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Structural Rigidification of *N*-Aryl-pyrroles into Indoles Active against Intracellular and Drug-Resistant Mycobacteria

Dorothy Semenya, Meir Touitou, Camila Maringolo Ribeiro, Fernando Rogerio Pavan, Luca Pisano, Vinayak Singh, Kelly Chibale, Georg Bano, Anita Toscani, Fabrizio Manetti, Beatrice Gianibbi, and Daniele Castagnolo*

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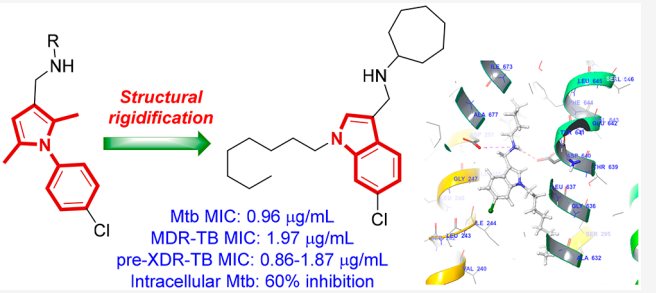
ABSTRACT: A series of indolyl-3-methyleneamines incorporating lipophilic side chains were designed through a structural rigidification approach and synthesized for investigation as new chemical entities against *Mycobacterium tuberculosis* (Mtb). The screening led to the identification of a 6-chloroindole analogue **7j** bearing an *N*-octyl chain and a cycloheptyl moiety, which displayed potent *in vitro* activity against laboratory and clinical Mtb strains, including a pre-extensively drug-resistant (pre-XDR) isolate. **7j** also demonstrated a marked ability to restrict the intracellular growth of Mtb in murine macrophages. Further assays geared toward mechanism of action elucidation have thus far ruled out the involvement of various known promiscuous targets, thereby suggesting that the new indole **7j** may inhibit Mtb via a unique mechanism.



KEYWORDS: *Tuberculosis, MDR-TB, XDR-TB, Indole, Pyrrole, Antimicrobial resistance*

Tuberculosis (TB) is an ancient scourge that blights millions of lives annually. In 1882, Robert Koch discovered *Mycobacterium tuberculosis* (Mtb) as the etiologic agent of TB, heralding multitudinous advances against its pathogenesis.[1] TB has ranked above HIV/AIDS as the leading deadliest infectious disease since 2007, which spotlights its implacable threat to public health security; this burden is further aggravated by the rampant emergence of drug resistance in Mtb.[2−4] In 2019, an estimated 10 million people developed TB and 1.4 million died. It is also estimated that close to a quarter of the global population is latently infected with Mtb, and the risk of progression to the symptomatic TB disease is about 5−10% over a lifetime.[2,5] First-line treatment of active TB hinges on gruelling six-month regimens comprising isoniazid, rifampicin, ethambutol, and pyrazinamide at a cost of around $40 USD per patient. MDR-TB that is resistant to isoniazid and rifampicin requires a more protracted course of treatment, which is not only inordinately expensive (≥$1000 USD per person) but also involves more toxic drugs and is vitiated by poor adherence.[2,6,7] Reports of more severe forms of TB, including pre-extensively drug-resistant (pre-XDR) TB that displays greater recalcitrance to the current anti-TB drug armamentarium and below-par therapy success rates, paint a grim picture.[8−11] These exigencies necessitate intensified and sustained TB mitigation efforts, all the more so in light of the unprecedented COVID-19 pandemic that has cast a shadow over strides made thus far.

