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PLOS ONE

# Improved BM212 MmpL3 Inhibitor Analogue Shows Efficacy in Acute Murine Model of Tuberculosis Infection

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## Abstract

1,5-Diphenyl pyrroles were previously identified as a class of compounds endowed with high *in vitro* efficacy against *M. tuberculosis*. To improve the physical chemical properties and drug-like parameters of this class of compounds, a medicinal chemistry effort was undertaken. By selecting the optimal substitution patterns for the phenyl rings at N1 and C5 and by replacing the thiomorpholine moiety with a morpholine one, a new series of compounds was produced. The replacement of the sulfur with oxygen gave compounds with lower lipophilicity and improved *in vitro* microsomal stability. Moreover, since the parent compound of this family has been shown to target MmpL3, mycobacterial mutants resistant to two compounds have been isolated and characterized by sequencing the *mmpL3* gene; all the mutants showed point mutations in this gene. The best compound identified to date was progressed to dose-response studies in an acute murine TB infection model. The resulting $ED_{99}$ of 49 mg/Kg is within the range of commonly employed tuberculosis drugs, demonstrating the potential of this chemical series. The *in vitro* and *in vivo* target validation evidence presented here adds further weight to MmpL3 as a druggable target of interest for anti-tubercular drug discovery.

**Citation:** Poce G, Bates RH, Alfonso S, Cocozza M, Porretta GC, et al. (2013) Improved BM212 MmpL3 Inhibitor Analogue Shows Efficacy in Acute Murine Model of Tuberculosis Infection. PLoS ONE 8(2): e56980. doi:10.1371/journal.pone.0056980

**Editor:** Riccardo Manganelli, University of Padova, Medical School, Italy

**Received** November 14, 2012; **Accepted** January 16, 2013; **Published** February 21, 2013

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**Funding:** This work was supported by Istituto Pasteur Fondazione Cenci-Bolognetti (Italy), MIUR-PRIN 2008 (Italy), MIUR 2011 (Progetti di Ricerca di Ateneo) (Italy) and Tres Cantos Open Lab Foundation. The research leading to these results also received funding from the European Union's 7th framework programme (FP7- 2007–2013) under grant agreement no 261378. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** Please note that RHB, LB, JR, and FA are affiliated to Diseases of the Developing World, GlaxoSmithKline. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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## Introduction

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), infects one third of the world's population and is the second leading cause of mortality worldwide [1]. The World Health Organization (WHO) estimated that there were 8.5–9.2 million cases of TB and 1.2–1.5 million deaths in 2010, including deaths from TB among HIV-positive people. The current 6-month chemotherapy regimen involves treatment with a combination of 4 drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) for 2 months followed by an additional 4 months with isoniazid and rifampicin alone [2]. Although this regimen can achieve 95% cure rates in clinical trials, global cure rates are much lower mainly because of poor patient compliance and poor quality drugs that give rise to drug-resistant strains and infection relapse cases. Almost 4% of all TB cases globally are estimated to be multi-drug resistant [3], with the number of drug resistant cases increasing annually. Thus, new drugs with shorter and simpler regimens and with bactericidal mechanisms that differ from those of current drugs are needed.

In this context, a class of 1,5-diphenyl-pyrrole derivatives endowed with potent antimycobacterial activity has been identified through whole-cell screening of a library of azole compounds [4–7]. Structure-activity relationship (SAR) studies and a pharmacophore-based ligand design approach allowed us to identify new chemical groups and substitution patterns on the pyrrole core responsible for activity [8]. Our initial SAR strategy evaluated a panel of four compounds **1** (BM212) [7], **2** (BM521) [9], **3** (BM533) [10], and **4** (BM579) [10] (Figure 1) as hits within this class of derivatives on the basis of their biological profiles. Herein, we report our efforts to develop new 1,5-diphenyl pyrrole derivatives with improved drug-like properties as potential new tuberculosis therapeutic agents.

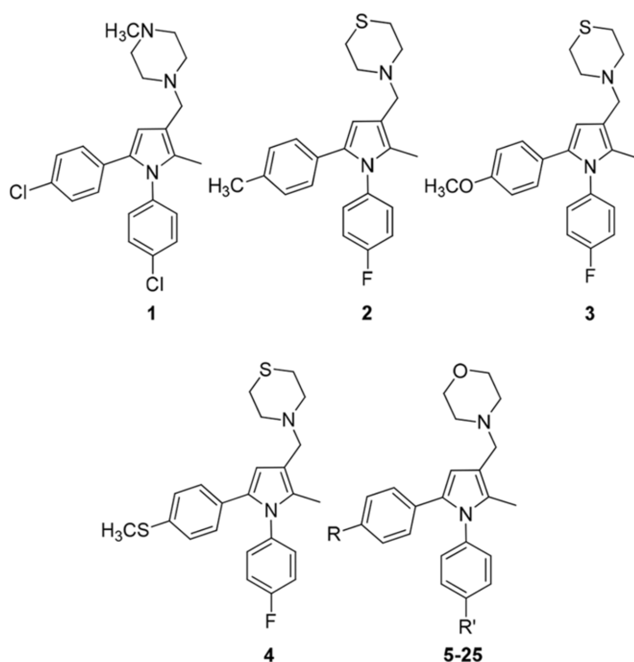


**Figure 1. Chemical structures of compounds 1–25.**
doi:10.1371/journal.pone.0056980.g001

## Materials and Methods

All procedures involving treatment of mice were performed with ethics approval of the Diseases of the Developing World (DDW-GSK) ethical committee. The animal research complies with Spanish and European Union legislation (European directive 86/609/EEC) on animal research and GlaxoSmithKline 3R policy on the care and use of animals: Replacement, Reduction and Refinement.

