The 7-phenyl benzoxaborole series is active against *Mycobacterium tuberculosis*

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**A B S T R A C T**

We identified a series of novel 7-phenyl benzoxaborole compounds with activity against *Mycobacterium tuberculosis*. Compounds had a range of activity with inhibitory concentrations (IC\(_{90}\)) as low as 5.1 \(\mu\)M and no cytotoxicity against eukaryotic cells (IC\(_{50}\) > 50 \(\mu\)M). Compounds were active against intracellular *Mycobacterium tuberculosis* cultured in THP-1 macrophages. We isolated and characterized resistant mutants with mutations in NADH dehydrogenase (Ndh) or the regulatory protein Mce3R. Mutations suggest that Ndh may be the target of this series.

Tuberculosis (TB) represents a serious threat to global health as one of the top 10 causes of death worldwide with increasing rates of new infections [1]. Current treatments suffer from long treatment time and a growing problem of multidrug resistance. There is therefore a need for new molecules with anti-tubercular activity as well as new targets for the development of treatments.

The combination of two privileged structures [2], the biphenyls and the benzoxaboroles, identified a novel 7-phenyl benzoxaborole series with activity against *Mycobacterium tuberculosis* in vitro. Three compounds with a 7-phenyl benzoxaborole structure were tested for activity against *Mycobacterium tuberculosis* (Fig. 1 and Supplementary Information). Compounds had activity in both liquid and solid medium (Table 1). We determined inhibitory concentrations under aerobic conditions as previously described [3]. Compounds were solubilized in DMSO and tested as 10-point two-fold serial dilutions. Bacteria were cultured in Middlebrook 7H9 medium supplemented with 10% v/v OADC (oleic acid, albumin, dextrose, catalase) and 0.05% w/v Tween-80. Assay plates were inoculated with *M. tuberculosis* H37Rv LP (ATCC 25618) [4] and incubated for 5 days at 37 °C. Growth was measured using OD\(_{590}\) and plotted using the Levenberg-Marquardt algorithm. The IC\(_{90}\) was defined as the concentration at which 90% growth was inhibited. All three compounds were active against replicating bacteria. AN11987 had good activity, with an IC\(_{90}\) of 5.1 \(\mu\)M (Table 1). AN6288 and AN6291 had less activity, with IC\(_{90}\) of 80 \(\mu\)M and 55 \(\mu\)M respectively (about 10-fold less active). Compounds were also active on solid medium. We determined the MIC\(_{99}\) (defined as the concentration required to prevent 99% growth) for each compound on Middlebrook 7H10 containing 10% v/v OADC. Again, AN11987 was the most active (MIC\(_{99}\) = 5 \(\mu\)M) as compared to AN6288 and AN6291 (MIC\(_{99}\) = 12.5 \(\mu\)M), although the difference was less pronounced (Table 1).

We also measured compound activity against intracellular bacteria. We infected the THP-1 human mononuclear cell line (ATCC-TIB202). Briefly THP-1 cells were maintained in RPMI-1640, 10% v/v FBS, 2 mM Corning glutagro, 1 mM sodium pyruvate and differentiated using 80 nM phorbol 12-myristate 13-acetate. Cells were infected at a multiplicity of infection of 1 with *M. tuberculosis* constitutively expressing luciferase [5], washed to remove extra-cellular bacteria and seeded into 96-well plates containing compounds. Infected cells were cultured for 72 h and bacterial viability assessed by RLU. The cytotoxicity of compounds was determined against uninfected THP-1 cells exposed to compounds for 72 h; cell viability was measured using CellTitert-Glo (Promega). For both assays, curves were fitted using the Levenberg-Marquardt algorithm. The IC\(_{50}\) and IC\(_{90}\) were defined as the concentrations that reduced cell/bacterial viability by 50% or 90% respectively. All three compounds showed good intracellular activity with no cytotoxicity (IC\(_{50}\) > 50 \(\mu\)M) (Table 1). The activity trend seen in axenic culture was preserved against intracellular bacteria, with AN11987 being the most active, IC\(_{90}\) = 1.8 \(\mu\)M AN6288 was the least active, with IC\(_{90}\) = 30 \(\mu\)M. AN6291 had intermediate activity.
Concentrations required to reduce bacterial/cell viability in the intracellular macrophage activity assay by 50% and 90% respectively. Intracellular activity is against *M. tuberculosis* whereas the cytotoxicity is against THP-1 cells.

**Biological activity of compounds.** Compounds were tested for activity in liquid or solid medium. MIC<sub>99</sub> is the concentration required to inhibit growth by 99%. IC<sub>50</sub> and IC<sub>90</sub> are the concentrations required to reduce bacterial/cell viability in the intracellular macrophage activity assay by 50% and 90% respectively. Intracellular activity is against *M. tuberculosis*, cytotoxicity is against THP-1 cells.

| Compound | Liquid (μM) | MIC<sub>99</sub> (μM) | IC<sub>50</sub> (μM) | IC<sub>90</sub> (μM) |
|----------|-------------|----------------------|---------------------|---------------------|
| AN6288   | 80 ± 30 (n = 4) | 12.5 | 15 | 50 > 50 |
| AN6291   | 55 ± 13 (n = 3) | 12.5 | 3.9 | 5.0 > 50 |
| AN11987  | 5.1 ± 1.5 (n = 4) | 5.0 | 0.69 | 1.8 > 50 |

Although the target for the 3-aminomethyl benzoxaboroles in *M. tuberculosis* is leucyl-RNA synthetase (LeuRS) [6] the target and/or mechanism of resistance for these 7-phenyl benzoxaboroles compounds is unknown. We isolated resistant mutants on solid medium [7]. *M. tuberculosis* was plated onto solid medium containing 5X or 10X the MIC<sub>99</sub>. Colonies were isolated and confirmed for resistance in solid or liquid medium. We isolated and confirmed resistant mutants for AN6291 (7 mutants), AN6288 (2 mutants) and AN11987 (3 mutants). We selected three mutants resistant to AN6291 and two mutants resistant to AN6288 for whole genome sequencing.