Drug discovery and development is central to TB research and innovation, but it is a laborious process that is faced with innumerable stumbling blocks, including, inter alia, a paucity of research funds and investments, high drug attrition rates, biological complexities of Mtb, and the lack of animal models that are representative of TB pathogenesis as seen in human hosts.[2,12] In addition to the acquisition of mutations in genes encoding drug targets or drug-activating enzymes, Mtb's inherent characteristics (e.g., the highly impermeable lipid-rich cell wall or the intrinsic activity of efflux pumps) confer resistance to antibiotics. Furthermore, Mtb is capable of altering its metabolic state from active growth to dormancy, effectuating phenotypic antibiotic resistance. This transition in itself is a challenge for the treatment of TB infections.[13−15] In spite of these challenges, 22 anti-TB drug candidates and various combination regimens were in clinical trials as of August 2020.[2,16]

Some candidates in the development pipeline represent novel scaffolds or appealing drug targets, *e.g.*, oxaborole GSK-

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3036656 that targets leucyl-tRNA synthetase, which is crucial for protein synthesis, and benzothiazinones (BTZ043 and macozinone) and ethylenediamine SQ109, which interfere with the cell wall assembly by blocking decaprenyl-phosphoribose-2′-epimerase (DprE1) and mycobacterial membrane protein large 3 (MmpL3), respectively. Moreover, three newly approved drugs have spurred renewed hope for tackling drug-resistant TB: bedaquiline (a diarylquinoline inhibiting ATP synthase) and two nitroimidazoles (delamanid and pretomanid) that target cell wall biosynthesis and cell respiration.[14,16−18] Despite this ray of hope, there remains a need to ramp up TB drug discovery and development efforts so as to launch medicines that are not only safe and effective but also affordable and accessible.

Nitrogen-containing heterocycles are considered privileged and versatile scaffolds that constitute the core frameworks of many bioactive molecules and have thus garnered notable attention in library design of prospective drugs.[19−21] In the recent past, we reported the discovery of a series of antitubercular *N*-aryl-2,5-dimethylpyrroles, including **1** and **2** (Figure 1) that were designed as molecular hybrids of the drugs BM212 and SQ109 and were endowed with activity against drug-resistant mycobacteria.[22−24] In an effort to interrogate the structure−activity relationship (SAR) of our pyrroles more extensively, and with the aim to improve their properties or to find similar potent compounds that exist in a novel chemical space, we adopted a structural rigidification strategy to design new antitubercular agents by substituting the *N*-aryl-2,5-dimethylpyrrole moiety of **1** and **2** with an indole nucleus. While our previous studies showed that replacing the sole pyrrole ring of **1** and **2** with other heterocyclic cores was detrimental to the antitubercular activity,[24] indole motifs have recently attracted interest as valuable scaffolds in the discovery of effective antimycobacterial agents.[25−30] Herein, we describe the design, synthesis, and biological evaluation of a series of structurally related indole compounds with potent activities against MDR-, pre-XDR-, and intracellular Mtb clinical isolates. In this respect, we also provide a preliminary account of their mechanism of action (MoA) (Figure 1).

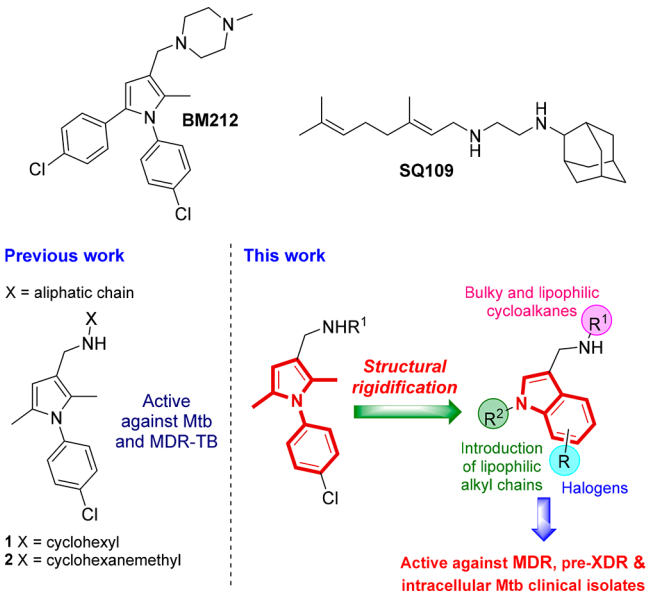


Figure 1. Previously identified antitubercular pyrroles **1** and **2** and the rationale behind this study.

