido instabilidade em pH 1,0 e 9,0; mostrando-se biodisponíveis e toleráveis. Por fim, comprovamos o quanto essas classes são promissoras *in vivo*, três compostos (dois pertencentes ao grupo de derivados furoxânicos e um derivado de benzofuroxânico) esterilizaram a infecção pulmonar com *Mtb* de camundongos BALB/c. Apresentamos assim, três novos compostos com

potencial para estudos clínicos e futuramente serem classes químicas presentes na terapia da doença.

Palavras-chaves: Tuberculose. Furoxanos. Benzofuroxanos. Quinoxalina. Novas moléculas.

ABSTRACT

The search for new drugs is necessary under two main points: the cure / treatment of the patient for the improvement of quality of life and the importance of treating it in order to eliminate outbreaks of the spread of infecto-contagious diseases. Tuberculosis (TB) is part of this scenario, since TB is an infectious disease with *Mycobacterium tuberculosis (Mtb)* as its main pathogen. The TB mortality rate has dropped by 45% since 1990 and the world is on track to meet the global 50% reduction target by 2015. The latest survey conducted in 2015 showed 6.3 million TB cases notified by national disease programs, of which 55% correspond to cases of HIV-TB co-infection. A recent problem has repercussions: resistance. This is proven by the estimated 580,000 new cases of MDR-TB in 2015 compared to 480,000 cases in 2014; actually 9.7% of these cases are TB-XDR. Based on these epidemiological data, we observed the need to search for new drugs in order to improve therapy and consequently the quality of life and cure of patients. In this sense, this thesis sought to investigate the biological potential *in vitro* and *in vivo* of twenty-two new n-oxide compounds: furoxanic, benzofuroxanic and quinoxaline derivatives. We also investigated the safety and preliminary data on the mechanism of action of these compounds. The compounds showed activity (MIC_{90}) in a ranging 0.40 to 62 μM. Among all the n-oxide studied, the benzofuroxan **(8)** was more promising (MIC_{90} values of 1.10 and 6.62 μM against the active and latent bacteria, respectively; and cytotoxicity against the MRC-5 cell line (IC50) was obtained at the concentration of 519.2 μM). This compound also showed activity against the monoresistant strains of important drugs of the therapy (isoniazid, rifampicin, moxifloxacin, bedaquiline, cycloserine and streptomycin) as well as demonstrated an intramacrophagic activity similar to the drug of rifampicin therapy - approximately 90% inhibition at all concentrations tested. For *in vivo* studies (oral bioavailability, tolerability and treatment) the compounds were incorporated into microemulsion due to instability at pH 1.0 and 9.0; being bioavailable and tolerable. Finally, we verified how much these classes are promising *in vivo*. Three compounds (two belonging to the group of furoxanic derivatives and a benzofuroxanic derivative) sterilize pulmonary infection with *Mtb* of BALB / c mice. Thus, we present three new compounds with potential for clinical studies and in future being chemical classes present in the therapy of the disease.

Key words: Tuberculosis. Furoxans. Benzofuroxans. Quinoxaline. New molecules.

LISTA DE FIGURAS

ABREVIATURAS

TB: tuberculose

a.C.: antes de Cristo

Mtb: *Mycobacterium tuberculosis*

BCG: Bacille Calmette-Guerin

OMS: Organização Mundial da Saúde

TB-MDR: tuberculose multi-fármaco resistente

TB-XDR: tuberculose extensivamente resistente

STM: estreptomicina

INH: isoniazida

RFP: rifampicina

PZA:pirazinamida

KAN: canamicina

AMK: amicacina

CAP: capreomicina

VIM:viomicina

CFX: ciprofloxacino

LFX: levofloxacino

MFX: moxifloxacino

OFX: ofloxacino

GFX: gatifloxacino

PAS: ácido paraamino salicílico

DCS: cicloserina

TRD: terizidona

ETO: ethionamida

PTO: prothonamida

THZ: tiacetazona

LZD: linezolida

CFZ: clofazimina

LZD: linizolida

AMX\CLV: amoxicilina\clavulanato

IPM\CLn: imipenem\clavulanato

CLR: claritromicina

STAND: *Shortening Treatment by Advancing Novel Drugs*

MEA: Mecanismo de Ação

POA: ácido pirazinóico

NO: óxido nítrico

RNS: espécies reativas de nitrogênio

CIM: concentração inibitória mínima

IC50: índice de citotoxicidade

CBM: concentração bactericida mínima

AST: aspartato aminotransferase

ALT: alanina aminotransferase

SUMÁRIO

1. INTRODUÇÃO

1.1 Dados gerais e epidemiológicos da tuberculose no mundo

As doenças infecciosas a cada dia crescem como uma preocupação da saúde pública mundial. Quando causada por microrganismo, como o caso da tuberculose (TB), a preocupação é maior pelo fato do aumento da resistência aos fármacos presentes no mercado ao longo dos últimos anos (WHO, 2013)

A TB é uma das doenças mais antigas do mundo. Evidências da presença da doença datam em documentos antigos no Egito, Índia e China à 5.000 a.c., 3.300 a.c. e 2.300 a.c., respectivamente (DANIEL, 2006).

A TB humana pode ser causada por membros do complexo *Mycobacterium tuberculosis* (*Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, Mycobacterium caprae, Mycobacterium microti, Mycobacterium pinnipedii e Mycobacterium canettii*) (ZUMLA et al., 2013). Sendo o *M. tuberculosis* (*Mtb*) a principal espécie causadora da TB, identificada em 1882 por Robert Koch.

Em termos de profilaxia, a única vacina contra TB é a BCG (Bacille Calmette-Guerin) produzida a partir de uma cepa atenuada de *M. bovis*, que apresenta eficácia a crianças contra formas disseminada da TB. Mas pouco eficaz em adultos, em especial para casos de TB pulmonar (TAUS et al., 2015).

A transmissão da TB ocorre pessoa-a-pessoa através de gotículas (aerossóis) contendo o bacilo, expelidas por indivíduos infectados e que manifestam a doença (forma ativa), ao tossir, falar ou espirrar. Essas gotículas são então inaladas por indivíduos sadios, podendo ou não estabelecer-se a infecção (JEREB et al., 1999). Cerca de 10 a 30% das pessoas expostas adoecem e outras 60-90% desenvolverão resposta imune celular eficaz para conter a infecção com sucesso (WARD et al., 2008; MANABE & BISHAI, 2000). O processo de TB primária ocorre quando macrófagos alveolares fagocitam os bacilos, mas não são capazes de eliminá-los. Os bacilos permanecem viáveis dentro das pequenas lesões granulomatosas pulmonares, mesmo após a ativação de células T específicas, permitindo sua disseminação. 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-replicante (“dormente”), considerado não infeccioso e que pode permanecer assim por anos. No entanto, razões relacionadas à resposta imunológica do indivíduo tais como: co-infecção ao HIV, deficiência nutricional, idade, consumo exagerado de álcool, entre outros; podem levar esses bacilos à sofrerem uma reativação, tornando-se infecciosos, o que resulta então no desenvolvimento da forma ativa da TB no indivíduo (RUSSEL, 2007).

A Organização Mundial da Saúde (OMS) estima 10,4 milhões de casos de TB no mundo somente no ano de 2015. Deste total 1,5 milhão representa o número de óbitos em decorrência da doença, dos quais 400.000 mortes correspondem a co-infecções TB-HIV (WHO, 2015). Em contrapartida, a taxa de mortalidade da TB caiu 45% desde 1990 e o mundo estaria a caminho de atingir a meta global de redução de 50% até 2015 (WHO, 2013).

Apesar da diminuição em números totais de casos da doença ao longo dos anos, o cenário da TB supostamente otimista não se apresenta tão real assim. Isto se comprova pelo aumento de casos de TB multi resistente a fármaco (TB-MDR) e o aparecimento de cepas de resistência estendida (TB-XDR).

A TB-MDR é definida como a resistência simultânea a pelo menos os fármacos isoniazida (INH) e rifampicina (RFP) que são de primeira escolha na terapia. Já a forma TB-XDR caracteriza-se além da resistência a INH e RFP a também qualquer das fluoroquinolonas além de um dos três fármacos injetáveis de segunda-linha (capreomicina, canamicina, e amicacina).

Somente no ano de 2014 a estimativa foi de 480.000 novos casos de TB-MDR e o relato de TB-XDR em 105 países; estima-se que 9,7% dos casos de TB-MDR sejam na verdade TB-XDR. Recentemente, a OMS estimou 1,5 milhão de casos de TB no mundo somente no ano de 2015 (WHO, 2015). Há relatos de casos de tuberculose totalmente resistente (TB-TDR), porém este termo ainda não se adequa pelo fato desses casos serem relatados no âmbito clínico e não ser realizado testes de sensibilidade para todos os fármacos possíveis do tratamento; carecendo assim esta nomenclatura mas não diminuindo a relevância (SLOMSKI, 2013).

Novas estratégias, melhores testes diagnósticos, novos regimes de medicamentos e vacinas estão entre as ferramentas urgentemente necessárias na luta contra a TB. As tecnologias empregadas para prevenir, diagnosticar e tratar a TB permanecem presas no século XX, enquanto que o patógeno *Mtb* continua a evoluir e desenvolver padrões de resistência complexos para o arsenal existente de fármacos disponíveis na terapia (FRICK & JIMÉNEZ-LEVI, 2013).

1.2 Tratamento da tuberculose

A estreptomicina (STM) produzida pela bactéria *Streptomyces griseus* e descoberta por Selman Waksman, em 1944, foi o primeiro fármaco efetivo para a TB. Oito anos decorreram (1952) para que a INH fosse insrida na terapia da TB, e posteriormente a RFP (1965). O etambutol (EMB) sintetizado em 1960, só passou a integrar a terapia da TB em 1968; assim como a pirazinamida (PZA) que apesar de sintetizada em 1936, somente em 1970 passa a compor a poliquimioterapia da doença (SOUZA & VASCONCELOS, 2005).

Para o tratamento básico a RFP, descoberta há mais de 50 anos, ainda representa o último modelo de fármaco introduzido no tratamento da TB. Juntamente com INH, EMB e PZA, a RFP compõem um regime de tratamento de seis meses sendo ineficaz contra TB-MDR e TB-XDR, apresentando ainda antagonismo com muitos antiretrovirais em casos de co-infecção TB/HIV (GANDHI et al., 2010).

Os fármacos utilizados para a terapia da TB são classificados em cinco grupos. Atualmente recomenda-se uma combinação de quatro fármacos para o tratamento de TB não resistente (Grupo 1) – chamados de fármacos de primeira linha ou primeira escolha. E para os casos de resistência, são denominados fármacos de segunda linha ou segunda escolha (Grupos 2, 3 e 4). Há ainda os fármacos de terceira linha ou terceira escolha (Grupo 5) que são fármacos que estão em investigação adicional quanto a sua segurança, tolerabilidade para tratamento de casos MDR-TB (ZUMLA et. al., 2013).

- Grupo 1 (Oral): isoniazida (INH), rifampicina (RFP), pirazinamida (PZA), etambutol (EMB);
- Grupo 2: aminoglicosídeos injetáveis – estreptomicina (STM), kanamicina

(KAN), amicacina (AMK); polipeptídeos injetáveis – capreomicina (CAP), viomicina (VIM);

- Grupo 3: fluoroquinolonas orais e injetáveis – ciprofloxacina (CFX), levofloxacina (LFX), moxifloxacina (MFX), ofloxacina (OFX), gatifloxacina (GFX);
- Grupo 4 (Oral): ácido para-amino-salicílico (PAS), cicloserina (DCS), terizidona (TRD), etionamida (ETO), prothionamida (PTO); tiacetazona (THZ), linezolida (LZD);
- Grupo 5: clofazimina (CFZ), linezolida (LZD), amoxicilina+clavulanato (AMX/CLV), imipenem+cilastatina (IPM/Cln), claritromicina (CLR).

De certa forma, subestimamos a capacidade evolutiva do *Mtb* e negligenciamos por anos a contenção da doença. O uso irracional dos antimicrobianos por várias gerações têm selecionado cepas cada vez mais resistentes aos fármacos encontrados no mercado ou até mesmo a substâncias inéditas que nunca foram apresentadas ao microrganismo. O *Mtb* torna esse desafio ainda mais difícil devido suas características morfológicas (espessa parede lipídica com ácidos graxos de alto peso molecular) (LEDERER, 1977), e suas características fisiológicas (a bactéria pode ser encontrada em estado ativo ou em estado de latência), além de bombas de efluxo (RUSSEL, 2007).

Diante da necessidade de uma nova molécula, o TMC-207 que era um dos compostos em fase clínica de apenas 5 (SQ-109 (Sequella Inc.), OPC-67583 (Otsuka Pharmaceutical Company), LL3858 (Lupin Ltda.), TMC 207 (Johnson & Johnson) e PA-824 (TB Alliance and Novartis) (Laloo e Ambaram, 2010; Lienhardt, Vernon *et al.*, 2010), foi rapidamente aprovado pelo FDA (*Food and Drug Administration)*, tornando-se hoje o primeiro fármaco contra TB resistente desde 1960, recebendo o nome de “Sirturo”. Seu uso é restrito a casos específicos (FDA, 2012).

Outro fármaco também aprovado recentemente - novembro de 2013 - pela Agência Européia de Medicamentos foi a delamanide (OPC 67683). Este fármaco atua bloqueando a síntese de ácidos micólicos que compõem a membrana do *Mtb* (WHO, 2015). Este fármaco é ativo não somente em bactérias sensíveis como resistentes; além de

demonstrar atividade em bactérias em estado ativo como em latência (ZANG *et al.*, 2013).

Uma segunda tentativa, atual, objetivou não somente a introdução de uma nova molécula ao esquema terapêutico, mas sim a total alteração do esquema terapêutico em vigor. A TB alliance financiada pela fundação Bill & Mellinda Gates propuseram recentemente um novo esquema terapêutico chamado de *STAND (Shortening Treatments by Advancing Novel Drugs)*, alterando toda a combinação terapêutica atual para uma nova que manteve apenas a pirazinamida. O esquema atual sugere a combinação de moxifloxacina, pretomanide (PA-824) e pirazinamida, ambos para tratamento de infecção sensível quanto resistente. O novo esquema encontra-se em fase de triagem clínica (TB ALLIANCE, 2014). Para conhecimento outras propostas de novos fármacos e/ou regimes terapêuticos são estudados por diversos grupos, tais como: *"Working group on new TB drugs"* (http://www.newtbdrugs.org/), "*International Union Against Tuberculosis and Lung Disease"* (http://www.theunion.org/what-we-do/research/clinical-trials), *CDC – Centers for Disease Control and Prevention* (https://www.cdc.gov/tb/topic/research/tbtc/).

1.3 Mecanismo de ação dos fármacos de primeira linha/escolha da tuberculose

Isoniazida (INH)

Em 1952 foi descoberta a atividade da INH frente ao *Mtb* (ROBITZEK & SELIKOFF, 1952). Este fármaco apresenta atividade bactericida sobre os bacilos de multiplicação rápida, no entanto, tem ação restrita sobre os bacilos de crescimento lento (intracelulares) e aqueles de multiplicação intermitente (extracelulares) (ZHANG, 2005). O mecanismo de ação (MEA) da INH envolve a inibição da biossíntese de ácidos micólicos, que é um importante componente da membrana celular do *Mtb* (WINDER & COLLINS, 1970). A isoniazida é um pró-fármaco, ativado pela enzima micobacteriana KatG. Após ativação, o metabólito produz radicais reativos de oxigênio, como superóxido, peróxido de hidrogênio e peroxinitrato, além de radicais orgânicos que inibem a formação de ácido micólico da parede celular, causando danos ao DNA e subsequente morte do bacilo. É também o principal fármaco utilizado para tratamento da

TB latente, indicada de maneira profilática, para grupos de alto risco como HIV positivos e outros indivíduos imunossuprimidos (TIMMINS; DERETIC, 2006; PETRI JR, 2011).

Rifampicina (RFP)

A RFP foi desenvolvida pela Dow-Lepetit Research Laboratories em Milão por modificação de rifamicinas, que são metabólitos naturais de *Nocardia mediterrane* (SENSI, 1983). As rifamicinas (rifampicina, rifabutina, rifapentina) constituem um grupo. Embora a atividade bactericida das rifamicinas seja inferior à da isoniazida, elas são os mais potentes agentes bactericidas disponíveis na quimioterapia da TB, continuando a matar bacilos persistentes durante toda a terapia. A atividade biológica das rifamicinas é atribuída a sua capacidade de inibir a transcrição ligando-se com alta afinidade a enzima micobacteriana RNA polimerase, formando um complexo fármaco-enzima estável, levando à supressão da iniciação da formação da cadeia na síntese de RNA (WEHRLI; STAEHELIN, 1971; KARAKOUSIS, 2012).

Pirazinamida (PZA)

A PZA é outro importante fármaco de primeira linha utilizado no tratamento da tuberculose, que contribuiu para a redução do tempo de tratamento de 12 para 6 meses. Assim como a INH, a PZA é um pró-fármaco que requer ativação para ser convertida ao ácido pirazinóico (POA) pelas enzimas nicotinamidase/pirazinamidase. POA é inicialmente formado no ambiente citoplasmático do *Mtb* e não apresenta atividade frente a bactéria. Somente após ser excretada para o meio ácido lisossomal e convertida no ácido conjugado, este tem a capacidade de entrar e se acumular na célula, promovendo a acidificação e a alteração do potencial de membrana, e como consequência a morte do bacilo (ZHANG, 2003; MITCHISON, 2003).

Etambutol (ETB)

O ETB é um dos quatro medicamentos de primeira linha para o tratamento da TB, e seu mecanismo de ação envolve diversas atividades celulares; o mecanismo mais provável é que o fármaco atue como um inibidor da biossíntese da parede celular. Este fármaco foi relatado pela primeira vez como um agente anti-TB em 1961. Assim como a

INH, o ETB é ativo principalmente em bacilos em fase de multiplicação, sendo um fármaco tuberculostático. O fármaco atua bloqueando as enzimas arabinosil transferases, envolvidas na biossíntese de arabinogalactano (PETRI JR, 2011; KOLYVA & KARAKOUSIS, 2012). Além disso, outros mecanismos celulares induzidos pelo EMB já foram relatados, como por exemplo, a inibição do metabolismo do RNA (FORBES; KUCK; PEETS, 1962, 1965), transferência de ácidos micólicos para a parede celular (TAKAYAMA et al., 1979) e síntese de fosfolipídios (CHEEMA & KHULLER, 1985).

1.4 Derivados Furoxânicos, benzofuroxânicos, quinoxalínicos e tuberculose

É real que a incidência de TB no mundo venha reduzindo e que a meta do STOP TB até o ano de 2015 foi relativamente cumprida no que se refere a casos de TB de cepas sensíveis. Entretanto, um problema maior vem surgindo que é o aumento da incidência de TB resistente.

Dentro deste desafio, esta tese refere-se aos resultados obtidos de derivados furoxânicos em colaboração iniciada em 2011 com o laboratório LAPDESF (Laboratório de Pesquisa e Desenvolvimento de Fármacos) da FCFAR/UNESP.

Os furoxanos compõem uma classe de compostos heterocíclicos doadores de óxido nítrico (NO). O NO é um mensageiro fisiológico que está presente em quase a totalidade dos tecidos humanos. O NO apresenta diversas ações fisiológicas potentes, dentre elas, a modulação de plaquetas e a adesão dos leucócitos ao endotélio. Ainda é também um dos efetores finais da resposta imune. As concentrações elevadas são produzidas, em particular, por macrófagos. Assim, difunde-se para a célula do parasita, onde exerce efeitos citotóxicos através de inúmeros mecanismos, incluindo a formação de espécies reativas de nitrogênio (RNS) e inibição de enzimas (GASCO et al., 2004).

De um lado, o *Mtb* suprime habilmente as vias envolvidas na produção de NO por parte do macrófago. De outro lado, esta classe de compostos é descrita na literatura tendo como característica a capacidade de doação de NO. Em suma, esta capacidade possivelmente vem ao encontro de auxiliar uma via que está suprimida ao longo da infeção até a manifestação da doença no caso da TB.

A síntese dos derivados furoxânicos foi realizada de acordo com a **Figura 1**. Inicialmente, foram sintetizados os derivados furoxânicos intermediários funcionalizados

contendo a função aldeído a partir de diferentes reagentes de partida (ácido metacrílico, estireno, tiofenol, etil 4-cloroacetoacetato, crotonaldeído e álcool cinâmico). A série dos benzofuroxânicos se iniciou com a síntese do derivado benzofuroxano aldeído a partir do 4-cloro-3-nitrobenzaldeido (**Figura 2**).

Posteriormente, os derivados furoxânicos e benzofuroxânico aldeídicos, foram submetidos a reações de condensação com a isoniazida em meio etanólico catalisada por ácido, levando a formação das moléculas híbridas finais (FERNANDES et al, 2016).