### Compound Synthesis

Compounds **5–25** were prepared following a previously described synthetic pathway [10]. Please see the Supporting Information for details.

### *Mycobacterium Tuberculosis* H37Rv Growth Inhibition Assay

The measurement of the minimum inhibitory concentration (MIC) for each tested compound was performed in 96 wells flat-bottom, polystyrene microtiter plates. Ten two-fold drug dilutions in neat DMSO starting at 200 μM were performed. Five μL of these drug solutions were added to 95 μL of Middlebrook 7H9 medium. Isoniazid was used as a positive control, 8 two-fold dilutions of Isoniazid starting at 160 μg/mL were prepared and 5 μL of this control curve was added to 95 μL of Middlebrook 7H9 medium (Difco). Five μL of neat DMSO were added 95 μL of Middlebrook 7H9 medium in row 12 (growth and Blank controls). The inoculum was standardized to approximately $1\times10^7$ cfu/mL and diluted 1 in 100 in Middlebrook 7H9 broth (Middlebrook ADC enrichment, a dehydrated culture media which supports growth of mycobacterial species available from Becton Dickinson), to produce the final inoculum of H37Rv strain (ATCC25618). One hundred μL of this inoculum was added to the entire plate but G-12 and H-12 wells (Blank controls). All plates were placed in a sealed box to prevent drying out of the peripheral wells and they were incubated at 37°C without shaking for six days. A resazurin solution was prepared by dissolving one tablet of resazurin (Resazurin Tablets for Milk Testing; VWR International Ltd) in 30 mL sterile PBS (phosphate buffered saline). Of this solution, 25 μL were added to each well. Fluorescence was measured (Spectramax M5 Molecular Devices, Excitation 530 nm, Emission 590 nm) after 48 hours to determine the MIC value.

### Low Oxygen Recovery Assay (LORA)

The LORA was performed as previously described [11] except that the low oxygen-adapted inoculum was prepared using a sealed, stirred culture instead of a fermentor. Briefly, low oxygen-adapted *M. tuberculosis* H37Rv *luxAB* was exposed to compounds in 7H12 medium contained in 96-well plates at 37°C under a low oxygen environment generated using an Anoxomat system. After 10 days incubation, plates were placed under normoxic conditions for 28 hours at 37°C and then luminescence was measured following the addition of *n*-decanal. The LORA MIC was defined as the lowest concentration resulting in a 90% reduction in luminescence signal relative to untreated cultures.

### Vero Cytotoxicity Assay

This experiment was carried out as previously described [10]. Briefly, Vero cells were grown and maintained in RPMI 1640 medium supplemented with 2 mM.

L-glutamine and 10% FCS. Cells were seeded in 96-well plates at a density of $1\times10^4$ cells/well. After 24 h, medium was replaced with fresh medium containing decreasing concentrations of the tested compound and incubated at 37°C in 5% $CO_2$. Morphological changes were observed at 24, 48 and 72 h of incubation. The effects on the proliferation of Vero cells were determined after 72 h by tetrazolium-based colorimetric MTT assay. The 50% cell-inhibitory concentration ($CC_{50}$) reduced by 50% the optical density values ($OD_{540,690}$) with respect to control no-drug treated cells.

### HepG2 Cytotoxicity Assay

Actively growing HepG2 cells were removed from a T-175 TC flask using 5 mL of Eagle's MEM (containing 10% FBS/1% NEAA/1% penicillin + streptomycin) and dispersed in the medium by repeated pipetting. Seeding density should be checked to ensure that new monolayers are not more than 50% confluent at the time of harvesting. Cell suspension was added to 500 mL of the same medium at a final density of $1.2\times10^8$ cells per mL. 25 μL of this cell suspension (typically 3000 cells per well) were dispensed into the wells of 384-well clear bottom Greiner plates using a Multidrop. Prior to addition of the cell suspension, these plates were dispensed with 250 nL of the screening compounds using an Echo 555. Plates were allowed to incubate at 37°C and a relative humidity of 80% for 48 hours in the presence of 5% $CO_2$. After the incubation period, the plates were allowed to equilibrate at room temperature for 30 minutes before proceeding to develop the luminescent signal. The signal developer, CellTiter- Glo™ (Promega) was equilibrated at room temperature for 30 minutes and added to the plates (25 μL per well) using a Multidrop. The plates were left for 10 minutes at room temperature for stabilization and were subsequently read using a ViewLux (Perkin Elmer).

### Physical Chemical Properties

Standard physical chemical property determination methods were used. Please see the Supporting Information for details.

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