We designed a new series of indoles **5a−l**, taking into consideration SAR deductions and molecular docking observations from our antimycobacterial pyrrole studies (Scheme 1 and Table 1).[22−24] In line with our previous SAR

Scheme 1. Main Synthetic Routes Followed for the Synthesis of the Desired Indole Derivatives 5a−l and 7a−m

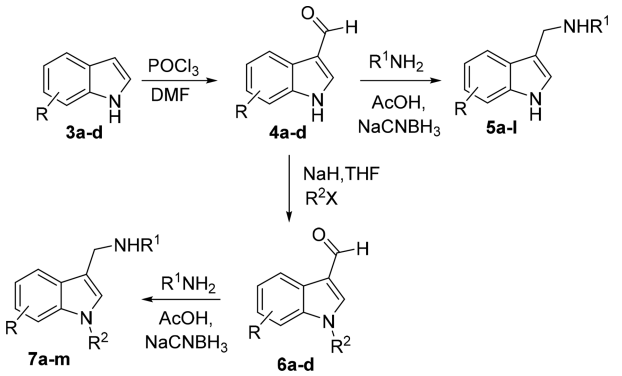
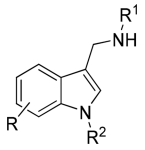


Table 1. Structures of the Indole Derivatives 5a−l and 7a−m and Activity against Mtb H37Rv[a]



compound	R	R^2	R^1	MIC (μg/mL)
5a	6-Cl	H	cyclohexyl	>25
5b	5-Cl	H	cyclohexyl	16.33 ± 0.01
5c	5-Me	H	cyclohexyl	>25
5d	6-Cl	H	cyclohexanemethyl	24.20 ± 0.93
5e	H	H	cyclohexanemethyl	>25
5f	6-Cl	H	cycloheptyl	17.14 ± 5.30
5g	5-Me	H	cycloheptyl	24.72 ± 0.32
5h	H	H	cycloheptyl	>25
5i	6-Cl	H	cyclooctyl	>25
5j	H	H	cyclooctyl	>25
5k	6-Cl	H	2-adamantyl	6.07 ± 0.22
5l	6-Cl	H	benzyl	22.83 ± 2.41
7a	6-Cl	Me	cyclohexyl	>25
7b	6-Cl	Me	cyclohexanemethyl	19.09 ± 4.91
7c	6-Cl	Me	cyclooctyl	23.25 ± 1.75
7d	6-Cl	Me	2-adamantyl	10.93 ± 1.38
7e	6-Cl	Me	benzyl	>25
7f	6-Cl	*i*Pr	cyclohexyl	23.87 ± 0.36
7g	6-Cl	*i*Pr	cyclohexanemethyl	6.09 ± 0.28
7h	6-Cl	*i*Pr	cycloheptyl	16.32 ± 4.98
7i	6-Cl	*i*Pr	cyclooctyl	14.00 ± 3.23
7j	6-Cl	Octyl	cycloheptyl	0.96 ± 0.22
7k	6-Cl	Octyl	benzyl	19.73 ± 0.09
7l	6-Cl	Octyl	cyclohexanemethyl	1.56 ± 0.24
7m	6-Cl	Geranyl	cycloheptyl	2.75 ± 0.79
1				0.2[22]
2				0.3[24]

[a]The results are the mean ± standard deviation of three independent tests.

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ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

considerations, the first series was designed by appending bulky and lipophilic amino-cycloalkanes to the C3 position of either unsubstituted or C5- or C6-substituted indole scaffolds to probe substituent effects. The synthesis of the derivatives is depicted in Scheme 1.[23,24] In brief, compounds **5a−l** were prepared via a facile two-step synthesis starting from the Vilsmeier−Haack formylation of commercially available indoles **3a−d**, followed by reductive amination of aldehydes **4a−d** using $NaCNBH_3$ as the reducing agent (Scheme 1).