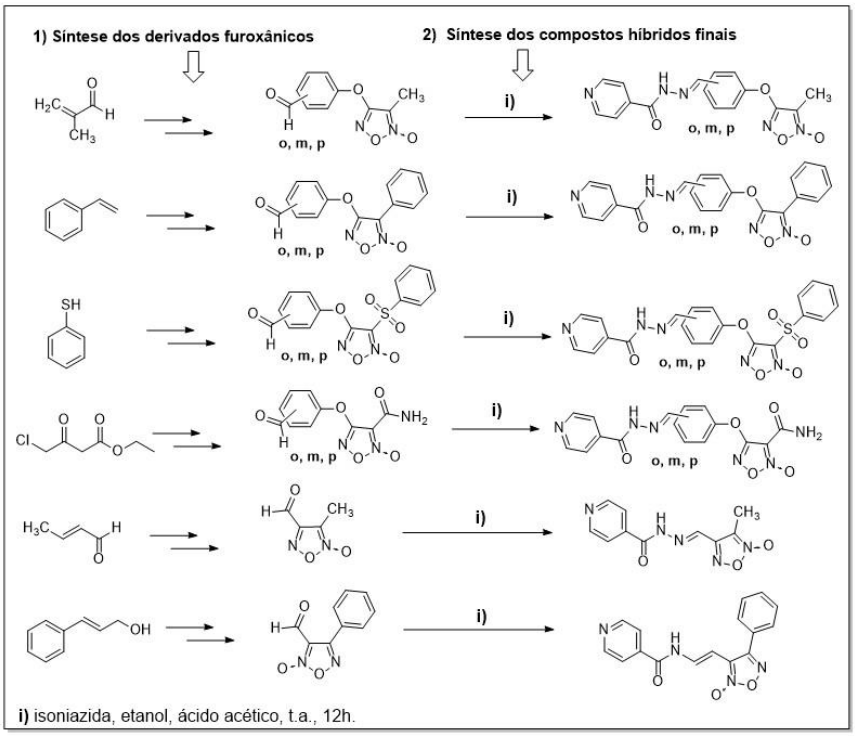

Figura 1. Esquema geral de síntese dos derivados furoxânicos.

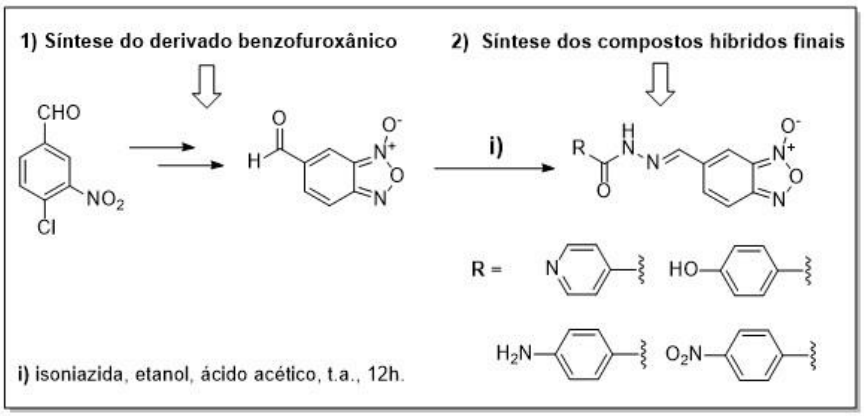

Figura 2. Esquema geral de síntese dos derivados benzofuroxânicos.

O primeiro relato sugerindo a utilização dos derivados quinoxalínicos para o tratamento da tuberculose foi realizado por Iland em 1948, partindo do princípio da semelhança desse heterocíclico com a vitamina K, que nos seus estudos apresentou atividade anti-TB (ILAND, 1948). A utilização dessa classe como potenciais agentes anti-TB se baseia na similaridade desse heterocíclico com as estruturas da pirazinamida e isoniazida (fármacos de primeira escolha na terapia da TB).

2. OBJETIVOS

2.1 Objetivo geral

Realizar estudos *in vitro* e *in vivo* de uma nova classe de derivados furoxânicos, benzofuroxânicos e quinoxalínicos para aplicação na terapêutica da Tuberculose.

2.2 Objetivos específicos

2.2.1 Determinação da concentração inibitória mínima (CIM) dos compostos frente à cepa H_{37}Rv sensível (ATCC 27294) em estado ativo;

2.2.2 Determinação da concentração inibitória mínima (CIM) dos compostos frente à cepa H_{37}Rv sensível (ATCC 27294) em estado latente;

2.2.3 Determinação da citotoxicidade (IC_{50}) dos compostos frente às linhagens celulares: J774A.1 (ATCC TIB-67) e MRC-5 (ATCC CCL-171) em 24h;

2.2.4 Determinação da concentração inibitória mínima (CIM) dos compostos frente às cepas monorresistentes rifampin (ATCC 35838); isoniazid (ATCC 35822); streptomycin (ATCC 35820); capreomycin, moxifloxacin and bedaquiline (*University of Illinois at Chicago - Institute for Tuberculosis Research*);

2.2.5 Determinação da concentração inibitória mínima (CIM) dos compostos frente as cepas clínicas resistentes;

2.2.6 Avaliação do potencial mutagênico;

2.2.7 Determinação da atividade intramacrofágica dos compostos frente macrófagos J774A.1 (ATCC TIB-67) infectados com *Mtb* H_{37}Rv sensível (ATCC 27294);

2.2.8 Determinação da concentração bactericida mínima (CBM) dos compostos frente à cepa H_{37}Rv sensível (ATCC 27294);

2.2.9 Determinação da concentração inibitória mínima (CIM) dos compostos frente à cepa H_{37}Rv sensível (ATCC 27294) em estado ativo em três diferentes condições (4% de albumina, 10% SFB, ph ácido);

2.2.10 Avaliação da tolerância máxima e toxicidade em camundongos BALB/c e análise histopatológica de fígado e rins;

2.2.11 Quantificação da atividade enzimática de aspartato aminotransferase (AST) e alanina aminotransferase (ALT) dos camundongos BALB/c;

2.2.12 Quantificação da enzima fosfatase alcalina (FA) no plasma dos camundongos BALB/c;

2.2.13 Quantificação da uréia no plasma dos camundongos BALB/c;

2.2.14 Avaliação da biodisponibilidade oral em camundongos BALB/c;

2.2.15 Infecção dos animais e determinação da eficácia dos compostos em camundongos BALB/c;

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Design, Synthesis and Characterization of *N*-oxide-containing Heterocycles with In vivo Sterilizing Antitubercular Activity

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ABSTRACT

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). The last surveys conducted by the World Health Organization (WHO) described TB as the infectious disease responsible for the largest number of deaths worldwide. Furthermore, an increased number of multidrug-resistant (MDR) and extensively-drug resistant (XDR) strains complicates further this scenario. *N*-oxide derivatives such as furoxan, benzofuroxan and quinoxaline have been previously described as promising scaffolds to be explored in new antitubercular drugs. Herein, twenty-two new *N*-oxide-containing compounds were synthesized and their *in vitro* and *in vivo* antitubercular potential against *Mtb* was evaluated. Moreover, their safety and preliminary mechanism of action was also explored. The compounds demonstrated MIC_{90} values ranging from 0.40 to 62 μM. Among the different heterocycles containing *N*-oxide, the benzofuroxan derivative **8** was the most promising compound, with MIC_{90} values of 1.10 and 6.62 μM against active and non-replicating *Mtb*, respectively. Cytotoxicity tests using MRC-5 cell line showed an IC_{50} value of 519.2 μM. Compound **8** was also active against monoresistant strains. In addition, similarly to rifampicin, a high bactericidalactivity was observed in macrophages infected with *Mtb*. Time-kill experiments with compound **(8)** showed bactericidal effect superior to the antibiotics used in the therapy. Initial mechanism of action studies employing microarray reveal up-regulation of a number of transcripts encoding protein belonging to both small and large subunits of the ribosome, suggesting that compound **(8)** blocks translation. Finally, we comfirmed with *in vivo* experiments that compound **(8)** is safe, orally bioavailable and high effective, reducing

the numbers of *Mtb* to undetected levels in a mouse model of infection. Altogether, these results show that benzofuroxan derivative (**8**) is a novel promising lead compound for the development of a novel chemical class of anti-tubercular drugs.

Keywords: *N*-oxide; furoxan, benzofuroxan, quinoxaline 1,4-di-*N*-oxide; tuberculosis; antituberculosis agents.

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB) in humans, was responsible for the highest number of deaths caused by infectious diseases worldwide in 2015, exceeding even human immunodeficiency virus-caused deaths (HIV). The World Health Organization (WHO) reported 9.6 million new cases and 2 million deaths worldwide in the same year.[1] It was estimated that one third of the world population are infected with the latent form of TB,[2] which treatment is often ineffective due to the lack of drugs that act in the dormant state of the mycobacteria.[3,4]

Furthermore, increased dissemination of multidrug-resistant (MDR), extensively drug-resistant (XDR) and totally drug-resistant (TDR) strains has become a huge challenge to be overcome throughout the world in the fight against TB.[5–8]

For the treatment, WHO recommends a combination of isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and pyrazinamide (PZA) for 6 months. In those cases involving resistance, the treatment can be extended up to 28 months and includes the use of second-line drugs, such as fluoroquinolones, aminoglycosides, D-cycloserine (DCS), linezolid (LZD) among others.[9–11] The current treatment presents several limitations,

including prolonged standard regimen, high rate of treatment discontinuation, adverse effects, toxicity, drug-drug interactions and lack of effectiveness against the latent mycobacteria.[2,12–16]

Over the past few years, limited but important progress in the development of drug candidates against TB was achieved. After a gap of more than 50 years without new drugs approved for TB, the United States Food and Drug Administration (FDA) approved bedaquiline (Bdq) (SIRTURO®; Janssen, Beerse, Belgium) in 2012 for the treatment of MDR-TB. Advances in the development of other compounds with potent antitubercular activity have been reported in the literature in the last five years[17–20] and several drug candidates were moved toward clinical trials, such as Q203, sutezolid (PNU-100480), posizolid (AZD-5847), delamanid (OPC-67683) and pretomanid (PA-824).[21,22] Nevertheless, *Mtb* strains resistant to these new compounds have already been reported,[23–25] reinforcing the urgency to develop a larger number of novel drugs for TB treatment.[9]

We have previously reported a series of furoxan derivatives with potent activity against *Mtb*, including MDR strains. Specifically, the compound (*E*)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**I**) **(Figure 1)** was able to inhibit 90% of *Mtb* H37Rv growth at 1 μM. In addition, we have characterized that the antitubercular activity of this furoxan derivative is related to its ability in generate nitric oxide (NO) after biotransformation.[26]

Motivated by preliminary promising results obtained with the furoxan derivatives, we designed new heterocycles analogous containing the *N*-oxide subunit including: amide-furoxan (series 1), benzofuroxan (series 2) and quinoxaline 1,4-di-*N*-oxide

(QdNO) (series 3) **(Figure 1)**. Furoxan, benzofuroxan and QdNO derivatives represent important scaffolds in medicinal chemistry due to their wide spectrum of biological activities,[27] including anti-tubercular activity.[28] The antimycobacterial activity of these compounds is thought to be related to reactive oxygen species (ROS) formed after their biotransformation.[29–31] Specifically for quinoxalines, it was reported that these compounds lead to an increased levels of ROS under hypoxic conditions, which could have interesting properties against latent-TB.[32–34]

ROS play an important role in the TB pathogenesis. Several studies have demonstrated the relationship between levels of ROS produced by immune cells and the susceptibility of patients to several species of the *Mycobacterium* genus.[35,36] Furthermore, ROS effects during TB are many-fold. For Example, increased ROS levels can inhibition of *Mtb* growth, damage to cellular components such as lipids, proteins and nucleic acids, and lead to macrophage inflammatory activity.[37–40] High levels of ROS can also induce macrophage apoptosis and prevent the growth and replication of the bacilli.[41] Therefore, the desing of new compounds that could act by the increasing the levels of ROS and perturbing mycobacterial redox homeostasis seems to be a promising strategy for the discovery of new agentes against TB.[42–44]

In a continuing effort to develop new drug candidates to TB treatment, we described herein the design, synthesis and biological activities of a series of heterocycles containing *N*-oxide designed as anti-tubercular compounds.

(Figure 1)

CHEMISTRY

Twenty-two new compounds containing the *N*-oxide subunit were prepared according to the synthetic methodologies presented in **Scheme 1**, **2** and **3**.

The synthesis of the amide-furoxan derivatives (**1, 2**) was achieved according to previously described methods.[45,46] The furoxan derivative (**2**) was then reacted with 2-, 3- or 4-hydroxybenzaldehyde in dichloromethane medium, using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base, leading to formation of the furoxan derivatives containing an aldehyde group (**3a-c**).[47] Then, a condensation reaction of the aldehyde derivatives with isonicotinohydrazide was carried out in ethanolic medium catalyzed by acid to provide the hybrid furoxan derivatives (**4a-c**) **(Scheme 1)**.

(Scheme 1)

The benzofuroxan derivative containing an aldehyde group (**7**) was obtained according to a previously reported methodology.[48] This compound (**7**) was reacted with different aromatic hydrazides through the same condensation reaction described above leading to formation of benzofuroxan derivatives (**8-17**) **(Scheme 2)**. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of these compounds showed protons and carbons' signals from the benzofuroxan nucleus as broad peaks, indicating a benzofuroxan tautomerism.[27,31]

(Scheme 2)

The dioxolan-benzofuroxan derivative (**18**) was obtained from the reaction between compound (**7**) and ethylene glycol.[49] Quinoxaline derivatives were obtained through a variation of the Beirut reaction,[50–52] wherein the dioxolan-benzofuroxan (**18**) was reacted with the appropriate nitrile derivatives in dichloromethane medium and potassium carbonate (K_2CO_3) as catalyst[53] leading to the formation of quinoxaline derivatives (**19-26**). Compound (**28**) was synthesized from quinoxaline (**19**), which was submitted to a cyclic acetal hydrolysis and then, reacted with isonicotinohydrazide through a condensation reaction **(Scheme 3)**.[54]

(Scheme 3)

All compounds were characterized by elemental analysis, infrared (IR) spectroscopy and ^{1}H and ^{13}C NMR. Furthermore, all compounds were analyzed by HPLC and their purity was confirmed to be superior to 98.5%. Experimental logP values and melting points were determined for all final compounds.

BIOLOGICAL STUDIES

Activity against *Mycobacterium tuberculosis*

Antitubercular activity of compounds containing *N*-oxide (**4a-c**, **8-17**, **19-26** and **28**) was performed using *Mtb* $H_{37}Rv$ ATCC 27294. A resazurin microtiter assay (REMA) method was employed as previously described.[26,55] The results were expressed as minimum inhibitory concentration (MIC_{90}) and compounds showing MIC_{90} values below 10 μM were characterized further. Evaluation of potential cytotoxicity was carried out

using MRC-5 cell line according to previously reported methodology[26,56] and the results were expressed as IC_{50} values. The selectivity index (SI) of the tested compounds was calculated through the ratio between IC_{50} and MIC_{90}. Compounds that showed SI $\geq$ 10 [57] were considered promising for further studies according to the cut-off established.[56] Potential anaerobic activity of best compounds was evaluated using the method described by Cho *et al.*, 2007.[58]

The spectrum of activity was performed through determination of MIC_{90} values against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) by measuring optical density (OD) at 570 nm (OD_{570}) after 16h and against *Candida albicans* (ATCC 10231) at OD_{570} nm after 48h.

Compounds were also tested against *Mtb* H_{37}Rv isogenic strains monoresistant to RMP (ATCC 35838); INH (ATCC 35822); streptomycin (SM) (ATCC 35820); capreomycin (CAP), moxifloxacin (MOX) and BDQ (strains from *University of Illinois at Chicago - Institute for Tuberculosis Research*), by microdiluition technique MABA.[59] After this step, we selected the best compound (compound **8**) for further studies.

Due to the ability of *Mtb* survive inside of macrophages we have investigated if compound (**8**) is able to inhibit growth of *Mtb* H37Rv in J774A.1 macrophages.

Different conditions of media culture were also tested by MABA: a) adjusting the culture medium for pH 6.0; b) including 4% BSA (bovine serum albumin) and; c) supplementation of 10% FBS (fetal bovine serum). The slightly acidic pH (pH 6) was chosen as *Mtb* experience acid pH when *Mtb*-containing phagosomes fused with lysozomes and acidify. Albumin is synthesized in the liver and one of its physiological functions is the transport of poorly soluble molecules of both endogenous and exogenous.

Origin.[60] Albumin-binding is a important pharmacological parameter that affects antibiotic action in humans.[61] FBS is used as it is presented in a number of conditions utilized to growth mammalian cells and might interfere with antitubercular action of some compounds.

Time-kill experiments were performed for up to 15 days to evaluate the bactericidal profile of compound (**8**).

In order to obtain an unbiased view of compound (**8**) action, microarray analysis was performed. Microarrays have been used to successful define mechanism-of-action (MOA) of antitubercular compouds.[62]

Mouse infection treatment experiments

In order to ensure greather stability and improve solubility, compound **(8)** was evaluated *in vivo* assays using pharmaceutical formulation. The nanostructured lipid system (ME) was prepared as described by us previously,[63] with the following composition: 10% oil phase (cholesterol), 10% surfactant (a mixture of soy phosphatidylcholine, sodium oleate and Eumulgin® HRE 40 (polyoxyl 40 castor oil - hydrogenated) 3: 6: 8) and 80% aqueous phase (phosphate buffer pH 7.4). The compounds studied were incorporated at the desired concentration for the *in vivo* experiments by mass solubilization at the respective volume and sonicated for 3 minutes in the batch mode and 15% of amplitude.

ME containing compound **(8)** was tested for tolerability and oral bioavailability as well treatment of female BALB/C infected with *Mtb*.

RESULTS AND DISCUSSION

*Mtb´*s ability to remain dormant is likely a major factor precluding sterilization with antibiotic therapy and promoting the development of antibiotic resistance.[64,65] An ideal drug should: (i) reduce the duration of treatment; (ii) be active against resistant strains; (iii) not interfere with other TB drugs and antiretrovirals; and (iv) be active against 'dormant' bacilli.[11]

Recently, we have identified the compound (*E*)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**I**) as a promising antitubercular drug candidate.[26] In order to optimize its antimycobacterial activity, we have designed novel heterocycles containing *N*-oxide analogues of compound (**I**), which comprises the following heterocyclic moieties: amide-furoxan (series 1, **4a-c**), benzofuroxan (series 2, **8**-**17**) and quinoxaline (series 3, **19**-**26**, **28**). The amide-furoxan derivatives (**4a-c**) were selected aiming to evaluate whether the replacement of the phenylsulfonyl group from compound (**I**) by an amide group would increase the antitubercular activity and/or decrease the cytotoxicity. The three amide-furoxan regioisomers (**4a-c**) presented an improved antitubercular activity than the parent compound (**I**).[26] Compounds from series 1 exhibited MIC_{90} values around 0.4 μM against actively growing *Mtb* $H_{37}Rv$ strains **(Table 1)**. Although the antitubercular activity of these compounds was originally attributed to potential NO release, we believe this is no longer the case, as low submicromolar concentrations of NO generated should not alter *Mtb* growth. An alternative mechanism is proposed below.

The benzofuroxan moiety (series 2) (**8**-**17**) was selected due to its ability to generate ROS after metabolism.[30] The structural design of this series was based on the

724 isosteric replacement of substituents attached at *para* position on the phenyl ring. Several
725 group substituents were used, such as hydrogen, nitro, *tert*-butyl, amino and hydroxyl
726 group. Furthermore, we also evaluated the substitution of the phenyl ring by a pyridine
727 ring, since this heterocyclic ring is present in the structure of several antituberculosis
728 drugs and bioactive compounds, such as INH, BDQ and ethionamide.[66] The replacement
729 of the phenyl ring of compound (**9**) by a pyridine ring in compound (**8**) led to a seven-
730 fold increase in the antituberculosis activity (MIC_{90} = 1.1 μM) **(Table 1)**.

731 Among the benzofuroxan series, we have identified compound (*E*)-6-((2-
732 isonicotinoylhydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (**8**) as the lead
733 benzofuroxan derivative with a MIC_{90} value of 1.1 μM (for actively growing *Mtb*); and
734 MIC_{90} value of 6.6 μM (for dormant *Mtb*). This small difference in MIC_{90} is an attractive
735 characteristic, as nearly equimolar effects against replicating and non-replicating *Mtb* are
736 rarely seen and highly desired.

737 Moreover, our data reveals that bulky groups such as *tert*-butyl (**10**) (MIC_{90} = 3.9
738 μM) and electron withdrawing groups such as nitro (**11**) (MIC_{90} = 5.3 μM) led to an
739 improvement in antitubercular activity. Compound (**9**), un-substituted phenyl, presented a
740 MIC_{90} value of 8.3 μM, while the hydroxyl regioisomers (**12**-**14**) presented MIC_{90} values
741 greater than 62 μM. Amino derivatives (**15**-**17**) also exhibited reduction in the
742 antitubercular activity in comparison to the non-substituted compounds (**9**); however,
743 those compounds have shown MIC_{90} values higher than those of hydroxyl substitution. It
744 was found MIC_{90} values ranging from 12.3 to 27.9 μM **(Table 1)**.

745 Concerning the quinoxaline 1,4-di-*N*-oxide series, we have evaluated the
746 influence of electron withdrawing and electron donating groups in the phenyl ring

regarding its antitubercular activity. We also have carried out an isosteric substitution of the phenyl ring by a furyl (**25**) and thenyl (**26**) moieties. For quinoxaline-phenyl derivatives (**19**-**24**), the MIC_{90} values ranged from 12.0 to 30.8 μM. The presence of substitution at *para* position of the phenyl ring seems to contribute to antituberculosis activity, since compound (**19**) that has no substituents and displays the lower potency among the phenyl quinoxaline derivatives (MIC_{90} = 30.8 μM). On the other hand, compound (**22**), which has a methoxyl group in *para* position, showed the lowest MIC_{90} value among the phenyl quinoxaline series. Nevertheless, we could not observe a clear and accurate structure-activity relationship regarding the electronic properties of the substituents, which supports the idea that NO release is not the mechanism.

For compounds (**25**, **26**), the isosteric replacement of the phenyl by a furyl or thenyl ring led to a significant increase in the antitubercular activity. Compounds (**25**) and (**26**) exhibited MIC_{90} values of 5.2 and 12.1 μM, respectively. Furthermore, we also have synthesized a quinoxaline derivative containing a *N*-acylhydrazone subunit (**28**) and the antitubercular activity of this compound decreased in comparison to the previous quinoxalines showing MIC_{90} value of 39.7 μM.