In our previous work, we postulated an MmpL3 inhibition mechanism for our *N*-aryl-pyrroles based on their structural similarity to the known inhibitors BM212 and SQ109.[31] Based on this premise, we hypothesized a similar MoA for the indole compounds and thus adopted a molecular docking-aided approach to generate a second series of derivatives bearing substituents at the N1 position, with the aim to improve their activity.

The *N*-substituted indoles were docked into a Mtb MmpL3 homology model[24,32,33] and their binding poses were compared to those of pyrrole **2** and SQ109 (a comparison of the best-scoring binding poses of both SQ109 and **7j** is reported in Figure S1, while the best scoring binding poses of **5f**, **7a**, **7h**, and **7m** are reported in Figure S3). The molecular docking study suggested that the introduction of lipophilic aliphatic groups of varying sizes at the N1 position could lead to a better interaction of the compounds with the hydrophobic binding pocket of MmpL3 (Figure 2). Thus, a series of *N*-substituted indoles **7a−m** were synthesized by alkylating 6-chloro-1*H*-indole-3-carbaldehyde **4a** with the appropriate alkylating agents in the presence of NaH and the subsequent reductive amination mentioned previously (Scheme 1).

First, we evaluated the minimal inhibitory concentration (MIC) of the indole derivatives **5a−l** and **7a−m** against drug-sensitive Mtb H37Rv using a resazurin-based microtiter assay in 96-well plates (Table 1).[34,35] As a general trend, most of the compounds exhibited good antimycobacterial activities. Indole **5k** bearing a 2-adamantyl moiety on the methyleneamine side chain at C3 was the most active among compounds **5a−l**, which lacked alkyl substituents at the N1 position of the scaffold.

The presence of a chloro substituent instead of a methyl group on the indole core appeared advantageous, as exemplified by compounds **5b**, **5c**, **5f**, and **5g**. Interestingly, the derivative **5d**, bearing the same functionalities as the pyrrole hit **2**, displayed a poorer activity. On the other hand, enhancing the lipophilic character of the analogues by introducing methyl, isopropyl, octyl, or geranyl groups on the N1 position proved to be favorable, leading to a pronounced improvement in activity.

An analysis of the biological data allowed us to define several SAR rules. Compounds with an eight-carbon-long hydrophobic side chain showed the best activity, and the saturated octyl appendage of **7j** was preferred to the unsaturated chain of the same length present in **7m** (0.96 and 2.75 μg/mL, respectively; the best docked pose of **7m** is represented in Figure S2). Among N1-octyl derivatives, the cycloalkyl analogues **7j** and **7l** showed similar activities (0.96 and 1.56 μg/mL, respectively), while the benzyl analogue **7k** showed a significant decrease in activity (19.7 μg/mL). This suggests that an aromatic portion is not tolerated in such a position. This was also confirmed by **5l** and **7e**, which showed activity values higher than 20. Reducing the size of the N1 substituent led to low-active or inactive compounds. As an example,

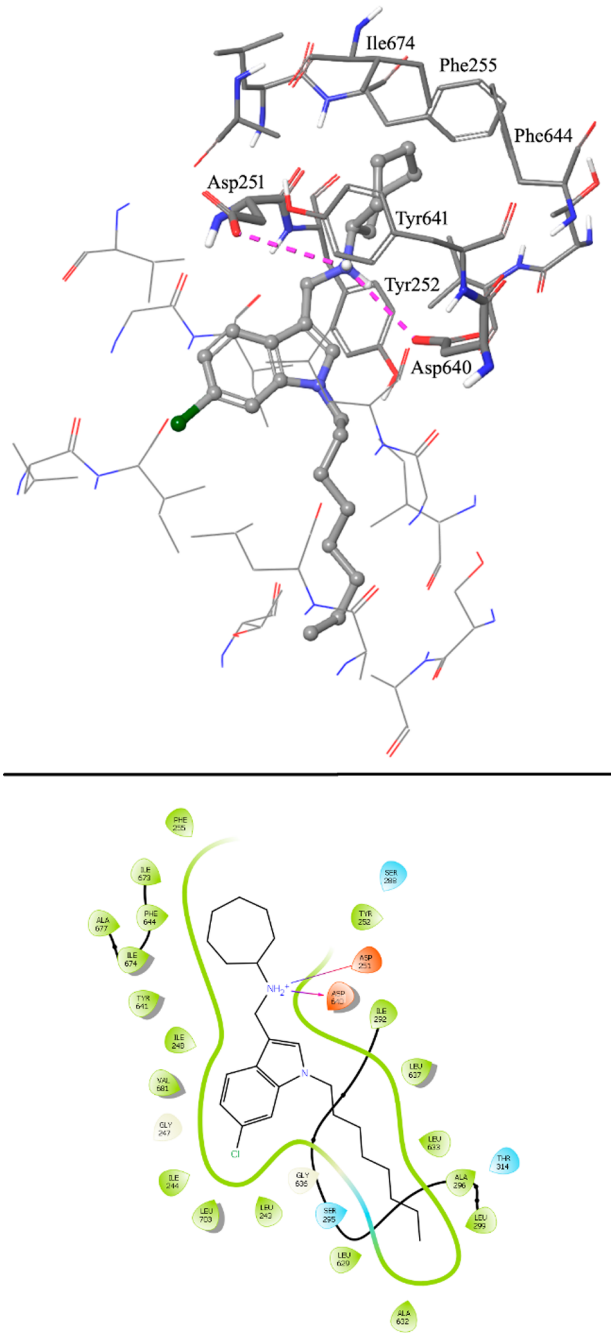

Figure 2. Best docked pose of *N*-alkyl indole **7j** into the Mtb MmpL3 homology model. Amino acids of the hydrophobic cage surrounding the heptylamino side chain are represented by thin tubes, and some of them are also labeled; Asp251 and Asp640, which are responsible for two salt bridges with the basic amino group of **7j**, are represented by thick tubes, while the remaining amino acids that constitute the ligand binding site are in wire representation. The ligand is in ball and stick representation.

among the C3-cyclohexanemethyl analogues, shortening the octyl chain of **7l** to isopropyl, methyl, and hydrogen (as in **7g**, **7b**, and **5d**, respectively) led to about 4−15-fold reduction in activity (from 1.56 to 6.09, 19.1, and 24.2 μg/mL, respectively). A very similar trend was observed among the cycloheptyl analogues, with activities ranging from 0.96 (found for the octyl derivative **7j**) to 16.3 (for the isopropyl analogue **7h**) and 17.1 μg/mL (for the N1 unsubstituted analogue **5f**).

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ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

Finally, the adamantyl derivatives **5k** and **7d** showed interesting antimycobacterial activities (6 and 10 μg/mL, respectively) that were independent of the presence of a long alkyl chain at N1.

The four derivatives with the highest *in vitro* antimycobacterial activities for each N1-susbstituted group, namely **5k**, **7d**, **7g**, and **7j**, were selected for additional screening. The four compounds were first screened against murine macrophages (J774A.1 cell line) to assess their cytotoxicity profiles and to determine their selectivity indices (SIs) (Table 2). Again, the most active compound **7j** showed a low cytotoxicity (IC_{50} = 10.62 μg/mL) and a SI greater than 10, thus turning out to be the best hit of the whole series.

Table 2. Cytotoxicity of Select Derivatives against Murine Macrophages[a]

compound	CLogP	IC_{50} (μg/mL)	MIC (μg/mL)	SI
5k	6.08	7.51 ± 2.58	6.07	1.24
7d	6.42	16.19 ± 4.26	10.93	1.48
7g	6.21	14.57 ± 3.50	6.09	2.39
7j	9.01	10.62 ± 4.02	0.96	11.06

[a]The results are the mean ± standard deviation of three independent tests. The selectivity index was calculated as a ratio between the IC_{50} and the MIC values observed against Mtb H37Rv.