We also evaluated the cytotoxicity of all final compounds against MRC-5 cell lines. This cell line is from health tissue and derived from human lung fibroblast and is widely used for phenotypic screening in antitubercular drug discovery.[67–69]

The data obtained in cytotoxicity studies of the amide-furoxan series (**4a-c**) indicates high selectivity against *Mtb*. All three regioisomers exhibit high IC_{50} values (> 854.0 μM), thereby leading to high SI values, which ranged from 2033 to 3205 **(Table 1)**.

769 Regarding the cytotoxicity studies of the benzofuroxan series, we observed IC_{50}
770 values ranging from 25 to 841 μM in MRC-5 cell lines. The most active benzofuroxan
771 derivative (**8**) presented IC_{50} value of 519 μM; however, compound (**17**) was the less
772 cytotoxic among the benzofuroxan series with IC_{50} of 841 μM. Compounds (**11-14)** did
773 not show promising antitubercular activity (MIC_{90} > 62 μM) and therefore they were not
774 included in cytotoxicity studies. Compound (**8**) was selected for further experiments, as it
775 possessed the highest potency against *Mtb* and the lowest cytotoxicity.
776 Compounds from quinoxaline series were significantly more cytotoxic in MRC-5
777 cells. The IC_{50} values for this series ranged from 13 to 67 μM, resulting in low SI (< 6.8)
778 values, making them less attractive as leads for antitubercular agents.[56]
779 The lipophilicity, usually expressed as logP, is related to the ability of a drug to
780 cross cell membranes by passive diffusion and it is one of the standard features identified
781 by Lipinski at the "Rule of 5" for drug-like molecules.[70,71] It is well established that
782 permeability of molecules through the cell wall of *Mtb* is a major limitation in the early
783 antitubercular drug discovery process, considering the constitution of this cell wall, which
784 is composed mainly by mycolic acids of high molecular weight, making it an efficient
785 barrier to hydrophilic molecules.[21,72] Thus, this psychochemical property plays an
786 important role in the early stages of anti-TB drug discovery, the higher logP, more
787 lipophilic. In this work, three amide furoxan regioisomers presented experimental logP
788 values of 1.3. In the benzofuroxan series, the logP values found ranged from 0.9 to 3.8.
789 The quinoxaline series display the logP values between 0.7 to 2.2 **(Table 1)**.
790
791 **(Table 1)**

(Table 2)

Antimicrobials are classified as possessing narrow, intermediate or broad spectrum of activity. Tuberculosis is a chronic infection where it is desired a drug with narrow spectrum, as example of INH. All amide-furoxan and benzofuroxan (**8, 11, 14** and **17**) derivatives were tested against *S. aureus*, *E. coli* and *C. albicans*. No antimicrobial activity was detected up to the maximum concentration of 200 μM.

In terms of cross-resistance, compound **(8)** showed equipotent activity (< 2-fold change in MIC_{90}) against all drug sensitive and monoresistant strains of *Mtb* tested suggesting a novel mechanism-of-action or that compound **(8)** inhibits a shared target in a distinct binding site **(Table 3)**. In contrast, amide-furoxan series (**4a-c**) were not actived against half of monoresistant strains tested and therefore they were not selected for further studies.

Microarray analysis of *Mtb* treated with compound (**8**) or vehicle control revealed a significant up-regulation in the vast majority of ribosomal genes as well as all genes encoding subunits of the ATP synthase **(Figure 2** and **Supplementary Table S1)**. This also includes EF-G, which induces GTP-dependent translocation of nascent peptide chains from the A- to the P-site in the ribosome and EF-Tu, which promotes GTP-dependent binding of aminoacylated tRNAs to the A-site in ribosomes. No up-regulation of heat shock proteins was observed. In fact, down-regulation of hsp, htrA and hspR was observed **(Supplementary Table S1)**. Similarly, up-regulation of ribosomal proteins has been observed when *Mtb* was treated with inhibitors of protein synthesis. Our data

indicates that a large number of operons as well as single genes encoding ribosomal proteins are up-regulated, which in turn indicates that compound (**8**) could affect protein synthesis by inhibiting the ribosome. Based on the work of Boshoff and coworkers (2004),[62] protein synthesis inhibitors can be further divided into two classes based on their effect on heat shock protein expression (either no effect or up-regulation). Compound (**8**) did not increase the abundance of transcripts encoding heat shock proteins, and in fact, it decreased levels of three transcripts (*hsp*, *htrA* and *hspR*). Regarding protein synthesis inhibition, these results indicate that compound (**8**) behaves more like an inhibitor of initiation, such as tetracyclines, rather than an inhibitor of protein synthesis that leads to mis-translation, such as aminoglycosides. Futher studies are required to define the exact mechanism-of-action of compound (**8**) and its binding site and inhibition of the *Mtb* ribosome and/or to ATP synthase, or other targets.

(Figure 2)

Using macrophage cell line J774A.1 we observed that compound **(8)** exhibited high intracellular inhibition in all concentrations tested (around 90%). Just like RMP, we could not verified dose-dependent inhibiton among the different concentrations **(Figure 3)**.

(Figure 3)

In time-kill kinetics, we observed that compound **(8)** is not only bactericidal, but also presented an early bactericidal effect. Noteworthy, compound **(8)** was able to sterilize cultures after 48 hours exposure (ca. of 6.7 log10) **(Figure 4)**.

(Figure 4)

While vehicle controls (CMC and ME) did not display any inhitory activity compound **(8)** inhibit *Mtb* growth on mice plasma **(Table 4)**.

(Table 4)

For toxicology studies, mice were monitored daily for 10 days, receiveing one daily oral dose (by gavage; 200 mg/kg body weight) and the behavior parameters (hippocratic screening) were evaluated. No significant variation in behavior was observed during the period of 10 days. Changes in organs weight (heart, lungs, spleen, kidneys and liver) was evaluated using analysis of variance (ANOVA) and Dunnett's test, establishing $P < 0.05$ as significance level. No statistically significant difference was observed between the drug-treated and control groups. To probe potential liver damage, levels of liver transaminases were tested in plasma. No significant differences were observed for alanine aminotransferase **(Figure 5a)**, aspartate aminotransferase **(Figure 5b)** and alkaline phosphatase **(Figure 5c)** between treated and control groups. In order to access potential changes in kidney function we evaluated levels of urea in blood samples. A significant

difference was observed for the group treated with RMP-ME, when compared to control group **(Figure 5d)**.

(Figure 5)

Histology of liver **(Figure 6)** and kidneys **(Figure 7)** reveals similar morphology in all groups indicate that no gross abnormalities were caused by treatments

(Figure 6)

(Figure 7)

In order to evaluate the effect of compound (**8**), we infected mice with *Mtb* H37Rv and subjected the infected animals to treatment with compound (**8**) or vehicle. Noteworthy, no CFUs were recovered from homogeneized lungs after the treatment **(Figure 8)**. Lower dilutions were plated in order to confirm this potential sterizing activity and again no CFUs were recovered from lung dilution 1:100 to 1:100,00. Control experiments with vehicles, RPM and RPM-CE behaved as expected **(Figure 8)**.

(Figure 8)

CONCLUSIONS

Twenty-two new *N*-oxide containing compounds were synthesized and their in vitro and in vivo antitubercular activity against *Mtb* were evaluated. The amide-furoxan series (**4a-c**) were the most promising compounds with MIC_{90} values around 0.40 μM against actively replicating *Mtb* and SI values ranging from 2033.3 to 3204.7. The benzofuroxan series (**8**-**17**) also presented promising antitubercular activity, especially compound (**8**), which presented MIC_{90} value of 1.1 μM against actively growing *Mtb* and 6.6 μM against non-replicating Mtb. Compound (**8**) also displayed high activity in the macrophage model of infection. In addition, in vivo studies employing the mouse model of infection showed sterilizing activity for compound (**8**). No detectable Mtb was observed in lungs lungs of mice treated, while controls displayed expected number of CFUs. This activity was superior to the activity of rifampicin. Altogether, these findings highlight the benzofuroxan derivative (**8**) as a novel lead compound for antitubercular drug design with sterilizing activity superior to rifampcine in the mouse model of infection.

EXPERIMENTAL SECTION

Chemistry

Melting points (mp) were measured using an electrothermal melting point apparatus (SMP3; Bibby Stuart Scientific) or in a Mettler FP82+FP80 apparatus (Greifense, Switzerland). Infrared spectroscopy (KBr disc) were performed on a FTIR-8300 Shimadzu or a Nicolet Nexu FTIR Thermo® spectrometer, and the frequencies are expressed in cm^{-1}. The NMR for ^{1}H and ^{13}C of all compounds were performed on a Bruker Fourier with Dual probe $^{13}C/^{1}H$ (300-MHz) NMR spectrometer or Bruker 400 Ultrashield^{TM} $^{13}C/^{1}H$ (400-MHz) NMR spectrometer using deuterated chloroform

904 ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6) as solvent and the chemical shifts were
905 expressed in parts per million (ppm) relative to tetramethylsilane. The signal
906 multiplicities are reported as singlet (s), doublet (d), doublet of doublet (dd), and
907 multiplet (m). Elemental microanalysis (C, H and N) was performed on a Perkin-Elmer
908 model 2400 analyzer or a CHN-900 Elemental Analyzer (LECO, Tres Cantos, Spain) and
909 the data were within ± 0.4% of the theoretical values. The compounds were purified on a
910 chromatography column with silica gel (60 Å pore size, 35-75-µm particle size) and the
911 following solvents were used as mobile phase: methanol, ethyl acetate, dichloromethane,
912 hexane and petroleum ether in a flow rate of approximately 20 mL/min. The reaction
913 progress of all compounds was monitored by thin-layer chromatography (TLC), which
914 was performed on 2.0- by 6.0-cm^2 aluminum sheets precoated with silica gel 60 (HF-254;
915 Merck) to a thickness of 0.25 mm and revealed under UV light (265 nm). All compounds
916 were analyzed by HPLC, and their purity was confirmed to be greater than 98.5%. HPLC
917 conditions: Shimadzu HPLC model CBM 20-A (Shimadzu®) equipped with UV-VIS
918 detector (model SPD-20A), quaternary pumping system mobile phase (model LC-20AT),
919 solvent degasser (model DGU-20As) and a Agilent® Eclipse XDB C-18 column (250mm
920 x 27 4,6mm; 5µm). For HPLC method it was used an isocratic flow [methanol:water
921 (75:25)]. Reagents and solvents were purchased from commercial suppliers. Compounds
922 **3a-c**,[45–47] **7**,[48] **18**, **19** and **28**[54] were prepared according to previously described methods.
923

924 **General procedure for the synthesis of compounds 20-26**

925 Compound (**18**) (0.3 g; 1.06 mmol) was dissolved in dichloromethane (15 mL) and then
926 cooled by placing it on ice batch. Next, was added the appropriate nitrile (0.15 g; 1.06

mmol) and potassium carbonate (0.18g; 1.32 mmol) in small portions. The reaction mixture was stirred at 40 ºC for 96 hours. After the reaction time, the solvent was evaporated under reduced pressure and the obtained solid was dissolved in 50 mL of ethyl acetate and washed with water. The organic phase was dried with anhydrous magnesium sulfate and the solvent was evaporated giving a yellow solid. The obtained solid was purified by silica gel column chromatography using hexane and ethyl acetate (70:30 v/v) as eluent to give the appropriate compound **20-26** with variable yields.

3-cyano-6-(1,3-dioxolan-2-yl)-2-(p-tolyl)quinoxaline 1,4-dioxide (20). Yellow powder; yield, 9%; mp, 179 to 180 °C. IR V_{max} (cm^{-1}; KBr pellets): 3090 (C-H aromatic), 2233 (CN nitrile), 1328 (N-O), 1073 (C-O ether). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) δ: 8.55 (2H; t; *J* = 18.7 Hz), 8.12 (1H; d; *J* = 8.8 Hz), 7.63 (2H; d; *J* = 8.0 Hz), 7.43 (2H; d; *J* = 7.9 Hz), 6.10 (1H; s), 4.09 (4H; m), 2.43 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 143.1, 143.0, 141.1, 136.7, 132.2, 130.1, 129.2, 129.1, 124.7, 121.0, 117.7, 101.1, 65.3, 21.1 ppm. Anal. Calcd. (%) for $C_{19}H_{15}N_3O_4$: C: 65.32; H: 4.33; N: 12.03. Found: C: 65.36; H: 4.32; N: 12.05.

2-(4-chlorophenyl)-3-cyano-6-(1,3-dioxolan-2-yl)quinoxaline 1,4-dioxide (21). Yellow powder; yield, 13%; mp, 177 to 178 °C. IR V_{max} (cm^{-1}; KBr pellets): 3091 (C-H aromatic), 2235 (CN nitrile), 1330 (N-O), 1093 (C-O ether). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) δ: 8.56 (2H; t; *J* = 16.0 Hz), 8.12 (1H; dd; *J* = 26.5 Hz), 7.75 (4H; q; *J* = 25.4 Hz), 6.11 (1H; s), 4.09 (4H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 144.8, 142.1, 138.8, 137.1, 136.0, 132.1, 130.8, 128.8, 126.5, 121.1, 117.7, 65.3 ppm.

Anal. Calcd. (%) for $C_{18}H_{12}ClN_3O_4$: C: 58.47; H: 3.27; N: 11.36. Found: C: 58.67; H: 3.36; N: 11.53.

3-cyano-6-(1,3-dioxolan-2-yl)-2-(4-methoxyphenyl)quinoxaline 1,4-dioxide (22). Yellow powder; yield, 26%; mp, 151 to 152 °C. IR V_{max} (cm^{-1}; KBr pellets): 3085 (C-H aromatic), 2236 (CN nitrile), 1335 (N-O), 1095 (C-O ether). ^1H NMR (400 MHz, DMSO-d_6, δ ppm) δ: 8.55 (2H; m), 8.05 (1H; dd), 7.71 (2H; d; J = 8.8 Hz), 7.17 (2H; d; J = 8.7 Hz), 6.09 (1H; s), 4.08 (4H; m), 3.87 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 161.2, 144.6, 142.9, 138.8, 132.4, 132.0, 120.6, 119.3, 118.0, 117.7, 113.9, 101.1, 65.2, 55.4 ppm. Anal. Calcd. (%) for $C_{19}H_{15}N_3O_5$: C: 62.46; H: 4.14; N: 11.50. Found: C: 62.51; H: 4.15; N: 11.52.

3-cyano-6-(1,3-dioxolan-2-yl)-2-(4-fluorophenyl)quinoxaline 1,4-dioxide (23). Yellow powder; yield, 30%; mp, 164 to 165 °C. IR V_{max} (cm^{-1}; KBr pellets): 3077 (C-H aromatic), 2234 (CN nitrile), 1330 (N-O), 1097 (C-O ether). ^1H NMR (400 MHz, DMSO-d_6, δ ppm) δ: 8.56 (2H; t; J = 17.5 Hz), 8.15 (1H; d; J = 10.4 Hz), 7.81 (2H; t; J = 14.1 Hz), 7.49 (2H; t; J = 17.7 Hz), 6.10 (1H; s), 4.09 (4H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 162.2, 143.2, 142.3, 139.1, 136.7, 133.0, 132.9, 132.3, 124.0, 121.0, 117.7, 115.7, 101.0, 65.3 ppm. Anal. Calcd. (%) for $C_{18}H_{12}FN_3O_4$: C: 61.19; H: 3.42; N: 11.89. Found: C: 61.18; H: 3.41; N: 11.90.

3-cyano-6-(1,3-dioxolan-2-yl)-2-(4-(trifluoromethoxy)phenyl)quinoxaline 1,4-dioxide (24). Yellow powder; yield, 3%; mp, 186 to 187 °C. IR V_{max} (cm^{-1}; KBr pellets): 3077

(C-H aromatic), 2236 (CN nitrile), 1333 (N-O), 1165 (C-O ether). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) δ: 8.57 (2H; t; J = 15.4 Hz), 8.15 (1H; d; J = 8.9 Hz), 7.89 (2H; d; J = 8.7 Hz), 7.66 (2H; d; J = 8.4 Hz), 6.11 (1H; s), 4.10 (4H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 149.9, 143.3, 141.9, 139.1, 136.8, 132.7, 132.4, 131.0, 126.7, 121.3, 121.0, 120.9, 117.7, 101.0, 65.3 ppm. Anal. Calcd. (%) for $C_{19}H_{12}F_3N_3O_5$: C: 54.42; H: 2.88; N: 10.02. Found: C: 54.47; H: 2.88; N: 10.06.

3-cyano-6-(1,3-dioxolan-2-yl)-2-(furan-2-yl)quinoxaline 1,4-dioxide (25). Orange powder; yield, 26%; mp, 177 to 179 °C. IR V_{max} (cm^{-1}; KBr pellets): 3087 (C-H aromatic), 2240 (CN nitrile), 1355 (N-O), 1107 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 8.57 (1H; dd; J = 5.2, 3.6 Hz), 8.49 (1H; d; J = 8.9 Hz), 8.24 (2H; d; J = 3.5 Hz), 8.01 (1H; dd; J = 8.9, 1.6 Hz), 6.95 (1H; dd; J = 3.6, 1.8 Hz), 6.08 (1H; d; J = 3.1 Hz), 4.09 (4H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 146.8, 144.7, 142.3, 140.2, 138.5, 135.6, 133.4, 132.3, 130.0, 120.9, 117.7, 116.8, 113.2, 111.1, 101.2, 65.3 ppm. Anal. Calcd. (%) for $C_{16}H_{11}N_3O_5$: C: 59.08; H: 3.41; N: 12.92. Found: C: 59.12; H: 3.42; N: 12.94.

3-cyano-6-(1,3-dioxolan-2-yl)-2-(thiophen-2-yl)quinoxaline 1,4-dioxide (26). Brown powder; yield, 15%; mp, 171 to 173 °C. IR V_{max} (cm^{-1}; KBr pellets): 3092 (C-H aromatic), 2241 (CN nitrile), 1357 (N-O), 1084 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 8.60 (1H; dd; J = 5.3, 3.6 Hz), 8.51 (1H; d; J = 8.8 Hz), 8.45 (1H; dt; J = 4.2, 1.2 Hz), 8.13 (1H; dd; J = 5.1, 1.0 Hz), 8.03 (1H; dd; J = 8.9, 1.7 Hz), 7.43 (1H; dd; J = 5.0, 4.2 Hz), 6.09 (1H; d; J = 3.8 Hz), 4.09 (4H; m) ppm. ^{13}C NMR (75

MHz, DMSO-d_6, δ ppm) δ: 145.1, 142.5, 138.1, 137.7, 136.8, 135.6, 132.5, 130.2, 127.0, 126.0, 120.7, 117.9, 112.6, 101.2, 65.1 ppm. Anal. Calcd. (%) for $C_{16}H_{11}N_3O_4S$: C: 56.30; H: 3.25; N: 12.31. Found: C: 56.31; H: 3.25; N: 12.30.

General procedure for the synthesis of compounds 4a-c and 8-17

A solution of compound **3a-c** or **7** (0.87 mmol) in 10 mL of ethanol and 3 drops of glacial acetic acid was stirred at room temperature for 20 min. Next, the appropriate aromatic hydrazide (0.106 g, 0.87 mmol) was added and the mixture was stirred for 12 h. The solvent was concentrated under reduced pressure and ice was added in order to precipitate the desired products. If necessary, the samples could be further purified by silica gel column chromatography using ethyl acetate-methanol (98:2 v/v) as eluent to give the appropriate compound **4a-c**, and **8-17** with variable yields.

(*E*)-3-carbamoyl-4-(2-((2-isonicotinoylhydrazono)methyl)phenoxy)-1,2,5-oxadiazole 2-oxide (4a). White powder; yield, 51%; mp, 226 to 229°C. IR V_{max} (cm^{-1}; KBr pellets): 3433 (NH_2 amide), 3203 (N-H), 1669 (C=O amide), 1587 (C=N imine), 1456 (N-O furoxan), 1344 (C-N aromatic), 1145 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.10 (1H; s; N-H), 8.79 (2H; d;), 8.51 (1H; s), 7.91 (1H; dd), 7.84 (2H; d), 7.46 (1H; m), 7.25 (1H, d), 7.11 (1H, t), 5.54 (2H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 161.55, 156.36, 155.65, 155.24, 150.30, 144.04, 140.47, 131.84, 125.85, 122.59, 121.87, 121.59, 113.36, 110.51 ppm. Anal. Calcd. (%) for $C_{16}H_{12}N_6O_5$: C: 52.18; H: 3.28; N: 22.82. Found: C: 52.25; H: 3.29; N: 22.90.

(*E*)-3-carbamoyl-4-(3-((2-isonicotinoylhydrazono)methyl)phenoxy)-1,2,5-oxadiazole 2-oxide (4b). White powder; yield, 59%; mp, 159 to 163°C. IR V_{max} (cm^{-1}; KBr pellets): 3439 (NH_2 amide), 3201 (N-H), 1712 (C=O amide), 1591 (C=N imine), 1485 (N-O furoxan), 1327 (C-N aromatic), 1147 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.09 (1H; s; N-H), 8.78 (2H; d), 8.46 (1H; s), 7.81 (3H; d), 7.41 (3H; m), 5.48 (2H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 156.96, 150.22, 147.97, 147.44, 142.63, 140.97, 132.70, 127.87, 122.48, 113.82, 113.14, 109.30, 105.24, 102.74 ppm. Anal. Calcd. (%) for $C_{16}H_{12}N_6O_5$: C: 52.18; H: 3.28; N: 22.82. Found: C: 52.27; H: 3.29; N: 22.90.