Encouraged by the potent *in vitro* activity of **7j**, it was further assayed against a panel of MDR and pre-XDR mycobacterial clinical isolates. Compound **7j** displayed excellent activities against all the drug-resistant clinical isolates CI1−4, with MICs below 2 μg/mL and in some respects a superior profile in comparison to those of the reference clinical antibiotics (Table 3). In particular, **7j** showed potent activity against the more recalcitrant pre-XDR Mtb CI3, with an MIC value of 0.86 μg/mL similar to that against the drug-susceptible Mtb H37Rv strain (0.96 μg/mL) and >10-fold more potent than that of the second-line drug moxifloxacin (Mox).

Countering Mtb's persistence, which is partly attributable to its ability to subvert host immune responses and survive in macrophages, is a priority of myriad anti-TB drug discovery campaigns in their infancy.[36,37] In this regard, indole **7j** was also assayed against murine macrophages infected with Mtb, where previously an incubation time of 72 h ensured 100% macrophage growth at the concentration tested, to assess its ability to traverse cell membranes and inhibit the replication of intracellular bacilli.

The activity against intracellular Mtb is presented as the Log_{10} colony forming unit (CFU) per milliliter, and the results of **7j** and the reference antibiotic moxifloxacin are compared with an untreated control. Interestingly, **7j** showed a reduction of 0.53 Log_{10} CFU/mL at a concentration 1 × MIC, and moxifloxacin only inhibited 0.11 Log_{10} CFU/mL under the same conditions (1 × MIC). (Figure 3).

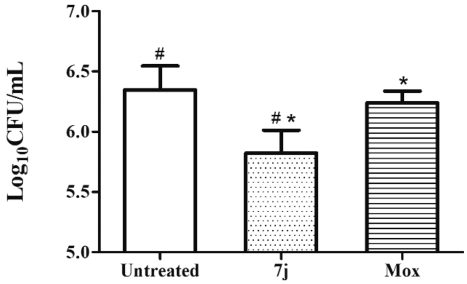

Figure 3. Intramacrophagic activity of **7j** and moxifloxacin (Mox) against Mtb-infected murine macrophages (J774A.1 cell line). Macrophages grew in the presence of **7j** and Mox at the concentration tested to ensure 100% cell growth. The concentration was 1 × MIC (0.17 μg/mL for Mox and 0.96 μg/mL for **7j**). According to one-way ANOVA with Newman-Keuls's post-test ($p < 0.05$), the pound sign (#) and asterisk (*) indicate significantly different results. The results are the mean ± standard deviation of at least two independent assays.

The indole derivatives **5** and **7** were designed as analogues of **1** and **2**, which were in turn synthesized as hybrids of the MmpL3 inhibitors BM212 and SQ109. Thus, a similar MoA was initially hypothesized for compound **7j**. However, with the aim to demonstrate experimentally the mechanism by which **7j** inhibits Mtb, we embarked on target deconvolution studies to elucidate its mechanistic profile. As a starting point, **7j** was tested in biology triage assays to assess whether known promiscuous targets are implicated in its MoA (Table S1). Compound **7j** did not show a positive signal in two standard bioluminescence reporter assays, namely PiniB-LUX, which detects the modulation of *iniB* expression if a test compound is implicated in Mtb cell wall damage, and PrecA-LUX, which detects modulation in *recA* expression as indicator of compounds that disrupt DNA integrity. Moreover, it did not show MIC modulation against mutant strains, including MmpL3 (G253E), DprE1 (C387S), CydA-KO, QcrB (A317T), and monoresistant strains of standard anti-TB drugs (isoniazid, rifampicin, ethionamide, and moxifloxacin).

Altogether, these results seemingly suggest that **7j** exerts its antimycobacterial action via a novel mechanism. However, given the good binding affinity of **7j** to MmpL3 observed in computational studies, it cannot be excluded that MmpL3 may play a role in the mechanism of the indole compounds, such as being involved in the transport of molecules from an extracellular space to an intracellular target. Mutagenesis and genome sequencing studies are currently in progress in our laboratories to ascertain the MoA of the newly identified antitubercular compound **7j**.