(*E*)-3-carbamoyl-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-1,2,5-oxadiazole 2-oxide (4c). White powder; yield, 67%; mp, 235 to 239°C. IR V_{max} (cm^{-1}; KBr pellets): 3.367 (NH_2 amide), 3255 (N-H), 1708 (C=O amide), 1612 (C=N imine), 1413 (N-O furoxan), 1255 (C-N aromatic), 1122 (C-O ether). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 11.95 (1H; s; N-H), 8.77 (2H; d;), 8.43 (1H; s), 7.80 (2H; d), 7.71 (2H; d), 7.14 (2H; d), 5.48 (2H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 162.96, 162.33, 159.91, 156.34, 150.86, 149.52, 141.14, 129.65, 128.08, 122.14, 115.84, 111.05 ppm. Anal. Calcd. (%) for $C_{16}H_{12}N_6O_5$: C: 52.18; H: 3.28; N: 22.82. Found: C: 52.20; H: 3.28; N: 22.84.

(*E*)-6-((2-isonicotinoylhydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (8). Yellow powder; yield, 85%; mp, 230 to 232 °C. IR V_{max} (cm^{-1}; KBr pellets): 3324 (N-H), 3045 (NH_2 amide), 1690 (C=O amide), 1600 (C=N imine), 1409 (N-O furoxan), 1273 (C-

N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.44 (1H; s), 8.80 (2H; d; J = 5.7 Hz), 8.51 (1H; s), 8.03 (3H; m), 7.84 (2H; d; J = 6.0 Hz) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 162.4, 150.6, 146.6, 140.2, 133.7, 130.8, 130.4, 130.1, 129.4, 121.8, 114.3 ppm. Anal. Calcd. (%) for $C_{13}H_9N_5O_3$: C: 55.13; H: 3.20; N: 24.73. Found: C: 55.22; H: 3.21; N: 24.78.

(*E*)-6-((2-benzoylhydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (9). Yellow powder; yield, 80%; mp, 220 to 221 °C. IR V_{max} (cm^{-1}; KBr pellets): 3389 (N-H), 3041 (NH_2 amide), 1641 (C=O amide), 1533 (C=N imine), 1487 (N-O furoxan), 1300 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.24 (1H; s), 8.52 (1H; s), 7.94 (5H; m), 7.58 (3H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 163.4, 145.0, 132.9, 132.1, 128.5, 127.8, 118.4, 113.5 ppm. Anal. Calcd. (%) for $C_{14}H_{10}N_4O_3$: C: 59.57; H: 3.57; N: 19.85. Found: C: 59.65; H: 3.56; N: 19.88.

(*E*)-6-((2-(4-(tert-butyl)benzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (10). Yellow powder; yield, 89%; mp, 189 to 191 °C. IR V_{max} (cm^{-1}; KBr pellets): 3341 (N-H), 3068 (NH_2 amide), 1659 (C=O amide), 1532 (C=N imine), 1486 (N-O furoxan), 1287 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.16 (1H; s), 8.51 (1H; s), 7.93 (4H; m), 7.56 (3H; d; J = 8.4 Hz), 1.32 (9H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 160.3, 155.0, 144.7, 130.2, 127.7, 125.3, 118.3, 113.3, 56.0, 34.8, 30.9 ppm. Anal. Calcd. (%) for $C_{18}H_{18}N_4O_3$: C: 63.89; H: 5.36; N: 16.56. Found: C: 63.95; H: 5.37; N: 16.59.

(*E*)-6-((2-(4-nitrobenzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (11). Yellow powder; yield, 78%; mp, 261 to 262 °C. IR V_{max} (cm^{-1}; KBr pellets): 3373 (N-H), 3073 (NH_2 amide), 1673 (C=O amide), 1525 (C=N imine), 1400 (N-O furoxan), 1357 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.50 (1H; s), 8.52 (1H; s), 8.39 (2H; d; J = 8.8 Hz), 8.17 (2H; d; J = 8.8 Hz), 7.95 (3H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 162.2, 149.4, 146.2, 138.7, 129.4, 123.7 ppm. Anal. Calcd. (%) for $C_{14}H_9N_5O_5$: C: 51.38; H: 2.77; N: 21.40. Found: C: 51.41; H: 2.77; N: 21.42.

(*E*)-6-((2-(2-hydroxybenzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (12). Yellow powder; yield, 79%; mp, 240 to 241 °C. IR V_{max} (cm^{-1}; KBr pellets): 3573 (O-H), 3332 (N-H), 3100 (NH_2 amide), 1638 (C=O amide), 1532 (C=N imine), 1484 (N-O furoxan), 1341 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.12 (1H; s), 11.64 (1H; s), 8.51 (1H; s), 7.95 (4H; m), 7.45 (1H; t; J = 7.5 Hz), 6.99 (2H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 164.8, 158.7, 145.7, 134.0, 129.0, 119.1, 118.4, 117.3, 116.3, 113.7 ppm. Anal. Calcd. (%) for $C_{14}H_{10}N_4O_4$: C: 56.38; H: 3.38; N: 18.79. Found: C: 56.41; H: 3.37; N: 18.80.

(*E*)-6-((2-(3-hydroxybenzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (13). Yellow powder; yield, 81%; mp, 265 to 266 °C. IR V_{max} (cm^{-1}; KBr pellets): 3578 (O-H), 3309 (N-H), 3118 (NH_2 amide), 1643 (C=O amide), 1532 (C=N imine), 1371 (N-O furoxan), 1293 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.15 (1H; s), 9.80 (1H; s), 8.50 (1H; s), 7.99 (3H; m), 7.33 (3H; m), 7.00 (1H; d; J = 6.9 Hz) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 163.4, 157.4, 144.9, 134.3, 129.6, 119.0, 118.3,

114.6, 113.3 ppm. Anal. Calcd. (%) for $C_{14}H_{10}N_4O_4$: C: 56.38; H: 3.38; N: 18.79. Found: C: 56.41; H: 3.38; N: 18.82.

(*E*)-6-((2-(4-hydroxybenzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (14). Yellow powder; yield, 85%; mp, 280 to 281 °C. IR V_{max} (cm^{-1}; KBr pellets): 3573 (O-H), 3332 (N-H), 3096 (NH_2 amide), 1638 (C=O amide), 1523 (C=N imine), 1371 (N-O furoxan), 1280 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.02 (1H; s), 10.21 (1H; s), 8.47 (1H; s), 8.01 (2H; m), 7.83 (2H; d; J = 8.7 Hz), 6.87 (3H; d; J = 8.7 Hz) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 163.0, 161.0, 152.8, 144.0, 136.3, 130.0, 128.8, 123.4, 118.2, 115.1, 114.8 ppm. Anal. Calcd. (%) for $C_{14}H_{10}N_4O_4$: C: 56.38; H: 3.38; N: 18.79. Found: C: 56.39; H: 3.39; N: 18.78.

(*E*)-6-((2-(2-aminobenzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (15). Yellow powder; yield, 77%; mp, 193 to 194 °C. IR V_{max} (cm^{-1}; KBr pellets): 3501 (NH_2 amine), 3319 (N-H), 3100 (NH_2 amide), 1668 (C=O amide), 1536 (C=N imine), 1477 (N-O furoxan), 1346 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 11.98 (1H; s), 8.44 (1H; s), 7.93 (3H; m), 7.59 (1H; d; J = 6.7 Hz), 7.22 (1H; t; J = 7.7 Hz), 6.76 (1H; d; J = 8.3 Hz), 6.58 (1H; d; J = 7.0 Hz), 6.45 (2H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 165.6, 152.8, 150.3, 143.8, 132.6, 128.5, 118.2, 116.5, 114.6, 112.7 ppm. Anal. Calcd. (%) for $C_{14}H_{11}N_5O_3$: C: 56.57; H: 3.73; N: 23.56. Found: C: 56.64; H: 3.74; N: 23.58.

(*E*)-6-((2-(3-aminobenzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (16). Yellow powder; yield, 82%; mp, 213 to 215 °C. IR V_{max} (cm^{-1}; KBr pellets): 3501 (NH_2 amine), 3232 (N-H), 3100 (NH_2 amide), 1638 (C=O amide), 1532 (C=N imine), 1416 (N-O furoxan), 1312 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 12.31 (1H; s), 8.86 (1H; s), 8.53 (1H; s), 8.10 (3H; m), 7.87 (3H; s), 7.64 (1H; t; *J* = 7.9 Hz), 7.57 (1H; d; *J* = 7.3 Hz) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 162.9, 159.6, 150.5, 148.9, 145.2, 134.1, 129.7, 128.9, 126.3, 124.6, 120.7, 114.7 ppm. Anal. Calcd. (%) for $C_{14}H_{11}N_5O_3$: C: 56.57; H: 3.73; N: 23.56. Found: C: 56.59; H: 3.73; N: 23.59.

(*E*)-6-((2-(4-aminobenzoyl)hydrazono)methyl)benzo[c][1,2,5]oxadiazole 1-oxide (17). Yellow powder; yield, 84%; mp, 218 to 200 °C. IR V_{max} (cm^{-1}; KBr pellets): 3478 (NH_2 amine), 3319 (N-H), 3118 (NH_2 amide), 1627 (C=O amide), 1500 (C=N imine), 1380 (N-O furoxan), 1252 (C-N aromatic). ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm) δ: 8.44 (1H; s), 7.92 (3H; m), 7.69 (2H; d; *J* = 8.6 Hz), 6.60 (2H; d; *J* = 8.6 Hz), 5.87 (2H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, δ ppm) δ: 165.3, 152.6, 142.9, 129.7, 118.9, 112.6 ppm. Anal. Calcd. (%) for $C_{14}H_{11}N_5O_3$: C: 56.57; H: 3.73; N: 23.56. Found: C: 56.58; H: 3.74; N: 23.57.

Biological activity

Determination of Minimal Inhibitory Concentration (MIC_{90}) against bacteria in active state

Resazurin Microtiter Assay (REMA) was used to determine the MIC_{90} values of all final compounds according to previously described method.[26,55] *Mtb* H_{37}Rv (ATCC 27294)

was used as the reference strain. The MIC_{90} value was defined as the lowest drug concentration at which 90 % of the cells are infeasible relative to the control. Samples were set up in three independent assays.

Cytotoxicity

All final compounds synthesized were evaluated for cytotoxicity (IC_{50}) against MRC-5 cell line according to previously described method.[26,55] The evaluation was performed with MRC-5 cells treated with compounds in a concentration ranging from 0.09 – 250 μg/mL. The IC_{50} value was defined as the highest drug concentration at which 50 % of the cells are viable relative to the control. Samples were set up in three independent assays.

Determination of the partition coefficient (logP)

Partition coefficient (logP) studies were performed according to previously described procedures.[26,73] A HPLC-based method was used to determine the logP of all final compounds. The following equipment and conditions were used: Shimadzu HPLC model CBM 20-A (Shimadzu®) equipped with UV-VIS detector (model SPD-20A) and a Agilent® Eclipse XDB C-18 column (250mm x 4,6mm; 5μm), isocratic flow rate [methanol:water (75:25)] at 1.0 mL/min, volume injected was 20.0 μL and the wavelength in the detector was 210 nm. Standards substances were used to construct the curve log K x log P. The capacity factor (log*K*) of the tested compounds was determined from their retention times and interpolated in the linearity curve log K x log P.

Determination of Minimal Inhibitory Concentration (MIC_{90}) against bacteria in latency state

The selective compounds (SI ≥ 10) were tested against the mycobacterium in latency state. The cultures were thawed, diluted in Middlebrook 7H12 broth, and sonicated for 5s. The cultures were diluted to obtain the concentration between $5x10^5$ to $2x10^6$ CFU/mL. Two-fold serial dilutions of compounds were prepared in a volume 100 mL in white 96-well microtiter plates, and 100 mL of the cell suspension was added. The microplate cultures were placed under anaerobic conditions (oxygen concentration, less than 0.16%) by using an Anoxomat model WS-8080 (MART Microbiology) and two cycles of evacuation and filling with a mixture of 10% H_2, 5% CO_2, and the balance N_2. An anaerobic indicator strip was placed inside the chamber to visually confirm the removal of oxygen. The plates were incubated at 37°C for 10 days and then transferred to an ambient gaseous condition (5% CO_2-enriched air) incubator for a 28h "recovery". On day 11 (after the 28-h aerobic recovery), we determined the luminescence and calculated the MIC_{90}.[58]

Spectrum activity

In order to establish the spectrum activity, we determinated MIC_{90} against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) by measuring optical density at 570 nm (OD570) after 16h of incubation in Mueller-Hinton II broth (Becton Dickinson, Sparks, MD, USA), and against *Candida albicans* (ATCC 10231) at OD570 nm after 48h in Cellgro RPMI 1640 medium (Mediatech Inc., Manassas, VA, USA). The

MIC was defined as the lowest concentration resulting in 90% reduction in absorption relative to that of untreated control cultures.

Determination of Minimal Inhibitory Concentration (MIC_{90}) against isogenic monoresistant strains

The MIC_{90} was determined against *M. tuberculosis* H37Rv isogenic strains monoresistant to rifampin (ATCC 35838); isoniazid (ATCC 35822); streptomycin (ATCC 35820); capreomycin, moxifloxacin and bedaquiline (*University of Illinois at Chicago - Institute for Tuberculosis Research*), via microdiluition technique MABA.[59]

Intramacrophage activity

The intramacrophage activity was determined by *in vitro* infection model with *M. tuberculosis* H37Rv (ATCC 27294). The suspension was then seeded on Middlebrook 7H10 (solid medium), supplemented with OADC, for CFU/mL counts. The J774A.1 (ATCC TIB-67) murine macrophage-like cell line was routinely maintained in RPMI-1640 complete medium (10% BFS) at 37 °C and 5% CO_2. After reaching confluence, the cells were detached and counted. For the intramacrophage activity, 5 x 10^5 cells/mL were seeded in a 24-well plate (TPP®) (1 mL per well) at 37 °C and 5% CO_2, for 24 h. The plates were incubated at 37 °C under a 5% CO_2 atmosphere for 24 h to allow cell adhesion. Bacterial suspensions were washed two times in phosphate-buffered saline (PBS) by centrifuging at 2200× *g* for 10 min and resuspended in RPMI-1640 complete medium. Cells were infected by incubation with 2 bacteria per cell for 1 h at 37 °C and then washed three times with Hanks balanced salt solution. The total number of cell-

associated mycobacteria was initially determined, as follows. J774A.1 cells were lysed by the addition of 1 mL of sterile distilled water containing 0.1% Triton X-100 per well to confirming the infection (TO). The compounds were diluted in RPMI-1640 complete medium at the following concentrations: 4 times higher than MIC, MIC value, and 4 times lower than MIC (1 mL per well at each concentration). Thus, the plates were incubated for 3 days at 37 °C and 5% CO_2 and then washed and lysed. The CFU was determined and the percent inhibition by each compound at each concentration was calculated, based on the positive control test. As a standard test, the intracellular inhibition by rifampin was determined. Each test was set up in triplicate.

Bactericidal kinects

To evaluate the bactericidal profile of a drug, bactericidal kinetics, also known as time-kill curve, is the methodology used. 50mL of the bacterial inoculum (aproximately 10^6 CFU/mL) was exposed to Compound 8 or antibiotics already used in the treatment individually for 15 days. Every 48 hours an aliquot was collected, diluted, and seeded on plates with solid medium. The initial concentration used was 2 fold MIC determined by REMA methodology: 0.72 µM for INH; 0.01 µM for RMP; 0.88 µM for MOX and 41.24 µM for Compound 8. The number of CFU was counted after 3 to 4 weeks and the values was expressed in Log_{10} CFU/mL by time.[74]

Microemulsion preparation

The nanostructured lipid system (ME) was prepared as described previously[63] with the following composition: 10% oil phase (cholesterol), 10% surfactant (a mixture of soy

phosphatidylcholine, sodium oleate and Eumulgin® HRE 40 (polyoxyl 40 castor oil - hydrogenated) 3: 6: 8) and 80% aqueous phase (phosphate buffer pH 7.4). The mixture was sonicated using the QSonica® Q700 stem sonicator with power of 700 watts, amplitude of 15%, batch mode, for 10 minutes at intervals of 30 seconds every two minutes, with ice bath throughout the process of sonication. After sonication, the MEs were centrifuged at 11,180 (x g) for 15 minutes to eliminate the titanium residues released by the sonicator stem. The compounds studied were incorporated at the desired concentration for the *in vivo* experiments by mass solubilization at the respective volume and sonicated for 3 minutes in the batch mode and 15% amplitude.

Tolerability test in BALB / c mice and toxicological analysis

The safety profile of the compounds was evaluated by the two assays quoted in mice (n = 4 animals per group). The experimental design established the need for 7 experimental groups (n = 4 animals / group), the 7 groups are described below. ME group: microemulsion control group; Rif-ME group: Rif is one of the primary first-line drugs of TB therapy, in this group it was administered with the microemulsion as a vehicle; CMC group: CMC (carboxymethylcellulose) (Sigma®) is a widely used vehicle for in vivo assays; Rif-CMC group: in this group Rif was administered with CMC (carboxymethyl cellulose) as the vehicle; Placebo Group: this group received filtered water; Compound **8** group: the compound was administered with the vehicle as the microemulsion.

Each compound was administered at a daily oral dose (gavage) at a concentration of 200 mg / Kg body weight (n = 4 animals / group) and the RMP at a concentration of 20 mg / Kg. The animals were monitored for 10 days and the behavioral parameters (hippocratic

screening) were evaluated. At the end of the 10 consecutive days elapsed, the animals were weighed, blood collected via the submandibular vein, euthanized in a CO_2 chamber. After euthanasia, the organs (kidneys and liver) were surgically removed, weighed and stored in cassettes that were stored in 10% formalin solution for histological analysis. Statistical analysis of animal organ weighing was performed using Graph Pad Prism Version 5.01 software. From the blood collection through the submandibular vein the samples were collected in heparinized collecting tubes, the tube was centrifuged at 10,000 rpm for 10 minutes. The plasma was separated and stored at -70 °C.

In order to analyze biochemical markers relevant to the main organs involved with metabolism and excretion (liver and kidney), the plasma was analyzed by biochemical kits from Labtest Diagnóstica S.A. The statistical analysis was performed by Graph Pad Prism Version 5.01 software, through Analysis of variance (ANOVA) and Dunnett's test, establishing $P < 0.05$ as significance level. Ethics Committee had previously approved the protocol (protocols: 09/2014 and 10/2014).

Histopathology analysis

For histological analysis, the biopsies were immediately immersed in 10% formalin for 72 hours at room temperature and submitted to routine processing. Serial 6-mm-thick histological sections obtained with a rotary microtome (820 Spencer Microtome, Spencer Products Co., Carson, CA, USA) were stained with hematoxylin and eosin and tehn evaluated under a light microscope (Olympus BX51, Olympus Optical do Brasil Ltda., São Paulo, SP, Brazil). Tissue reaction was examined by a pathologist blinded to the experimental and control groups. A descriptive analysis of the histological characteristics

of the tissue was carried out and representative micrographs (64 x and 250x magnifications) were obtained for every experimental and control groups. Ethics Committee had previously approved the protocol (protocols: 09/2014 and 10/2014).

Oral bioavailability

As pharmacokinetic screening, one can estimate the amount of the compound or its active product in the bloodstream by calculating the amount required to be administered orally for the compound to remain in sufficient doses active in the bloodstream. Determination of the *in vivo* bioavailability of the compounds was performed according to the methodology suggested by Gruppo et al. (2006).[75] The mice (n = 6 mice per group) received a single oral dose per gavage of each compound (300 mg / Kg body weight). At the time of 20 minutes, 1 hour, 2 hours and 4 hours, the animals had blood collected via the submandibular vein.[76] From the blood collection through the submandibular vein, the samples were conditioned in heparinized collecting tubes, then, the tube was centrifuged at 10,000 rpm for 10 minutes. The plasma was separated and stored at -70 °C. Serial dilutions of the plasma were prepared in a concentration between 10% and 0.039% and the inhibitory percentage of the compounds present in the serum was determined according to the REMA technique against the *Mtb* H37Rv. Plasma concentrations above 12.5% may inhibit the growth of *Mtb*, so only lower concentrations were considered for the study. An estimate of the concentration of the compound in the plasma (in μg/mL) was obtained using MIC data (previously determined) of the compounds analyzed. Ethics Committee had previously approved the protocol (protocols: 09/2014 and 10/2014).

Infection and treatment

Female 20 g BALB/c mice were infected by aerosol with a low dose ($5x10^6$ CFU/mL) of *M. tuberculosis* Erdman[77] in an aerosol infection chamber (Glas-Col, Terra Haute, IN). The compounds and RMP group were prepared in microemulsion and the other RMP control group by suspension in 0.5% (wt/vol) carboxymethylcellulose (CMC) such that the target dosages were obtained by once-daily dosing by oral gavage of a 200 µL suspension. Groups of 7 mice were dosed for 5 consecutive days each week. Mice were sacrificed 3 days after the final dose to minimize carryover from the lung homogenates to the plating medium. Both lungs were homogenized and diluted in Hanks' balanced salt solution (HBSS)-Tween, and aliquots will be plated on Middlebrook 7H11 medium. CFU will be determined after 3 weeks of incubation at 37 °C. Ethics Committee had previously approved the protocol (protocols: 09/2014 and 10/2014).

Microarray studies

For the microarray assay, *M. tuberculosis* H37Rv strain was treated with the tested compounds and DMSO (negative control) for 4 hours reaching the final concentration of 2 x MIC_{90}, always respecting the limit of 1% of DMSO in culture. For each treatment the bacteria was grown in 7H9 medium in triplicate. The treatment was initiated after the cultures reach OD_{600nm}=1.0. The RNA extraction was performed with RNeasy Mini Kit (QIAGEN), following the instructions of the manufacturer. Next, for cDNA synthesis with Cy3/Cy5 labeling, was prepared one reaction for Cy3 labeling and another one for Cy5 labeling (Dye-Swap technique). Briefly, 1 µL of RNA was added to 1 µL of random primers (3 µg/µL) and water to complete a final volume of 11 µL. The microtube was

placed in dry bath equipment (70 °C) for 2 minutes and quickly cooled in ice before the centrifugation step (8000 rpm / 5 seconds). After centrifugation were added for each combination: 5 µL of First Strand Buffer (5x, Invitrogen), 2.5 µL of DTT (100 mM), 2.3 µL de dNTPs (5 mM dA/G/TTP e 2 mM dCTP), 1.7 µL of Cy3 or Cy5 dCTP (GE Healthcare) and 2.5 µL of the SuperScript III enzyme (200 U/µL). Following, the samples were incubated in the dark at 25 °C for 10 minutes and then at 50 °C for 90 minutes. Next, the purification of Cy3/Cy5 labeled cDNA was performed as follows: the sample treated with the compound and labeled with Cy3 was mixed with the sample treated with DMSO and labeled with Cy5. In the Dye-Swap reaction the sample treated with the compound and labeled with Cy5 was mixed with the sample treated with DMSO and labeled with Cy3. After this mixture step, each final sample underwent a purification process using Qiagen MinElute Purification (catalog number 28004), following the instructions of the manufacturer. Finally, in a new microtube were added for each reaction: 18 µL of the cDNA mixture, 4.5 µL of *Agilent 10x Blocking Agent* and 22.5 µL of *Agilent 2x Hibridization Buffer*. The microtubes were incubated at 95 °C for 5 minutes e centrifuged at 13000 rpm for 1 minute. Following, 40 µL of the hybridization mixture were pipetted in the respective array of the slide. Carefully, the array slide was placed over the coverslide, avoiding bubble formation. The slides were incubated at 65 °C for 12 hours with a vertical rotation of 20 rpm. After the incubation, the slides proceeded to the washing step with *Oligo aCGH Wash Buffer* 1, 2 and 3 (Agilent Technologies), following the manufacturer´s instructions. To finish the assay, the microarray slides were scanned with *Agilent High Resolution Microarray Scanner* and analysis was performed using the *GeneSpring 13.1* software (Agilent Technologies).

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SUPPORTING INFORMATION

The supporting information contains spectral characterization data and microarrays data. The Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS USED

1,8-diazabicyclo[5.4.0]undec-7-ene (DBU); Dichloromethane (DCM); Extensively drug-resistant (XDR); Food and Drug Administration (FDA); Human immunodeficiency virus (HIV); Infrared spectroscopy (IR); Multidrug-resistant (MDR); Minimum bactericidal

concentration (MBC); *Mycobacterium tuberculosis* (*Mtb*); Nuclear magnetic resonance (NMR); Quinoxaline 1,4-di-*N*-oxide (QdNO); Reactive oxygen species (ROS); Resazurin microtiter assay (REMA); rifampicin (RMP); Selectivity index (SI); Totally drug-resistant (TDR); Tuberculosis (TB); World Health Organization (WHO).

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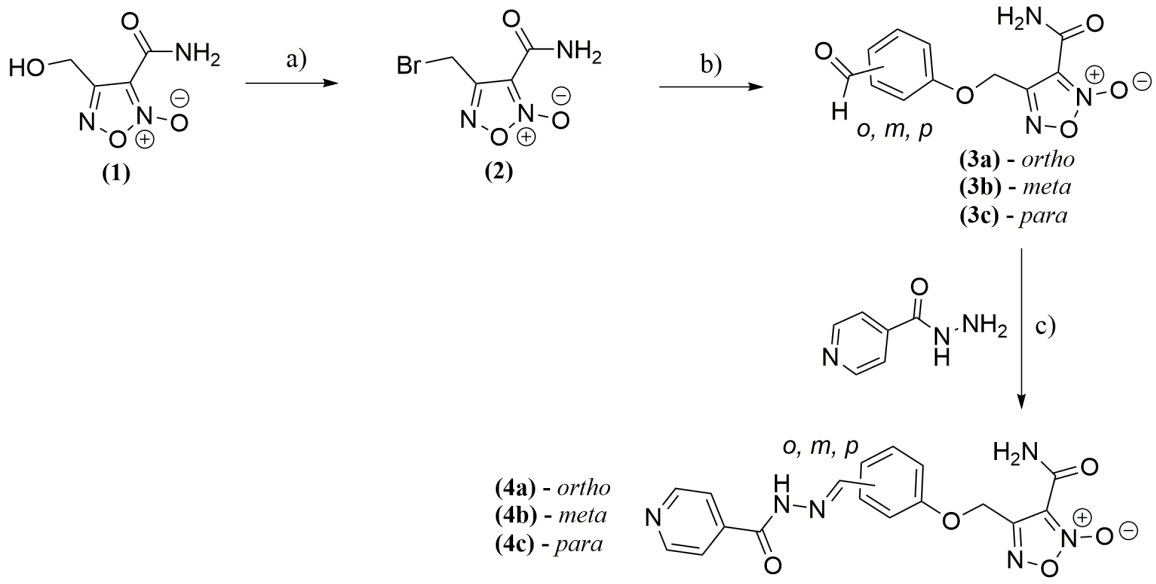
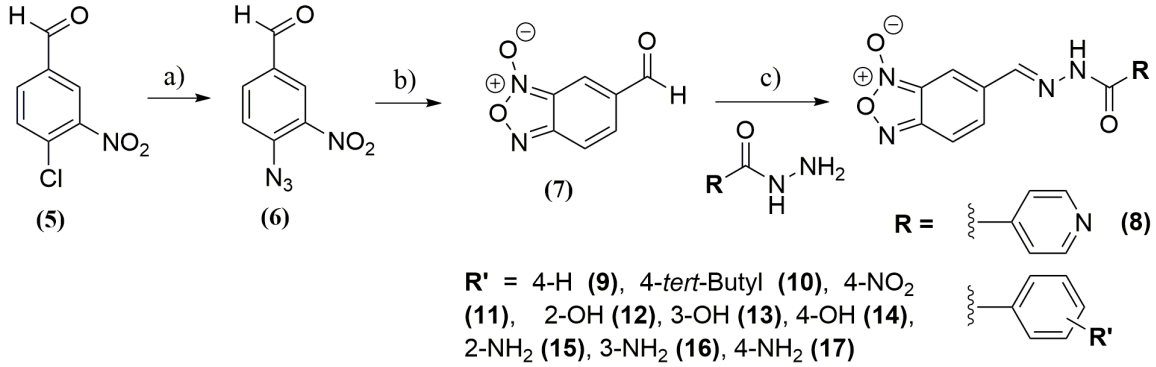
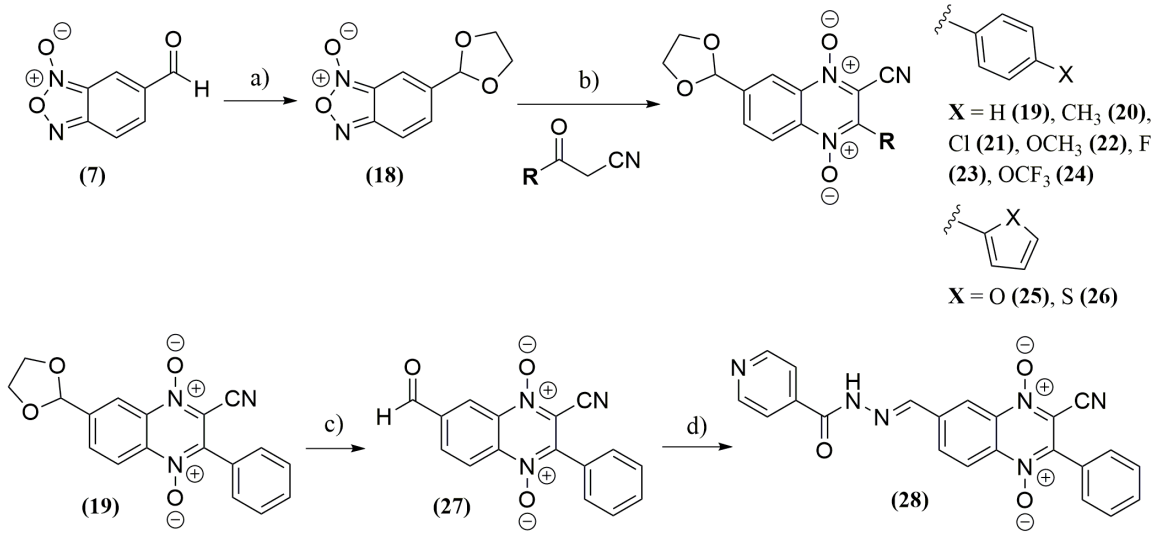
Scheme 1. Series 1 compounds. Reagents and conditions: **(a)** thionyl bromide, DMF, r.t., 30 min; **(b)** DBU, 2, 3 or 4- hydroxybenzaldehyde, DCM, r.t., 1 h; **(c)** ethanol, acetic acid, r.t., 12 h.



Scheme 2. Series 2 compounds. Reagents and conditions: **(a)** NaN_3, DMSO, 75 ºC, 1 h; **(b)** toluene, reflux, 2h; **(c)** aromatic hydrazide, ethanol, acetic acid, r.t., 12 h.



Scheme 3. Series 3 compounds. Reagents and conditions: **(a)** toluene, ethylene glycol, *p*-toluenesulfonic acid, reflux, 12 h; **(b)** DCM, K_2CO_3, 40 ºC, 96 h; **(c)** acetone, HCl, r.t., 48 h; **(d)** isonicotinohydrazide, ethanol, acetic acid, r.t., 12 h.



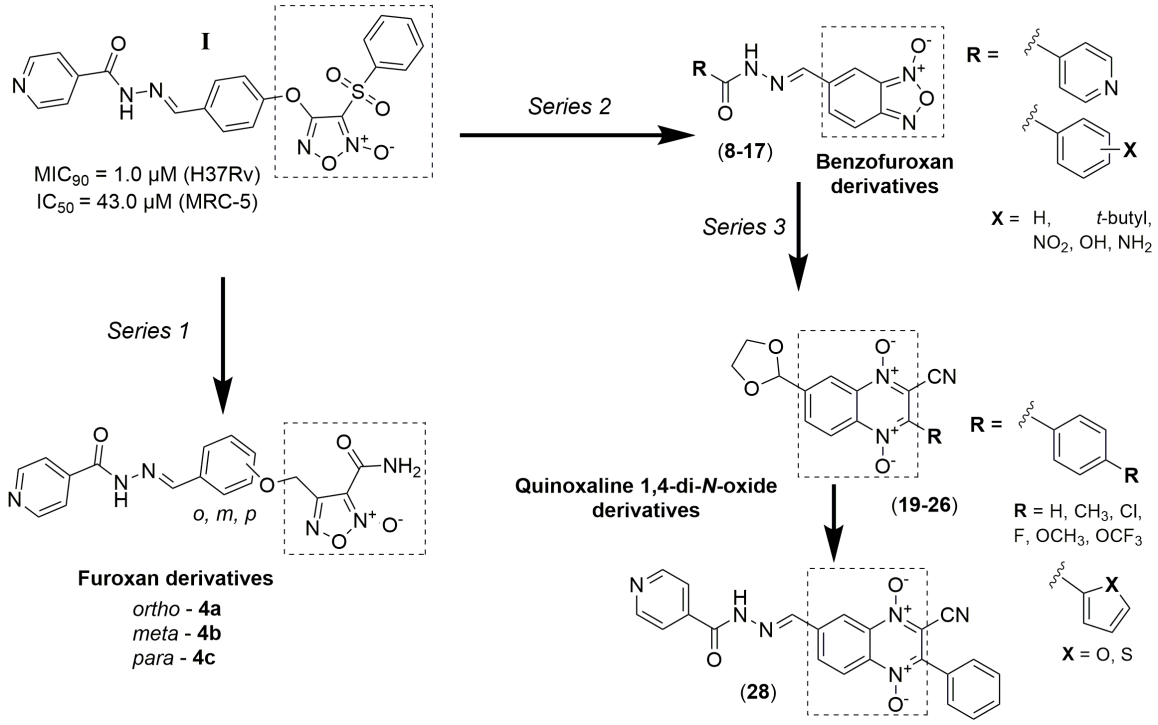
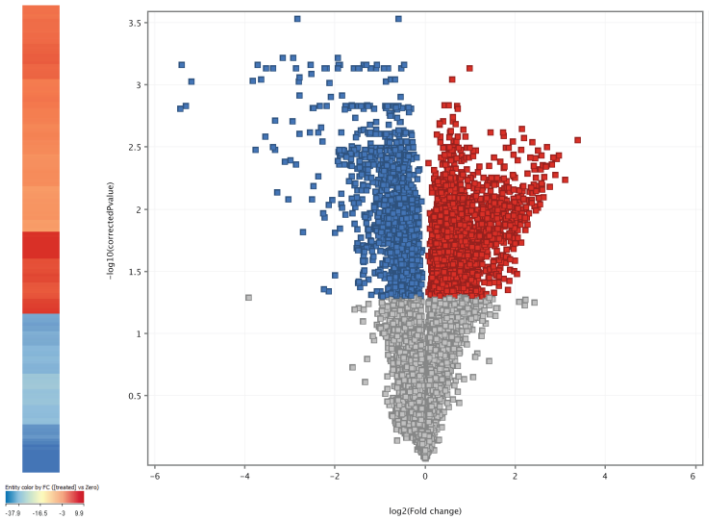


Figure 1. Design of the *N*-oxide containing heterocycles derivatives.

(a) (b) (c)



Gene number	Gene name	FC Av (Log_2)	Number of probes detected	Gene number	Gene name	FC Av (Log_2)	Number of probes detected
Rv0053	*rpsF*	1.60	2	Rv1015	*rplY*	1.11	2
Rv0054	*rpsR1*	1.74	1	Rv1630	*rpsA*	1.12	1
Rv0055	*rpiI*	1.71	3	Rv1643	*rplT*	1.64	2
Rv0634B	*rpmG2*	1.85	1	Rv2055	*rpsR2*	1.03	1
Rv0640	*rplK*	1.65	2	Rv2057	*rpmG1*	1.50	1
Rv0641	*rplA*	1.33	2	Rv2058	*rpmB2*	1.33	1
Rv0651	*rplU*	1.30	3	Rv2412	*rpsT*	1.49	2
Rv0652	*rplL*	1.92	2	Rv2441	*rpmA*	1.51	3
Rv0682	*rpsL*	1.92	4	Rv2442	*rplU*	1.77	2
Rv0683	*rpsG*	2.24	3	Rv2785	*rpsO*	1.14	2
Rv0684	*fusA*	2.09	3	Rv2889	*tsf*	1.30	2
Rv0685	*tuf*	2.33	3	Rv2890	*rpsB*	1.68	1
Rv0700	*rpsJ*	2.77	3	Rv2904	*rplS*	1.55	2
Rv0701	*rplC*	2.65	3	Rv2909	*rpsP*	1.51	2
Rv0702	*rplD*	2.18	3	Rv3442	*rpsI*	1.71	2
Rv0703	*rplW*	2.26	3	Rv3443	*rplM*	1.95	3
Rv0704	*rplB*	2.54	2	Rv3456	*rplQ*	1.61	3
Rv0705	*rpsS*	2.86	2	Rv3458	*rpsD*	1.64	3
Rv0706	*rplV*	2.09	3	Rv3459	*rpsK*	1.58	2
Rv0707	*rpsC*	2.28	2	Rv3460	*rpsM*	1.60	3
Rv0708	*rplP*	2.68	1	Rv3461	*rpmI*	1.70	1
Rv0709	*rpmC*	2.79	2	Rv3924	*rpmH*	1.77	2
Rv0710	*rpsQ*	2.23	3				
Rv0714	*rplN*	1.86	3				
Rv0715	*rplX*	2.12	3	Rv1304	atpB	1.12	1
Rv0716	*rplE*	2.25	2	Rv1305	atpE	1.15	2
Rv0717	*rpsN*	1.33	1	Rv1306	atpF	1.31	1
Rv0717B	*rpsZ*	2.30	2	Rv1306/7	atpFH	1.40	2
Rv0718	*rpsH*	2.04	3	Rv1308	atpA	1.77	3
Rv0719	*rplF*	1.95	3	Rv1309	atpG	1.81	2
Rv0720	*rplR*	1.99	2	Rv1310	atpD	1.87	3
Rv0721	*rpsE*	2.34	3	Rv1311	atpC	1.35	2
Rv0722	*rpmD*	2.12	2				
Rv0723	*rplO*	2.03	2				

Figure 2. Genes expression after 4 hours of exposure to compound **(8)** at 2 times of MIC by microarray. (a) Heatmap and (b) Volcano plot, illustrating the effect of compound (**8**) versus vehicle on gene expression in M. tuberculosis H37Rv. (c) Table showing two examples of up-regulated genes associated with the ribosome or ATP synthase.

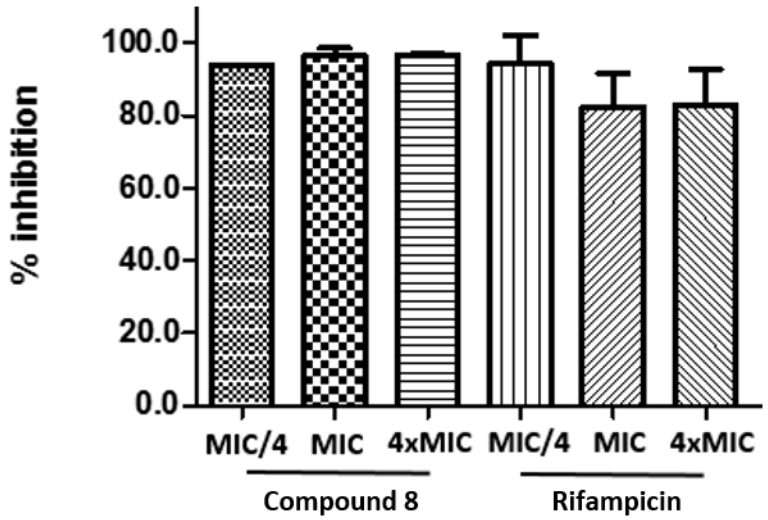


Figure 3. Intramacrophage activity of compound **(8)** and RMP after infection of J774A.1 macrophages with *M. tuberculosis* H37Rv (ATCC 27294). The percentage of inhibition was determined as the mean of three independent assays. The compounds concentrations were: Compound (**8**) 23.86 µg/mL (4xMIC), 5.84 µg/mL (MIC), 1.46 µg/mL (MIC/4); positive control (RMP) 0.064 µg/mL (4xMIC), 0.016 µg/mL (MIC), 0.04 µg/mL (MIC/4). Bars: Means±S.D.

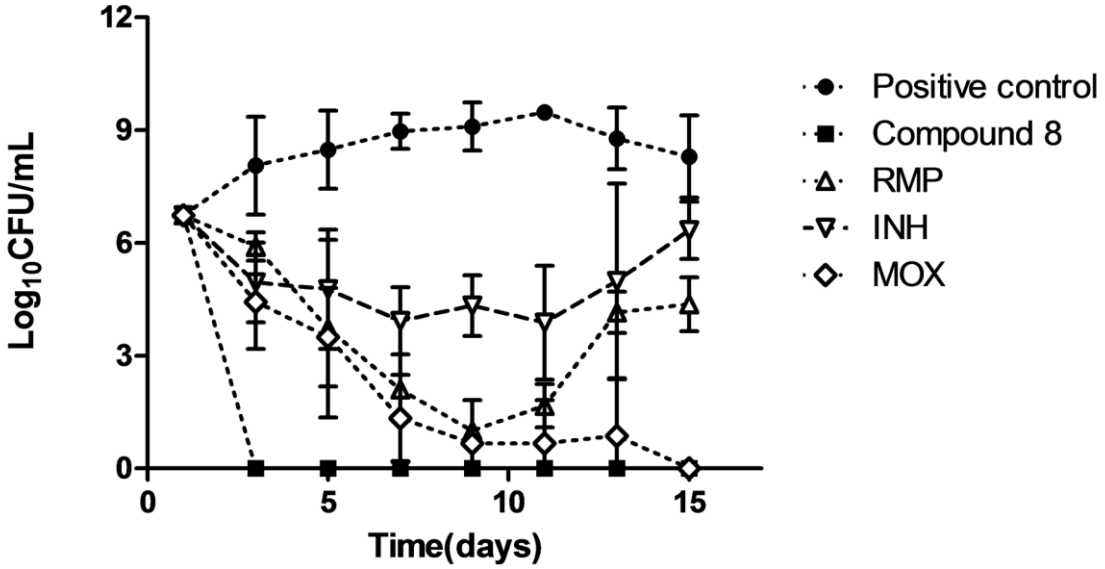


Figure 4. Time-kill curves of the Compound **(8)**, rifampicin, isoniazid and moxifloxacin: results in Log_{10} CFU/mL of the *M. tuberculosis* H37Rv (ATCC 27294) according to time (days). The CFU count was determined as the mean of three independent assays.The compounds concentrations were: 0.72 µM for INH; 0.01 µM for RMP; 0.88 µM for MOX and 41.24 µM for Compound 8. Bars: Mean ± S.D.