Table 3. MICs (μg/mL) of 7j and Clinical Antibiotics against Mtb H37Rv and Four Clinical Isolates[a]

compound	H37Rv	clinical isolate CI1	clinical isolate CI2	clinical isolate CI3	clinical isolate CI4
7j	0.96 ± 0.22	1.97 ± 0.44	1.87 ± 0.68	0.86 ± 0.20	0.69 ± 0.04
rifampicin (RIF)	<0.10	>25	0.65 ± 0.16	>25	<0.10
isoniazid (INH)	0.30 ± 0.07	>25	>25	>25	0.25 ± 0.07
amikacin (AMK)	0.27 ± 0.06	0.32 ± 0.08	23.13 ± 2.49	>25	0.14 ± 0.05
moxifloxacin (Mox)	0.17 ± 0.08	0.20 ± 0.06	0.19 ± 0.09	10.26 ± 1.83	<0.10
classification	susceptible laboratory strain	MDR-TB	resistant to INH and AMK	pre-XDR-TB	susceptible clinical isolate

[a]The results are the mean ± standard deviation of three independent tests.

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In conclusion, a new series of indoles (**5a−l** and **7a−k**) were designed through a structural rigidification approach as analogues of the antitubercular *N*-aryl-2,5-dimethylpyrroles **1** and **2** in an effort to explore their chemical space more exhaustively. The set of derivatives were synthesized and screened against Mtb, which led to the identification of compound **7j** with outstanding *in vitro* potency against the drug-sensitive strain H37Rv as well as MDR and pre-XDR clinical isolates. **7j** also proved to be effective against intracellular Mtb bacilli, showing approximately 60% growth inhibition at 1 × MIC. Finally, preliminary MoA studies have suggested that known promiscuous targets such as DprE1 are likely not implicated in the mechanism of **7j**. Further investigations are currently underway to fully elucidate its MoA and ascertain its biological target.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00431.

General procedures for the synthesis and biological evaluation of the compounds and full characterizations of compounds **5** and **7** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Daniele Castagnolo − *School of Cancer and Pharmaceutical Sciences, King's College London, London SE1 9NH, United Kingdom;* orcid.org/0000-0002-7517-5732; Email: daniele.castagnolo@kcl.ac.uk

Authors

Dorothy Semenya − *School of Cancer and Pharmaceutical Sciences, King's College London, London SE1 9NH, United Kingdom*

Meir Touitou − *School of Cancer and Pharmaceutical Sciences, King's College London, London SE1 9NH, United Kingdom*

Camila Maringolo Ribeiro − *Tuberculosis Research Laboratory, School of Pharmaceutical Sciences, Sao Paulo State University (UNESP), 14800-903 Araraquara, Brazil*

Fernando Rogerio Pavan − *Tuberculosis Research Laboratory, School of Pharmaceutical Sciences, Sao Paulo State University (UNESP), 14800-903 Araraquara, Brazil*

Luca Pisano − *School of Cancer and Pharmaceutical Sciences, King's College London, London SE1 9NH, United Kingdom*

Vinayak Singh − *Drug Discovery and Development Centre (H3D) and South African Medical Research Council Drug Discovery and Development Research Unit, Department of Chemistry and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch 7701, South Africa;* orcid.org/0000-0001-9002-2489

Kelly Chibale − *Drug Discovery and Development Centre (H3D) and South African Medical Research Council Drug Discovery and Development Research Unit, Department of Chemistry and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch 7701, South Africa;* orcid.org/0000-0002-1327-4727

Georg Bano − *School of Cancer and Pharmaceutical Sciences, King's College London, London SE1 9NH, United Kingdom*

Anita Toscani − *School of Cancer and Pharmaceutical Sciences, King's College London, London SE1 9NH, United Kingdom*

Fabrizio Manetti − *Dipartimento di Biotecnologie, Chimica e Farmacia, Università di Siena, I-53100 Siena, Italy;* orcid.org/0000-0002-9598-2339

Beatrice Gianibbi − *Dipartimento di Biotecnologie, Chimica e Farmacia, Università di Siena, I-53100 Siena, Italy*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsmedchemlett.1c00431

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; MDR, multidrug-resistant; XDR, extensively drug-resistant; MIC, minimum inhibitory concentration

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