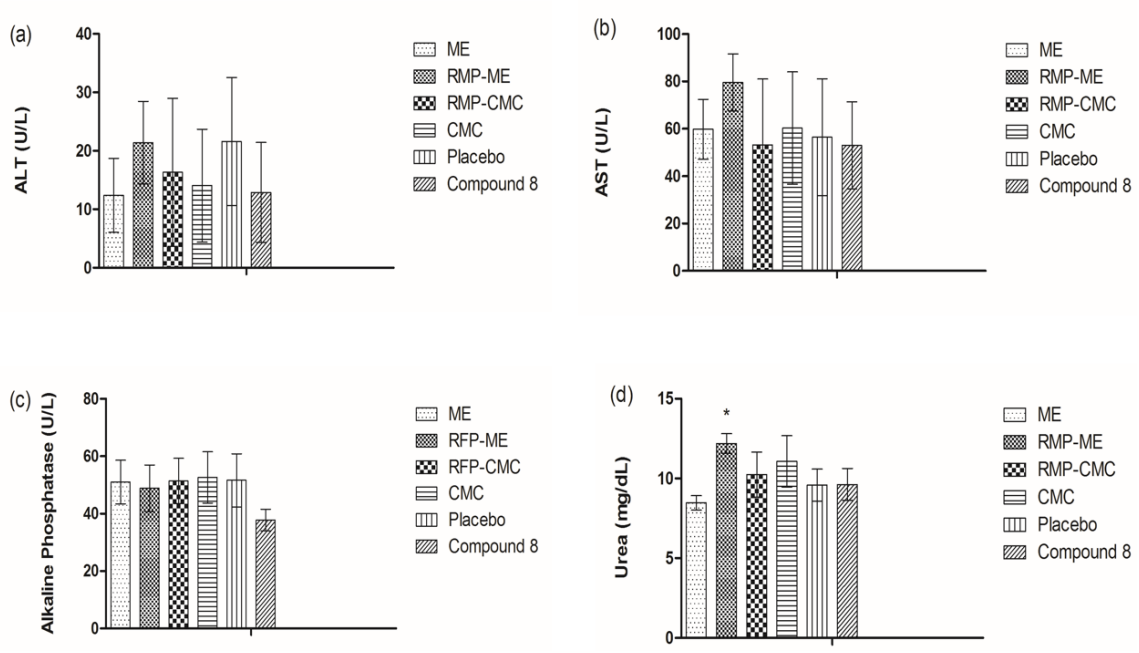


Figure 5. Results of alanine aminotransferase (**4a**), aspartate aminotransferase (**4b**), alkaline phosphatase enzyme activity determination (**4c**) and of urea quantification (**4d**) in the plasma of BALB / c mice. Each compound was administered at a daily oral dose (gavage) at a concentration of 200 mg / kg body weight (n = 4 animals / group) and the Rif at a concentration of 20 mg / kg. The statistical analysis was performed by Graph Pad Prism Version 5.01 software, through Analysis of variance (ANOVA) and Dunnett's test, establishing $P < 0.05$ as significance level. Bars: S.D.

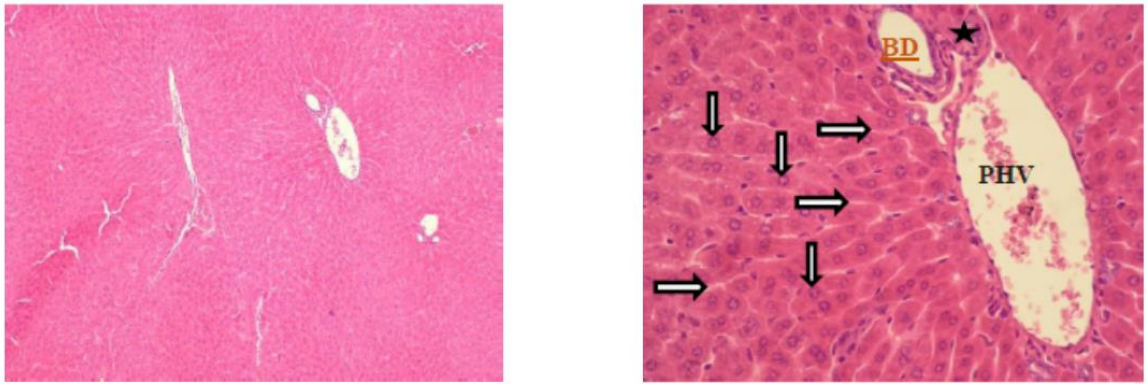


Figure 6. Histological section of liver stained with H / E from the group treated with compound **(8)**. In these groups, histological sections stained with H / E showed, in general, presence of hepatocytes with polyhedral morphology, grouped in strings separated by sinusoids, forming well-defined hepatic lobes. The sinusoids characterized as wall capillaries coated by typical endothelial cells, had some Kupffer cells. In each border of the hepatic lobe there was the presence of well-organized structures called portal-space, which is characterized mainly by the presence of portal hepatic vein (PHV), arteriole (A) and bile duct (BD). This set was surrounded by a layer of intact and continuous connective tissue, which appeared cut off by channels that discharged blood into the sinusoids, which flow into the central lobular vein.

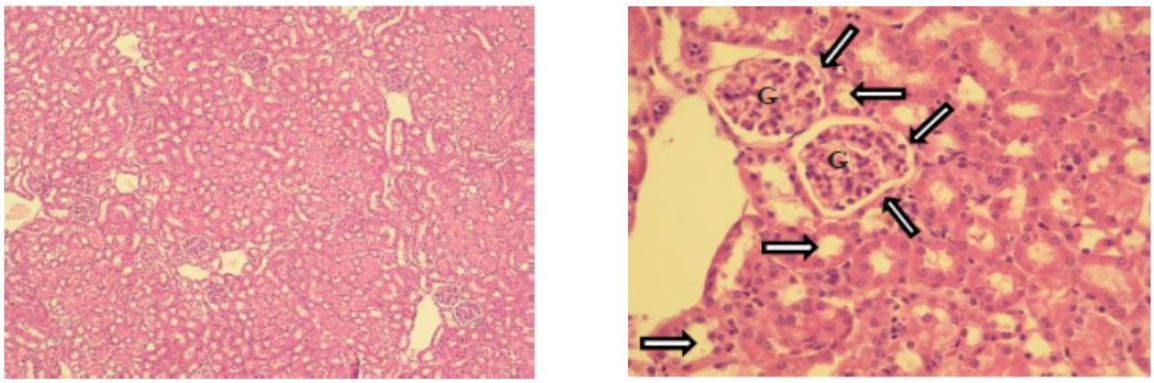


Figure 7. Histological section of kidneys stained with H / E from the group treated with compound **(8)**. Histological sections stained with H / E generally exhibited normal, well established and morphologically normal functional units of the kidney. Each nephron had the Malpighi corpuscle, characterized by the following structures: 1) Bowman's capsule, formed by simple pavement epithelium, and 2) glomerulus (G), characterized by a set of capillaries of fenestrated type. Near the Malpighi corpuscle, it was possible to observe proximal convoluted tubules formed by a simple layer of high cuboidal epithelial cells, which have brush edges facing the light of the tubules. Also adjacent to the Malpighi corpuscles were distal tubules formed by low cuboidal cells.

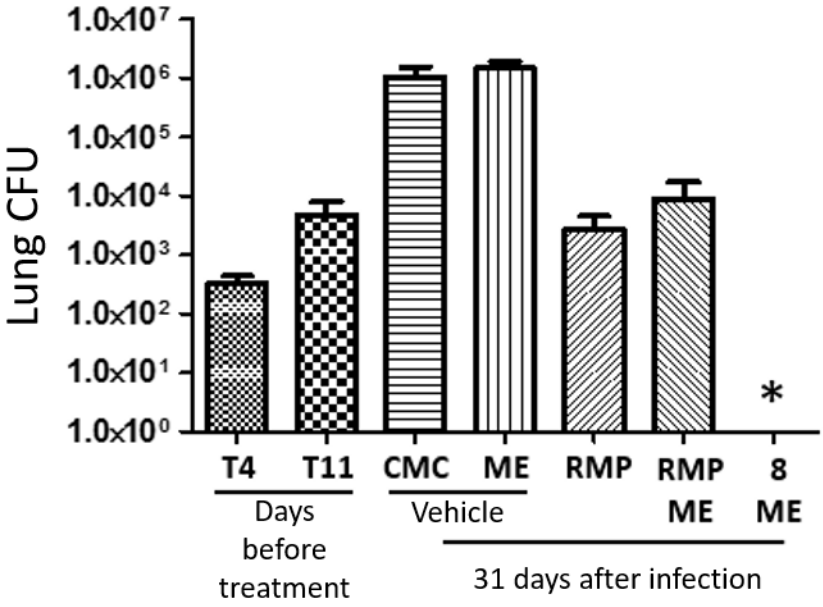


Figure 8. Eficacy of compound **(8)** in microemulsion and controls administrated once-daily by oral gavage of a 200 µL suspension. Efficacy of 200 mg/kg of compound (8) against acute TB in mice. Female 20g BALB/c mice were infected by aerosol with a low dose ($5x10^6$ CFU/ml) of M. tuberculosis Erdman. Start of treatment commenced at 10 days postinfection and terminated at 29 days postinfection. Dosages were obtained by once-daily dosing by oral gavage. Groups of 7 mice were dosed for 5 consecutive days each week. CFU were determined, after 3-day washout period, at day 31 postinfection. Both lungs were homogenized and diluted in Hanks' balanced salt solution (HBSS)-Tween, and aliquots were plated on Middlebrook 7H11 medium. Bars: S.D. *Sterilize effect.

1891 **Table 1**. Antitubercular activity of *N*-oxide containing heterocycles against actively
1892 replicating and dormant *Mycobacterium tuberculosis* $H_{37}Rv$ (MIC_{90}); cytotoxicity against
1893 MRC-5 cell line (IC_{50}); selectivity index (SI) and experimental logP [a].

Class	Compound	MIC_{90}		Dormant MIC_{90}		Cytotoxicity IC_{50}		SI[1]	LogP [a]
		µg/mL	µM	µg/mL	µM	µg/mL	µM		
Furoxan	**4a**	0.16	0.42	2.95	7.72	326.70	854.00	2033.30	1.3
	4b	0.15	0.40	1.60	4.20	490.10	1281.90	3204.70	1.3
	4c	0.16	0.43	0.78	2.04	443.30	1159.50	2696.50	1.3
Benzofuroxan	**8**	0.31	1.10	-	6.62	147.10	519.20	472.0	1.5
	9	2.40	8.30	-	-	36.80	130.40	15.60	2.2
	10	1.30	3.90	-	-	8.50	25.20	6.30	3.8
	11	1.73	5.29	-	-	-	-	-	0.9
	12	16.40	> 62.0	-	-	-	-	-	1.3
	13	> 25.0	> 62.0	-	-	-	-	-	1.2
	14	> 25.0	> 62.0	-	-	-	-	-	1.2
	15	3.70	12.30	-	-	36.40	122.40	9.90	2.0
	16	5.30	17.80	-	-	24.40	82.10	4.60	1.4
	17	3.16	10.66	>10.0	>10.0	250.0	841.0	78.90	1.2
Quinoxaline	**19**	10.30	30.80	-	-	10.70	31.90	0.90	0.7
	20	5.70	16.50	-	-	6.20	17.20	1.10	1.6
	21	6.40	16.20	-	-	5.00	12.60	0.80	1.8
	22	4.40	12.00	-	-	5.50	15.00	1.20	1.4
	23	8.60	24.30	-	-	7.70	21.80	0.90	1.3
	24	6.50	15.40	-	-	28.00	66.80	4.30	2.2
	25	1.70	5.20	-	-	11.60	35.70	6.80	2.0
	26	4.10	12.10	-	-	5.90	17.30	1.40	1.9
	28	16.30	39.70	-	-	8.60	21.00	0.50	1.0
Standard Drugs	Isoniazid	0.014	0.1	-	1.108.4	-	-	-	-
	Rifampicin	0.082	0.1	-	0.1	N.D.	N.D.	N.D.	N.D.

1894
1895 [a] Determined by partition coefficient (*n*-octanol/water), HPLC method [73].
1896 [b] Abbreviations: SI[1], ratio between IC_{50} for MRC-5 and MIC_{90}; dash (-) means not determined.
1897

Table 2. Results of MIC (µM) determinations with compound **8** against *M. tuberculosis* H37Rv (ATCC 27294) in three different conditions.

Compound	Normal*[1]		Acid pH*[2]		FBS*[3]		BSA*[4]	
	MIC (µM)	S.D	MIC (µM)	S.D	MIC (µM)	S.D	MIC (µM)	S.D
8	5.47	1.01	3.41	1.52	11.79	0.93	9.81	1.21
Rifampicin	0.05	0.03	0.02	0.02	0.10	0.08	0.10	0.03

*[1] Normal: normal pH media= 6.8
*[2] acid pH: adjusted pH media for 6.0
*[3] BFS: 10% bovine fetal serum
*[4] *BSA: 4%* bovine serum albumin

Table 3. Antitubercular activity of *N*-oxide containing heterocycles against *Mycobacterium tuberculosis* monoresistant strains

Class	Compound	INHr	RMPr	MOXr	BDQr	CAPr	SMr
		MIC (µM)					
Furoxan	***4a***	>261.71	0.44	0.81	0.81	>261.71	27.4
	4b	>261.71	2.31	1.22	2.56	>261.71	>261.71
	4c	>261.71	1.99	0.66	6.38	>261.71	>261.71
Benzofuroxan	***8***	8.59	3.78	5.72	1.20	15.25	16.98
Standard Drugs	RIF	0.01	>1.00	0.10	0.04	0.21	0.03
	INH	>5.0	0.35	0.28	0.23	>5.00	>5.00
	MOX	0.23	0.12	>8.00	0.26	0.35	0.36
	BDQ	0.01	0.01	0.06	1.70	0.06	0.06
	CAP	-		-	-	60.46	1.72
	SM	-		-	-	2.55	>100

Abreviations: (INHr) isoniazid resistant; (RMPr) rifampicin resistant; (MOXr) moxifloxacin resistant; (BDQr) Bedaquiline resistant; (CAPr) capreomycin resistant and (SMr) streptomycin resistant. Dash (-) means not determined.

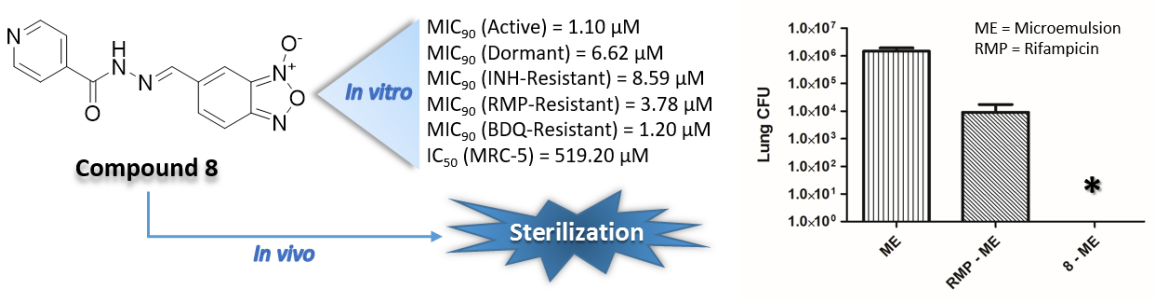
Table 4. Plasma levels of compound (**8)** (300 mg/kg dose), RFP-ME and RFP-CMC (dose of 20 mg/kg) following a single administration through oral route at times: 0,3h; 1h; 2h and 4h. Experiments were carried out in BALB/c mice.

Compound	**Standard**[1]	**Drug Dose**	**Mice Data**[2]							
			0,3 h		1 h		2 h		4 h	
	MIC (µg/mL) predetermined	(mg/kg/body)	Inhibition (%)	Estimate (µg/mL)	Inhibition (%)	Estimate (µg/mL)	Inhibition (%)	Estimate (µg/mL)	Inhibition (%)	Estimate (µg/mL)
Compound (**8**)-ME	5.84	300	65.67	42.61	79.00	51.26	76.00	49.31	51.33	33.30
RMP-ME	0.015	20	66.33	0.11	63.00	0.31	76.33	0.37	91.00	0.15
RMP-CMC	0.015	20	73.67	0.11	84.00	0.14	62.27	0.10	68.00	0.11

*[1] predetermined by *Resazurin Microtiter Assay (REMA) in vitro*;

*[2] determined using plasma mice by *REMA in vitro*, estimative in plasma.

TABLE OF CONTENTS GRAPHIC



ANEXO I

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Research paper

Synthesis and biological activity of furoxan derivatives against Mycobacterium tuberculosis

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abstract

Tuberculosis (TB) remains a serious health problem responsible to cause millions of deaths annually. The scenario becomes alarming when it is evaluated that the number of new drugs does not increase pro-portionally to the emergence of resistance to the current therapy. Furoxan derivatives, known as nitric oxide (NO) donors, have been described to exhibit antitubercular activity. Herein, a novel series of hybrid furoxan derivatives (1,2,5-oxadiazole 2-N-oxide) (compounds 4a-c, 8a-c and 14a-c) were designed, synthesized and evaluated in vitro against Mycobacterium tuberculosis (MTB) $H_{37}Rv$ (ATCC 27294) and a clinical isolate MDR-TB strain. The furoxan derivatives have exhibited MIC_{90} values ranging from 1.03 to 62 mM ($H_{37}Rv$) and 7.0e50.0 mM (MDR-TB). For the most active compounds (8c, 14a, 14b and 14c) the selectivity index ranged from 3.78 to 52.74 (MRC-5 cells) and 1.25e34.78 (J774A.1 cells). In addition, it was characterized for those compounds $logP_{o/w}$ values between 2.1 and 2.9. All compounds were able to release NO at levels ranging from 0.16 to 44.23%. Among the series, the phenylsulfonyl furoxan de-rivatives (compounds 14a-c) were the best NO-donor with the lowest MIC_{90} values. The most active compound (14c) was also stable at different pHs (5.0 and 7.4). In conclusion, furoxan derivatives were identified as new promising compounds useful to treat tuberculosis.

1. Introduction

Tuberculosis, caused mainly by Mycobacterium tuberculosis (MTB), is the infectious disease responsible for the largest number of deaths in the world, exceeding even human immunodeficiency virus (HIV). The latest surveys conducted by World Health Orga-nization (WHO) in 2014 showed 9.6 million of new cases around the world and 1.5 million of deaths annually [1]. The emergence of drug resistant strains, including multidrug resistant (MDR), extremely drug resistant (XDR) and the recently cases of totally drug resistant (TDR) increase the challenges to eliminate TB worldwide. Furthermore, WHO estimates that one third of the world population are infected by latent TB [2], whose treatment is unavailable due to the lack of new drugs [3e5].

The current treatment against MTB have shown limitations which include: high toxicity [6e10], drug-drug interactions [11], long-term therapy and low efficacy against resistant strains. After a gap of 50 years without any new antitubercular drugs, bedaquiline (SIRTURO®; Janssen, Beerse, Belgium) was approved by the United States Food and Drug Administration (FDA) for the treatment of MDR-TB; however, resistant strains to this drug are already re-ported [12]. After bedaquiline, there was a noteworthy increase in the number of papers describing compounds with potent antitu-bercular activity [13e16].

In order to find new antitubercular drugs, we have established a phenotypic-based screening program with more than five thou-sand compounds present in our current library. From these data, we have identified (hydroxybenzylidene)isonicotinohydrazide de-rivatives active against MTB. Specifically, the compound (E)-N′-(4-hydroxybenzylidene)isonicotinohydrazide (I) (Fig. 1) have exhibi-ted MIC_{90} value of 1.0 mM against MTB $H_{37}Rv$ and selective index against VERO and J774A.1 cell lines superior to 100.

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0223-5234/

Notwithstanding, this molecule did not show antitubercular ac-tivity against MDR strains, presenting MIC_{90} values superior to 62 mM.

In this work, using the molecular hybridization approach, we designed new analogues of (E)-N'-(4-hydroxybenzylidene)iso-nicotinohydrazide (I) containing the furoxan moiety [17] (Fig. 1). Furoxan derivatives represent an important class of compounds that exhibit a variety of biological activities, such as, anti-mycobacterial [18], antichagasic [19] and antileishmanicidal [20]. The wide spectrum of biological activities of furoxan derivatives have been associated to its ability to generate nitric oxide after biotransformation [21,22]. NO is an important mediator produced by macrophages during MTB infection and has an essential role to eliminate MTB [23]. It has been demonstrated that NO can disrupt bacterial DNA, proteins, signaling mediators, and/or induction of macrophage apoptosis [24]. Nitric oxide is also increased in mac-rophages during the infection and its inhibition promotes MTB growth [25]. MTB infected mice treated with nitric oxide synthase inhibitors exhibited higher mortality rates and pathological tissue damages compared to control group without treatment [26].

Not only endogenous, but also exogenous sources of NO have demonstrated effectiveness to reduce the number of bacilli. Some works have demonstrated that low levels of NO-donors can kill the mycobacteria [27e29]. These data suggested that strategies aiming to raise NO levels seem to be promising as antitubercular therapy. Therefore, in a continuing effort to develop new drug candidates to treat TB infection, we report herein the synthesis, NO-donor release, experimental logP values, antitubercular and cytotoxic activities of furoxan derivatives (4a-c, 8a-c, and 14a-c) (Fig. 1). The antimycobacterial activity against a clinical isolate of MDR strain (resistant to isoniazid, rifampicin, streptomycin and ethambutol) was characterized for the most active compounds. Moreover, for the most potent compound, we also studied the chemical stability at different pHs (1.0; 5.0; 7.4 and 9.0).

2. Results

2.1. Chemistry

The synthetic routes for the preparation of furoxan derivatives (4a-c, 8a-c, and 14a-c) derivatives are summarized in Schemes 1 and 2.

Compounds 2, 6, and 12 were synthesized according to a pre-viously described methodology [20,30e32]. The 2-, 3- or 4-hydroxybenzaldehyde was reacted with compounds 2, 6, and 12 in dichloromethane medium, using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base, to provide the furoxan derivatives 3a-c, 7a-c, and 13a-c, in yields varying between 20% and 67%.

The last step to obtain all furoxan derivatives (4a-c, 8a-c, and 14a-c) involves the coupling reaction between aldehyde function present in the furoxan derivatives and isonicotinic hydrazide in order to obtain the target compounds in excellent yields varying between 83% and 95% (Schemes 1 and 2). All chemical structures were established by infrared (IR) spectroscopy, elemental analysis and ^{1}H and ^{13}C nuclear magnetic resonance (NMR). The analysis of ^{1}H NMR spectra of all acyl hydrazone derivatives (compounds 4a-c, 8a-c, and 14a-c) have shown a single signal referring to ylidenic hydrogen attributed to the E-diastereomer [33e36]. All compounds were also analyzed by high-performed liquid chromatography (HPLC), and their purity was confirmed to be greater than 98.5%.

2.2. Antitubercular activity

The antitubercular activity of hybrid furoxan derivatives (4a-c, 8a-c, and 14a-c) and intermediates (3a-c, 7a-c, and 13a-c) were determined against Mycobacterium tuberculosis $H_{37}Rv$ ATCC 27294 and a clinical isolate MDR strain resistant to isoniazid, rifampicin, streptomycin and ethambutol. Among the furoxan intermediates, only compounds from the phenylsulfonyl (13a-c) series were active against MTB; the MIC_{90} for these compounds ranged from 2.89 to 26.01 mM, while the methyl (3a-c) and phenyl (7a-c) series pre-sented MIC_{90} values superior to 88 mM.

In the assays, hybrid furoxan derivatives (4a-c, 8a-c, and 14a-c) showed similar biological activity than those exhibited for in-termediates (3a-c, 7a-c, and 13a-c). Phenylsulfonyl (14a-c) series were the most activity compounds with MIC_{90} ranging from 1.03 to 8.60 mM. Furthermore, the para isomer (compound 8c) from the phenyl series was also active against MTB with MIC_{90} value of 11.82 mM. Interestingly, in the presence of a nitric oxide scavenger (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide - PTIO) all compounds have shown MIC_{90} superior to 62 mM. This data demonstrates the importance of NO to antitubercular activity for these derivatives.

The four more active compounds (8c and 14a-c) were also evaluated against a clinical isolate MDR strain and have showed MIC_{90} values ranging from 7.0 to 50.0 mM (Table 2). Compounds 4a-c and 8a-b showed MIC_{90} superior to 62 mM (Table 1) and were not considered promising for the determination of cytotoxicity.

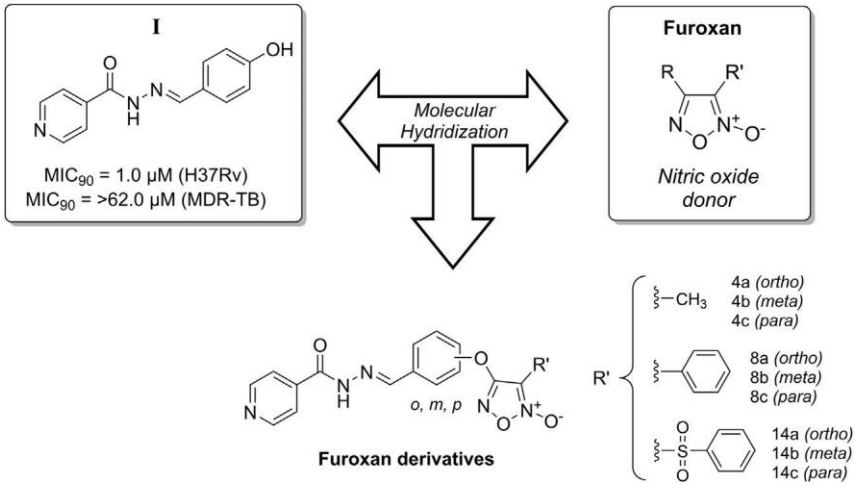
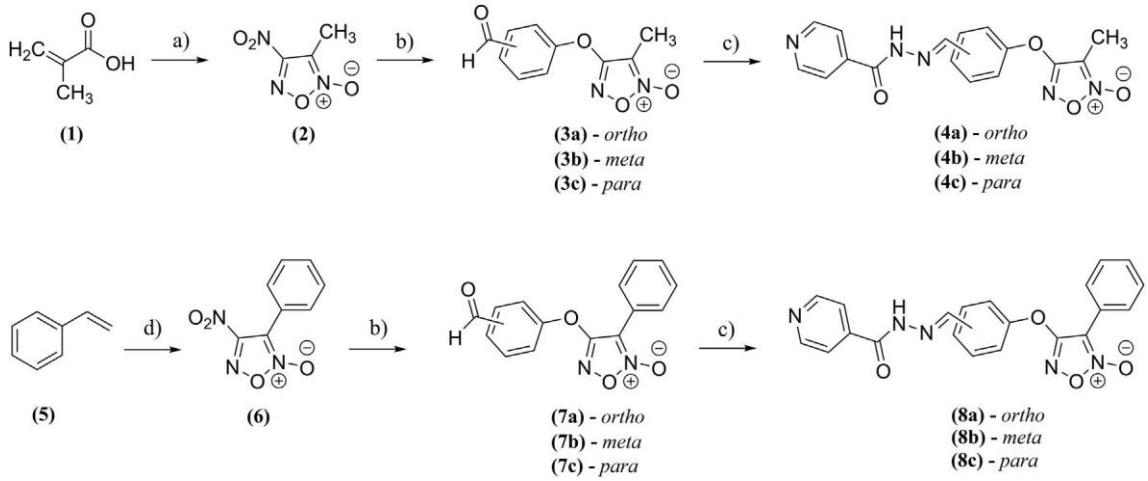
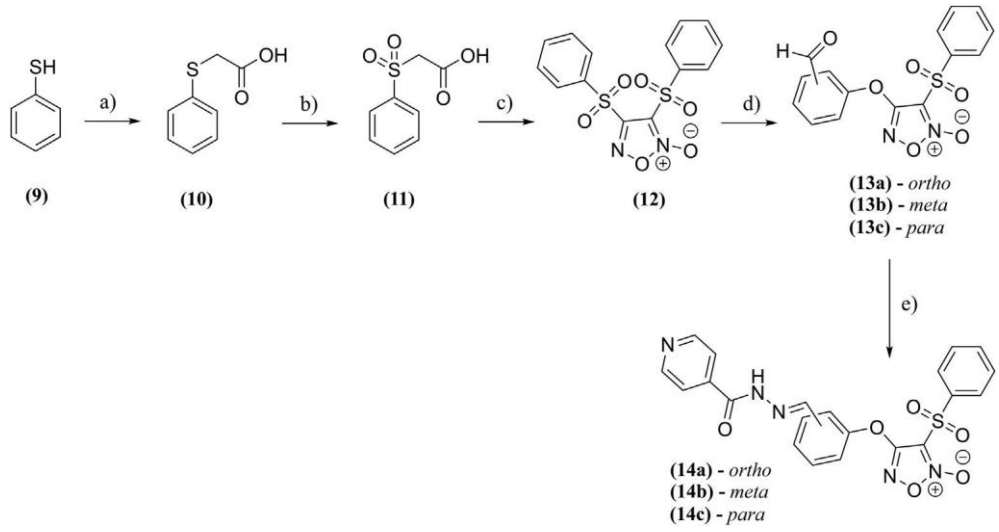


Fig. 1. Design of the hybrid furoxanyl N-acylhydrazone derivatives.



Scheme 1. Reagents and conditions: (a) 1,2-dichloroethane, H_2SO_4 60%, $NaNO_2$, 50 C, 30 min; (b) 2, 3 or 4-hydroxybenzaldehyde, 1,8-diazabicycloundec-7-ene (DBU), anhydrous dichloromethane, r.t., 2 h; (c) isonicotinic hydrazide, ethanol, acetic acid, r.t., 12 h; (d) acetic acid, hydrochloric acid, dichloromethane, $NaNO_2$, r.t, 12 h.



Scheme 2. Reagents and conditions: (a) monochloroacetic acid, NaOH, H_2O, 110 C, 3 h; (b) hydrogen peroxide 30%, acetic acid, r.t., 24 h; (c) fuming nitric acid, acetic acid, 110 C, 1 h; (d) 2, 3 or 4-hydroxybenzaldehyde, 1,8-diazabicycloundec-7-ene (DBU), anhydrous dichloromethane, r.t., 2 h; (e) isonicotinic hydrazide, ethanol, acetic acid, r.t., 12 h.

2.3. Determination of cytotoxicity

Cytotoxicity studies were performed using two different cell lines: MRC-5 and J774A.1. The selectivity index (SI) represents the ratio between IC_{50} and MIC_{90}. For this assay, the furoxans in-termediates (7c and 13a-c) have exhibited cytotoxic effect and low selectivity index against J774A.1 cell line. On the other hand, hybrid furoxan (8c and 14a-c) have shown IC_{50} values ranging from 34.4 to 623.4 mM (MRC-5) and 4.30e408.97 mM (J774A.1), respectively. For these compounds, SI values ranged from 3.78 to 52.74 (MRC-5) and 1.25 to 34.78 (J774A.1) (Table 1).

2.4. Nitric oxide release

The nitrite production resulted from the oxidative reaction of nitric oxide, oxygen and water for the hybrid compounds (4a-c, 8a-c, and 14a-c) was quantified through Griess reaction [37e39]. The results, expressed as percentages of nitrite (NO_2 ; mol/mol), are summarized in Table 1. Isosorbide dinitrate (DNS), used as a posi-tive control, induced 7.5% of nitrite formation. All furoxan derivatives (4a-c, 8a-c, and 14a-c) were able to induce nitrite for-mation at levels ranging from 0.16% to 43.55%.

2.5. Partition coefficient study

The partition coefficients were characterized by HPLC method [40] for all hybrid furoxan derivatives. The $logP_{o/w}$ values of the furoxan derivatives were positive and the values ranged from 1.2 to 2.9 (Table 1).

2.6. In vitro stability study

Chemical hydrolysis was performed for the most active com-pound (14c) in order to characterize chemical stability at different pHs (1.0; 5.0; 7.4 and 9.0). At extreme pHs (1.0 and 9.0) the com-pound was unstable. After 1 h, at pH 1.0, compound 14c have un-dergone 90% of degradation; while, for pH 9.0 the compound was reduced by 50%. However, at pH 5.0 and 7.4, the compound 14c have shown better stability. After 6 h, it was not detected significant chemical degradation at pH 5.0; while a reduction of 15% at pH 7.4

Table 1
Antitubercular activity of compounds against Mycobacterium tuberculosis $H_{37}Rv$; cytotoxicity against MRC-5 and J774A.1 cell lines (IC_{50}); selectivity index (SI); NO release data and experimental LogP.[d]

Compounds	MIC_{90} (mM) e $H_{37}Rv$	MIC_{90} (mM),[a] $H_{37}Rv$, PTIO	IC_{50} (mM) for MRC-5	SI[1]	IC_{50} (mM) for J774A.1	SI[2]	% NO_2 (mol/mol),[b, c] L-Cys, 50 10^4 M	LogP[d]
Intermediate furoxans								
3a	>62.0	e	e	e	e	e	0	e
3b	>62.0	e	e	e	e	e	0	e
3c	>62.0	e	e	e	e	e	0	e
7a	>62.0	e	e	e	e	e	25.10 ± 0.07	e
7b	>62.0	e	e	e	e	e	23.10 ± 0.40	e
7c	>62.0	e	e	e	e	e	19.44 ± 0.70	e
13a	20.23	e	e	e	7.4	0.4	21.19 ± 4.12	e
13b	20.89	e	e	e	5.6	2	27.05 ± 3.83	e
13c	26.01	e	e	e	2.2	0.1	24.45 ± 3.94	e
Hybrid furoxans								
4a	>62.0	e	e	e	e	e	0.35 ± 1.71	1.4
4b	>62.0	e	e	e	e	e	0.16 ± 2.13	1.3
4c	>62.0	e	e	e	e	e	2.02 ± 1.36	1.3
8a	>62.0	e	e	e	e	e	11.22 ± 0.5	2.7
8b	>62.0	e	e	e	e	e	6.87 ± 0.66	2.9
8c	11.82	>62.0	623.44	52.74	408.97	34.78	7.33 ± 1.77	2.9
14a	8.60	>62.0	34.40	3.78	10.75	1.25	44.23 ± 0.81	2.2
14b	1.61	>62.0	30.10	14.13	4.30	3.00	38.49 ± 4.05	2.3
14c	1.03	>62.0	43.01	20.29	10.75	11.98	43.55 ± 4.26	2.1
RIF	0.5	e	e	e	e	e	0	e
INH	0.11	e	e	e	e	e	0	e
DNS	e	e	e	e	e	e	7.17 ± 0.54	e

Abbreviations: DNS, isosorbide dinitrate (DNS possesses two ONO_2 groups that may release NO); SI[1], ratio between IC_{50} for MRC-5 and MIC_{90}; SI[2], ratio between IC_{50} for J774A.1 and MIC_{90}; RIF, rifampicin and INH, isoniazid (reference drugs); dash ( ) means not determined.

a Determined using the REMA methodology [41] in the presence of an equimolar concentration of the PTIO reagent (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), a nitric oxide scavenger.

b Mean ± standard error of the mean.

c Determined by Griess reaction, after incubation for 1 h at 37 C in pH 7.4 buffered water, in the presence of a 1:50 molar excess of L-cysteine.

d Determined by partition coefficient (n-octanol/water), HPLC method [40].

Table 2
Antitubercular activity of the most activity compounds against a clinical isolate MDR-TB strain (MIC_{90}).

Compound	MIC_{90} (mM) e MDR-TB[a]
8c	24.3
14a	50.0
14b	21.3
14c	7.0
RIF	Res
INH	Res

a Resistance to isoniazid, rifampicin, streptomycin and etham-butol [42]. Res, resistant.

was observed. After 24 h, a reduction of 20% of compound 14c was quantified at pHs 5.0 and 7.4 (Fig. 2).

3. Discussion

From a phenotypic-based screening containing more than five thousand compounds present in our current library, we have identified the (E)-N'-(4-hydroxybenzylidene)isonicotinohydrazide (I) (Fig. 1), (MIC_{90} ¼ 1 mM), as a promising scaffold for molecular modifications (supplementary material). In our drug design, we have used the molecular hybridization between this compound and furoxan derivatives. The furoxan derivatives were selected due to its anti-mycobacterial effect [18], related in parts, to its ability to release NO after biotransformation [22].

In vivo, NO is produced as a result of cytokines and chemokines stimulation [43]. The antimycobacterial effects of NO were firstly demonstrated in murine macrophage infected with bacilli [44]. Accurately, iNOS / mutated mice have exhibited higher sus-ceptibility to MTB infection and early death compared to non-mutated mice [45]. Exogenous NO has been shown as a useful strategy to kill the bacilli. It is established that compounds, such as pretomanid, can kill the MTB by induce NO intracellular after metabolism [46]. Furthermore, different NO-donors such as diethylenetriamine nitric oxide adduct (DETA/NO) [46] and S-nitrosothiols [47] are also examples of NO donors presenting antitubercular activity. Nitric oxide, as well as others reactive ni-trogen intermediates, can alter mycobacterial DNA by generating abasic sites and strand breaks. Additional NO mycobacterial-induced toxicity include interaction with proteins resulting in enzymatic inactivation and/or structural modifications [48].

In this work, it was characterized that furoxan derivatives methyl (4a-c) and phenyl (8a-b) did not exhibit activity against MTB. For these compounds, it was found MIC_{90} values superior to 62 mM. However, three isomers from the phenylsulfonyl furoxan series: ortho (14a), meta (14b) and para (14c) have shown promising activity against MTB with MIC_{90} values below 8.6 mM. Moreover, a phenyl furoxan derivative (8c) also exhibited a promising MIC_{90} value of 11.82 mM. The MIC_{90} values of these four compounds (14a-c; 8a) were greater than several first and second line antitubercular drugs, such as pyrazinamide (>48 mM), cycloserine (245 mM) and kanamycin (3.4 mM) [49]. We also evaluated the furoxan in-termediates (3a-c, 7a-c and 13a-c) against MTB $H_{37}Rv$, however, these compounds showed MIC_{90} values superior to 20.0 mM.

Our data suggest a direct effect in the pattern of substitution in the furoxan ring and the antitubercular activity. For phenylsulfonyl series (14a-c), the most active compounds were those in that N-acylhydrazone was substituted at para position (14c), followed by meta (14b) and ortho (14a) substitution. A similar effect can be observed in the phenyl series, wherein the para substituted deriv-ative (8c) was the most potent compound among its regioisomers.

We also measured the levels of nitrite in the medium as an in-direct method to quantify NO release by compounds. The results demonstrated that NO release is dependent of the presence of a

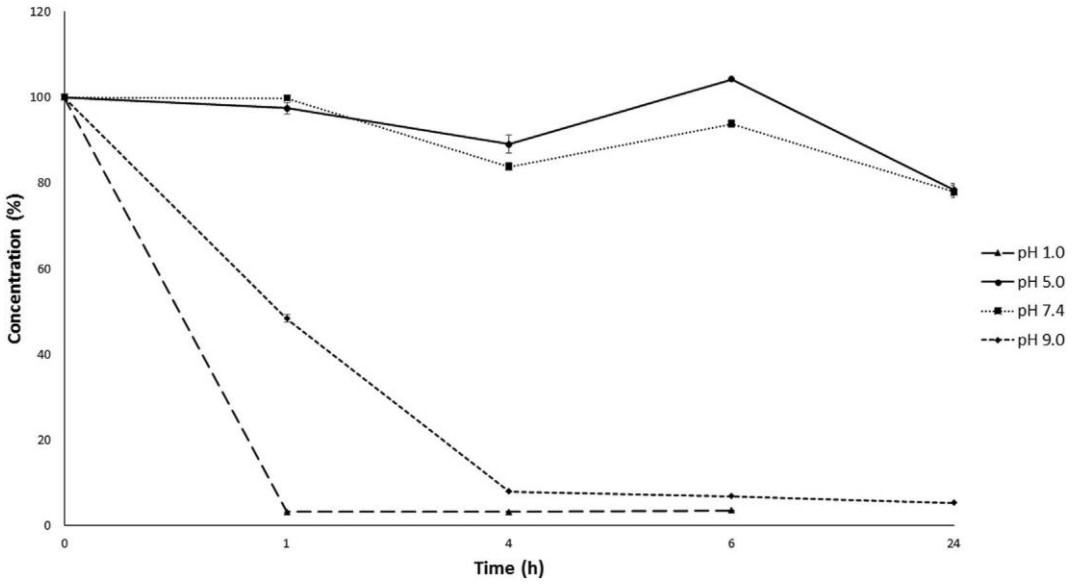


Fig. 2. In vitro chemical stability. Hydrolytic profile of compound 14c in buffer (pH 1.0; 5.0; 7.4 and 9.0) (data are represented as means ± SEMs and expressed as %).

large excess of L-cysteine (1:50), since conditions without this aminoacid were not able to release NO (results not shown) [37]. All furoxan compounds were capable to generate nitrite in the me-dium at values ranging from of 0.16%e44.23%. Our findings appoint that the antitubercular activity seems to be related in part, to the ability to release nitric oxide by the furoxan subunit. It was observed that phenylsulfonyl series (14a-c) showed the best anti-tubercular activity and generated high levels of nitric oxide, while the methyl series (4a-c), with low NO-release profile, demonstrated inferior antitubercular activity. Moreover, we evaluated the anti-tubercular activity of these four promising compounds in the presence of an equimolar concentration of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), a nitric oxide radical scavenger [50], in order to verify the importance of nitric oxide for antitubercular activity. The results showed that in the presence of PTIO, the four promising compounds have shown MIC_{90} values higher than those found in the assay without the PTIO re-agent, confirming the influence that nitric oxide plays in the anti-tubercular activity of these compounds.

The ability to release NO by furoxan derivatives is directly related to the substitution in the carbon atom at 3 position (C-3), neighboring to N-oxide function [20,39]. Furoxan derivatives with electron-withdrawing substituents at position C-3 (i.e., phenylsulfonyl-substituted (14a-c) derivatives) was able to release NO at high levels than methyl (4a-c) or phenyl (8a-c) series.

The most promising compounds (8c; 14a-c) identified in the primary screening were also evaluated against MRC-5 and J774A.1 cell in order to characterize their respective cytotoxicity. These cells were selected because MRC-5 is widely used for phenotypic screening of drugs to be regarded as a normal cell derived from lung human and J774A.1 is a macrophage murine cell. Compounds (8c; 14a-c) have demonstrated IC_{50} against MRC-5 at values ranging from 30.10 to 623.44 mM and SI values between 3.78 and 52.74. Regarding the J774A.1 cell line, it was found IC_{50} values ranged from 4.30 to 408.97 mM with SI values ranging from 1.25 to 34.78. Phenylsulfonyl derivatives (14a-c) were more cytotoxic than phenyl furoxan derivative (8c) against both cell lines; however, among the phenylsulfonyl derivatives it was not observed a rela-tionship between NO-donor release and cytotoxic effect. The furoxan intermediates (3a-c, 7a-c and 13a-c) have shown cytotoxicity with IC_{50} ranging from 2.2 to 113.2 mM and SI values between 0.1 and 2 against J774A.1 cell.

After the initial screening, we selected the four promising compounds (8c; 14a-c) to be evaluated against a clinical isolate MDR strain. This strain was phenotypically and genotypically characterized and it exhibited resistance to isoniazid, rifampicin, streptomycin and ethambutol [42]. Specifically, for this MDR strain it was characterized a mutation in the inhA gene, responsible for encode the NADH-dependent enoyl-ACP reductase of the FAS II system [51]. Compounds (8c; 14a-c) showed MIC_{90} values ranging from 7.0 to 50.0 mM, being the compound 14c (MIC_{90} 7.0 mM) the most promising among the series. These data suggest that furoxan moiety improved the antitubercular activity against MDR-TB, considering that compound (I) did not showed antitubercular ac-tivity against MDR-TB strains.

Therefore, we selected the most promising compound (14c) to analyze its chemical stability using an in vitro assay. We carried out the stability study under four conditions, (pHs 1.0, 5.0, 7.4 and 9.0) in order to mimic the acidic stomach (pH 1.0), the macrophage phagolysosome (pH 4.5e6.2) [52e54], the neutral plasma (pH 7.4) environments and a basic condition (pH 9.0), respectively. Com-pound 14c were unstable at pH 1.0 and 9.0 being degraded around 90% and 50% after the first hour, respectively. Despite of that, it was not detected significant chemical degradation at pH 5.0 (0%) and 7.4 (15%) after 6 h. After 24 h, a reduction of 20% was observed at both pHs 5.0 and 7.4, showing a relative stability of compound 14c in these pHs values. The degradation rates of compound 14c were calculated by HPLC-MS/MS and the degradation products were not characterized (supplementary material).

Moreover, it is well established that the permeability through the peptidoglycan-arabinogalactan-mycolic core in the MTB is a great limitation for antitubercular drug development [16]. There-fore, the lipophilicity, mostly expressed as $logP_{o/w}$ (the logarithm of the partition coefficient in a specific solvent ($P_{octanol}/P_{water}$)), is an important physico-chemical property that must be evaluated for new compounds during drug discovery [55,56]. Recently, we have identified that for the most active antitubercular compounds described in the literature between 2012 and 2014 (MIC_{90} inferior to 7), cLogP values ranged from 2 to 6 [16]. Hybrid furoxan de-rivatives reported here showed $logP_{o/w}$ values ranging from 1.3 to

2.9 (Table 1). We did not find a direct relationship between logP and antitubercular activity for all compounds; however, for those more active (8c and 14a-c) we have observed $logP_{o/w}$ values superior to 2.1, comparable to those values reported in literature [16].

4. Conclusion

In conclusion, a novel series of hybrid furoxan derivatives was synthesized and characterized. The furoxan derivatives have demonstrated nitric oxide release properties at levels ranging from 0.16% to 44.23%. Among the nine hybrid furoxan derivatives, com-pounds (8c and 14a-c) showed MIC_{90} values ranging from 1.03 to 11.82 mM and SI ranging from 3.78 to 52.74 (MRC-5) and 1.25 to 34.78 (J774A.1). Moreover, the four selected compounds (8c and 14a-c) presented activity against a clinical isolate MDR-TB strain with MIC_{90} values ranging from 7.0 to 50.0 mM. In vitro hydrolysis studies have demonstrated that compound 14c is stable at pH 5.0 and 7.4 until 6 h. The results described here pointed out compounds 8c and 14a-c as novel lead compounds for the treatment of TB infection, including against resistant strain.

5. Experimental section

5.1. Chemistry

Melting points (mp) were measured using an electrothermal melting point apparatus (SMP3; Bibby Stuart Scientific) in open capillary tubes. Infrared spectroscopy (KBr disc) were performed on an FTIR-8300 Shimadzu spectrometer, and the frequencies are expressed per cm^{-1}. The NMR for ^{1}H and ^{13}C of all compounds were scanned on a Bruker Fourier with Dual probe $^{13}C/^{1}H$ (300-MHz) NMR spectrometer and a Bruker Ascend (600-MHz) NMR spec-trometer using dimethyl sulfoxide (DMSO-d_6) as solvent. Chemical shifts were expressed in parts per million (ppm) relative to tetra-methylsilane. The signal multiplicities are reported as singlet (s), doublet (d), doublet of doublet (dd), and multiplet (m). Elemental analyses (C, H and N) were performed on a Perkin-Elmer model 2400 analyzer, and the data were within ±0.4% of the theoretical values. The compounds were separated on a chromatography col-umn with silica gel (60 Å pore size, 35e75-mm particle size) and the following solvents were used as mobile phase: dichloromethane, hexane, ethyl acetate and petroleum ether. The reaction progress of all compounds was monitored by thin-layer chromatography (TLC), which was performed on 2.0- by 6.0-cm^2 aluminum sheets pre-coated with silica gel 60 (HF-254; Merck) to a thickness of 0.25 mm and revealed under UV light (265 nm). All compounds were analyzed by HPLC, and their purity was confirmed to be greater than 98.5%. Reagents and solvents were purchased from commer-cial suppliers and used as received.

Compounds 2, 6, and 12 were synthesized according to a pre-viously described methodology (Schemes 1 and 2) [20,30e32]. Isonicotinohydrazide were purchased commercially.

5.2. General procedure for the synthesis of compounds 4a-c, 8a-c, 14a-c and 23a-c

A solution of compound 3a-c, 7a-c or 13a-c (0.87 mmol) in 10 mL of ethanol and 3 drops of hydrochloric acid was stirred at for 20 min at room temperature (r.t.). Next, isonicotinohydrazide (0.106 g, 0.87 mmol) was added, and the mixture was stirred at r.t. for 12 h. The reactions were monitored by TLC (98:2, ethyl acetate: methanol). The solvent was concentrated under reduced pressure, and 8 mL of ice water was added in order to precipitate the desired products. If necessary, the samples could be further purified through column chromatography (silica gel), using ethyl acetate-methanol (98:2) as the mobile phase to give the compounds 4a-c, 8a-c and 14a-c with variable yields (83e95%).

5.2.1. (E)-4-(2-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-methyl-1,2,5-oxadiazole 2-oxide (4a)

White powder; yield, 83%; mp, 196e198 C. IR V_{max} (cm^{-1}; KBr pellets): 3.203 (NeH), 3.030 (CeH aromatic), 1.689 (C]O amide), 1639 (C]N imine), 1485 (NeO furoxan), 1448 (CH_3), 1.284 (CeN aromatic), 1.143 (CeO ether). ^{1}H NMR (300 MHz, DMSO-d_6) d: 12.08 (1H; s), 8.78 (2H; d; J ¼ 5.8), 8.60 (1H; s), 7.98 (1H; d; J ¼ 8.8), 7.81 (2H; d; J ¼ 6.0), 7.57 (2H; m), 7.47 (1H; t; J ¼ 16.1), 2.24 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 163.53, 161.69, 150.54, 150.39, 143.50, 140.27, 131.91, 128.29, 127.22, 125.46, 121.66, 121.55, 107.48,
7.07 ppm. Calculated analysis (%) for $C_{16}H_{13}N_5O_4$: C: 56.6; H: 3.8; N: 20.6. Found: C: 56.7; H: 3.8; N: 20.5.

5.2.2. (E)-4-(3-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-methyl-1,2,5-oxadiazole 2-oxide (4b)

White powder; yield, 89%; mp, 149e154 C. IR V_{max} (cm^{-1}; KBr pellets): 3.217 (NeH), 3.049 (CeH aromatic), 1.633 (C]O amide), 1548 (C]N imine), 1446 (NeO furoxan), 1413 (CH_3), 1.305 (CeN aromatic), 1.159 (CeO ether). ^{1}H NMR (300 MHz, DMSO-d_6) d: 8.78 (2H; d; J ¼ 5.9), 8.49 (1H; s), 7.82 (3H; d; J ¼ 5.9), 7.69 (1H; d; J ¼ 7.6), 7.60 (1H; t; J ¼ 15.7), 7.51 (1H; d; J ¼ 8.4), 2.16 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 163.20, 161.82, 153.01, 151.59, 150.40, 147.64, 140.35, 136.28, 130.78, 125.59, 121.59, 117.72, 107.61,
6.99 ppm. Calculated analysis (%) for $C_{16}H_{13}N_5O_4$: C: 56.6; H: 3.8; N: 20.6. Found: C: 56.5; H: 3.8; N: 20.5.

5.2.3. (E)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-methyl-1,2,5-oxadiazole 2-oxide (4c)

White powder; yield, 85%; mp, 214e217 C. IR V_{max} (cm^{-1}; KBr pellets): 3.236 (NeH), 3.078 (CeH aromatic), 1.666 (C]O amide), 1604 (C]N imine), 1485 (NeO furoxan), 1408 (CH_3), 1.305 (CeN aromatic), 1.155 (CeO ether). ^{1}H NMR (300 MHz, DMSO-d_6) d: 12.11 (1H; s), 8.78 (2H; d; J ¼ 5.7), 8.50 (1H; s), 7.85 (2H; d; J ¼ 8.7), 7.82 (2H; d; J ¼ 5.9), 7.51 (2H; d; J ¼ 8.6), 2.14 (3H; s) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 162.76, 161.68, 154.06, 150.35, 147.80, 140.41, 131.96, 129.05, 121.53, 119.98, 107.59, 6.97 ppm. Calculated analysis
(%) for $C_{16}H_{13}N_5O_4$: C: 56.6; H: 3.8; N: 20.6. Found: C: 56.7; H: 3.8; N: 20.6.

5.2.4. (E)-4-(2-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-phenyl-1,2,5-oxadiazole 2-oxide (8a)

White powder; yield, 85%; mp, 209e211 C. IR V_{max} (cm^{-1}; KBr pellets): 3.184 (NeH), 3.045 (CeH aromatic), 1.674 (C]O amide), 1600 (C]N imine), 1435 (NeO furoxan), 1.300 (CeN aromatic),
1.149 (CeO ether), 769 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) d: 12.06 (1H; s), 8.74 (2H; d; J ¼ 6.0), 8.65 (1H; s), 8.14 (2H; d; J ¼ 7.9), 8.03 (1H; d; J ¼ 7.8), 7.75 (2H; d; J ¼ 6.0), 7.63 (5H; m), 7.50 (1H, t; J ¼ 15.9) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 162.31, 161.39, 150.50, 150.08, 142.69, 139.99, 131.72, 130.75, 128.91, 127.30, 127.23, 126.47, 125.57, 121.77, 121.43, 121.24, 107.92 ppm. Calculated anal-ysis (%) for $C_{21}H_{15}N_5O_4$: C: 62.8; H: 3.7; N: 17.4. Found: C: 62.9; H: 3.7; N: 17.3.

5.2.5. (E)-4-(3-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-phenyl-1,2,5-oxadiazole 2-oxide (8b)

White powder; yield, 92%; mp, 210e213 C. IR V_{max} (cm^{-1}; KBr pellets): 3.324 (NeH), 3.068 (CeH aromatic), 1.660 (C]O amide), 1604 (C]N imine), 1483 (NeO furoxan), 1.323 (CeN aromatic),
1.157 (CeO ether), 769 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) d: 8.78 (2H; d; J ¼ 5.6), 8.51 (1H; s), 8.10 (2H; d; J ¼ 7.1), 7.94 (1H; s), 7.82 (2H; d; J ¼ 5.7), 7.72 (1H; m), 7.62 (5H, m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 154.31, 145.39, 130.29, 123.46, 123.24, 121.39,

120.27, 119.00, 118.61, 113.94, 100.65 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_4$: C: 62.8; H: 3.7; N: 17.4. Found: C: 62.8; H: 3.7; N: 17,4.

5.2.6. (E)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-phenyl-1,2,5-oxadiazole 2-oxide (8c)

White powder; yield, 88%; mp, 198e202 C. IR V_{max} (cm ^{1}; KBr pellets): 3.250 (NeH), 3.066 (CeH aromatic), 1.651 (C]O amide), 1610 (C]N imine), 1438 (NeO furoxan), 1.332 (CeN aromatic), 1.205 (CeO ether), 756 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) d: 8.78 (2H; d; J ¼ 5.8), 8.51 (1H; s), 8.07 (2H; d; J ¼ 6.6), 7.88 (2H; d; J ¼ 8.7), 7.82 (2H; d; J ¼ 5.9), 7.63 (5H; m) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 153.62, 149.33, 126.38, 124.00, 123.26, 121.41, 118.97, 113.81, 112.77, 100.72 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_4$: C: 62.8; H: 3.7; N: 17.4. Found: C: 62.9; H: 3.7; N: 17.3.

5.2.7. (E)-4-(2-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (14a)

White powder; yield, 85%; mp, 199e202 C. IR V_{max} (cm ^{1}; KBr pellets): 3.280 (NeH), 3.068 (CeH aromatic), 1.651 (C]O amide), 1645 (C]N imine), 1454 (NeO furoxan), 1.354 (CeN aromatic), 1.161 (S]O sulfone), 1.083 (CeO ether), 744 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) d: 8.78 (2H; d; J ¼ 5.1), 8.50 (1H; s), 8.07 (2H; d; J ¼ 7.9), 7.93 (1H; t; J ¼ 7.3), 7.85 (4H; m), 7.78 (1H; t; J ¼ 7.7), 7.71 (1H; d; J ¼ 7.5), 7.60 (1H; t; J ¼ 7.9), 7.51 (1H; d; J ¼ 8.9) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 154.08, 150.83, 145.10, 139.80, 132.57, 129.15, 128.56, 123.07, 122.31, 120.90, 118.31, 113.97, 113.84, 110.05, 103.58 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_6S$: C: 54.2; H: 3.2; N: 15.1. Found: C: 54.3; H: 3.2; N: 15.0.

5.2.8. (E)-4-(3-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (14b)

White powder; yield, 95%; mp, 202e204 C. IR V_{max} (cm ^{1}; KBr pellets): 3.182 (NeH), 3.003 (CeH aromatic), 1.680 (C]O amide), 1604 (C]N imine), 1444 (NeO furoxan), 1.357 (CeN aromatic), 1.166 (S]O sulfone), 1.083 (CeO ether), 742 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) d: 8.79 (2H; d; J ¼ 6.0), 8.48 (1H; s), 8.07 (2H; d; J ¼ 7.3), 7.95 (1H; t; J ¼ 13.8), 7.83 (4H; m), 7.77 (1H; m), 7.71 (1H; d; J ¼ 7.6), 7.61 (1H; t; 15.7), 7.51 (1H; d; J ¼ 9.3) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 154.08, 150.87, 145.12, 142.66, 139.79, 132.59, 129.16, 128.27, 123.08, 122.32, 120.94, 118.33, 113.99, 113.85, 110.08, 103.61 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_6S$: C: 54.2; H: 3.2; N: 15.1. Found: C: 54.1; H: 3.2; N: 15.1.

5.2.9. (E)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (14c)

White powder; yield, 92%; mp, 194e197 C. IR V_{max} (cm ^{1}; KBr pellets): 3.238 (NeH), 3.068 (CeH aromatic), 1.664 (C]O amide), 1610 (C]N imine), 1450 (NeO furoxan), 1.359 (CeN aromatic), 1.165 (S]O sulfone), 1.082 (CeO ether), 750 (aromatic). ^{1}H NMR (300 MHz, DMSO-d_6) d: 8.78 (2H; d; J ¼ 6.0), 8.50 (1H; s), 8.05 (2H; d; J ¼ 8.6), 7.88 (2H; d; J ¼ 8.8), 7.82 (2H, d; J ¼ 6.0), 7.77 (3H, t; J ¼ 15.6), 7.53 (2H, d; J ¼ 8.7) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) d: 153.98, 150.42, 146.23, 142.65, 139.97, 132.66, 129.12, 128.58, 124.62, 122.33, 121.32, 120.89, 113.83, 112.44, 103.63 ppm. Calculated analysis (%) for $C_{21}H_{15}N_5O_6S$: C: 54.2; H: 3.2; N: 15.1. Found: C: 54.3; H: 3.2; N: 15.0.

5.3. Biological activity

5.3.1. Determination of minimal inhibitory concentration (MIC_{90}) The antitubercular activity of all compounds was determined through the REMA methodology according procedures described by Palomino and coworkers [57]. Stock solutions of the tested compounds were prepared in DMSO and diluted in Middlebrook 7H9 broth (Difco) supplemented with 10% OADC enrichment (dextrose, albumin, and catalase) using a Precision XS™ (BioTek®), to obtain final drug concentration ranging from 0.09 to 25 mg/mL. Rifampicin and isoniazid were used as a control drugs. A suspen-sion of the MTB H_{37}Rv ATCC 27294 or the clinical isolate MDR-TB strain was cultured in Middlebrook 7H9 broth supplemented with 10% OADC and 0.05% Tween 80. The culture was frozen at 80 C in aliquots. The concentration was adjusted to 2 10^{5} UFC/mL and 100 mL of the inoculum was added to each well of a 96-well microtiter plate together with 100 mL of the com-pounds. Samples were set up in three independent assays. The plate was incubated for 7 days at 37 C. After 24 h, 30 mL of 0.01% resazurin in distilled water was added. The fluorescence of the wells was read using a Cytation™ 3 (BioTek®) in which were used excitations and emissions filters at wavelengths of 530 and 590 nm, respectively. The MIC_{90} value was defined as the lowest drug con-centration at which 90% of the cells are infeasible relative to the control.

5.3.2. Cytotoxicity assay

In vitro cytotoxicity assays (IC_{50}) were performed on MRC-5 (ATCC® CCL-171) and J774A.1 (ATCC® TIB-67), as described by Pavan and colleagues [58]. The cells were routinely maintained in complete medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) plus amphotericin B (2 mg/L) and gentamicin (50 mg/L) at 37 C, in a humidified 5% CO_2 atmosphere. After reaching confluence, the cells were detached, counted and adjusted to 1 10^{5} cells/mL. The cells were seeded in 200 mL of complete medium in 96-well plates. The plates were incubated under the same conditions for 24 h to allow cell adhesion prior to drug testing.

From the stock solutions described above the compounds were diluted using a Precision XS™ (BioTek®), to obtain final drug con-centration ranging from 0.09 to 250 mg/mL. Then, the cells were exposed to compounds for 24 h and 30 mL of 0.01% resazurin in distilled water was added. The fluorescence of the wells was read using a Cytation™ 3 (BioTek®) in which were used excitations and emissions filters at wavelengths of 530 and 590 nm, respectively. The IC_{50} value was defined as the highest drug concentration at which 50% of the cells are viable relative to the control. Samples were set up in three independent assays.

5.3.3. Nitric oxide release

Nitric oxide has a short half-life, therefore, the quantification of NO metabolites like nitrite and nitrate is a useful method to quantify this molecule in the medium [59,60]. The amount of NO released was indirectly detected by Griess reaction through the measurement of nitrites in the medium. A volume of 98 mL of a phosphate buffer (pH 7.4) solution containing 5 mM of L-cysteine was added in triplicate in a 96-well, flat-bottomed, polystyrene microtiter plate. After loading the plate with the phosphate buffer (98 mL), it was added 2 mL of solution containing the appropriate compound diluted in DMSO (3 mL). The final concentration of the compound in each well was 1 10^{4} M. After 1 h of incubation at 37 C, it was added 100 mL of the Griess reagent (4 g of sulfanil-amide, 0.2 g of N-naphthylethylenediamine dihydrochloride, 85% phosphoric acid [10 mL] in distilled water [final volume, 100 mL]). After 10 min at room temperature, the absorbance was measured at 540 nm using a BioTek® microplate reader spectrophotometer. Standard sodium nitrite solutions (0.5e100 nmol/mL) were used to construct the calibration curve. The yields of nitrite are expressed as % NO_2 (mol/mol). No production of nitrite was observed in the absence of L-cysteine [37e39].

5.3.4. Partition coefficient (n-octanol/water) measured by HPLC method

The partition coefficient was characterized using the HPLC method according procedures described by OECD Guidelines for the Testing of Chemicals [40]. The equipment used was a Shimadzu HPLC model CBM 20-A (Shimadzu®) equipped with UV-VIS detec-tor (model SPD-20A), quaternary pumping system mobile phase (model LC-20AT), solvent degasser (model DGU-20As) and a Agi-lent® Eclipse XDB C-18 column (250 mm 4,6 mm; 5 mm). For HPLC method it was used an isocratic flow [methanol:water (75:25)] at 1.0 mL/min. The volume injected was 20.0 mL and the wavelength in the detector was 210 nm. The following substances were used as standards to construct the curve log K log P: acetanilide, ben-zonitrile, nitrobenzene, toluene, naphthalene, biphenyl and phen-anthrene. The capacity factor (logK) of the hybrid compounds was determined from their retention times and interpolated in linearity curve log K log P.

5.3.5. In vitro stability study

In vitro hydrolysis was performed by HPLC method. The com-pound 14c was separated using a Phenomenex Luna reverse-phase C_{18} (2)-HTS column (2.5-mm particle, 2 by 50 mm). The isocratic flow was 50:50 (water: 0,1% formic acid-acetonitrile, v/v) and the flow rate was 0.25 mL/min. The HPLC was coupled to a API 2000 triple quadrupole mass spectrometer equipped with a heated electrospray ionization interface (H-ESI) operated in the positive ionization mode at capillary voltage 5200 V, source temperature 350 C, nitrogen gas flow 65 units.

For hydrolysis, an appropriate solution of compound 14c was diluted in acetonitrile at 1000 mM. Then, this solution was diluted to 10 mM using four different PBS buffer (phosphate-buffered saline) in order to provide the following pHs: 1.0; 5.0; 7.4 and 9.0. During the assay, all samples were maintained at constant agitation using a shaker (400 rpm) at 37 C. Aliquots were taken from the solution at the following times: 0, 1, 4, 6, and 24 h. The injection volume in the HPLC was 5 mL. All analyses were conducted in triplicate, and the results were expressed as the averages of the concentrations in percentages (±standard error of the mean [SEM]).

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Appendix A. Supplementary data

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