Whole genome sequencing and mutation identification was conducted as described in Ioerger et al. 2010 [4] using an Illumina HiSeq 2500 and either 72 × 72bp paired-end reads or 54 × 54 paired-end reads. We identified a number of single nucleotide polymorphisms (SNPs) and a large deletion. Resistant mutants raised against AN6288 contained mutations within *ndh*. Colonies were isolated and confirmed resistant mutants for AN6291 (7 mutants), AN6288 (2 mutants) and AN11987 (3 mutants). We selected three mutants resistant to AN6291 and two mutants resistant to AN6288 for whole genome sequencing.

**Mutations identified in resistant isolates.** Strains were isolated on 5X or 10X MIC on solid medium and were confirmed as resistant by determining MICs. AN6291-RM1 had a large deletion encompassing mce3R. Mutations were identified by whole genome sequencing in five strains (indicated by an asterisk). Mutations in the remaining strains were identified by PCR amplification and sequencing of the gene.

| Compound | Strain | Gene(s) | Mutation | Amino Acid change |
|----------|--------|---------|----------|-------------------|
| AN6288   | RM1<sup>*</sup> ndh | 667C > T | A         | P223T             |
| AN6288   | RM2<sup> </sup> ndh | 1262 T > C | A         | F421S             |
| AN6291   | RM1<sup>*</sup> Rv1948c-Rv1970 | deletion | deletion | deletion         |
| AN6291   | RM2<sup> </sup> mce3R | 334 T > C | A         | W112R             |
| AN6291   | RM3<sup> </sup> mce3R | +149–150 deletion | deletion | W112R             |
| AN6291   | RM4<sup> </sup> mce3R | 334 T > C | A         | W112R             |
| AN6291   | RM5<sup> </sup> mce3R | 563G > T | A         | C188F             |
| AN6291   | RM6<sup> </sup> mce3R | 555C > A | A         | Y185<sup>*</sup> |
| AN6291   | RM7<sup> </sup> mce3R | +649–936 deletion | deletion | V332 M             |
| AN11987  | RM1<sup> </sup> ndh | 994G > A | A         | V332 M             |
| AN11987  | RM2<sup> </sup> ndh | 994G > A | A         | V332 M             |
| AN11987  | RM3<sup> </sup> ndh | 998C > T | A         | A333V             |

Table 2 was confirmed by PCR amplification and sequencing from genomic DNA. We sequenced *ndh* and mce3R in the remaining isolates. All the strains isolated against AN6291 had mutations in mce3R, whereas the strains isolated against AN6288 and AN11987 had mutations in *ndh* (Table 2).

We tested selected strains for cross-resistance; MICs were determined on solid medium using a modified version of the MIC<sub>99</sub> using 96-well plates and a smaller inoculum to conserve compound. Again, we saw the same trend of activity with AN11987 being most active (MIC = 0.2 μM) (Table 3).

**Cross-resistance profile of resistant strains.** MICs were measured on solid medium in 96-well format. MICs were defined as the minimum concentration required to inhibit growth completely. *AN6291-RM1 had a large deletion encompassing mce3R.

| Strain | Gene | Mutation | Amino Acid change | MIC solid (μM) |
|--------|------|----------|-------------------|----------------|
| AN6288 | ndh  | 667C > A | P223T             | 12.5 12.5 0.2 |
| AN6288 | RM1<sup> </sup> ndh | 1262 T > C | F421S             | 50 50 25    |
| AN6291 | ndh  | 667C > A | P223T             | 12.5 12.5 0.2 |
| AN11987| ndh  | 667C > A | P223T             | 50 50 25    |
| AN6291 | mce3R | 563G > T | C188F             | 50 100 3.1  |
| AN11987| mce3R | 555C > A | Y185<sup>*</sup> | 50 100 3.1  |

Fig. 1. Synthetic pathway of 7-phenyl benzoxaborole compounds. R = H, X = Br; R = Me, X = I; a) (CH2O)n, MgCl2, TEA, MeCN, reflux, overnight; b) MOM3, DIPEA, DCM, 0 °C, 1 h, 90%; c) 2-cyanophenylboronic acid, Pd(PPh3)2Cl2, Na2CO3, 1,4-dioxane, H2O, 80 °C, 12 h, 46–56%; d) 2 N HCl (aq), THF, 50 °C, 4 h, 64–93%; e) TEO, pyridine, DMAP, DCM, rt, 2 h, 45–70%; f) PIN1B2, KOAc, Pd(PPh3)2Cl2, 1,4-dioxane, Na2CO3, 80 °C, 6 h, 67–87%; g) NaNH4, MeOH, THF, rt, 0.5 h; 6 N HCl (aq), rt, 0.5 h, 56–66%; h) NaOH, H2O, 80 °C, 8 h, 32–64%.
Mce3R is a TetR transcriptional repressor that negatively regulates genes involved in lipid metabolism and redox reactions [16]. The Mce3R mutations were inactivating mutations, including premature stop codons, a frameshift and a large deletion covering mce3R (Table 2). This suggests Mce3R might control genes involved in resistance, since deletion of Mce3R activity would lead to upregulation of regulon members. Genes controlled by Mce3R include a putative epoxide hydrolase (Rv1938), a monoxygenase (Rv1936), a methyltransferase (Rv1498c) and a succinate-semialdehyde dehydrogenase (Rv0234c) as well as several synthetases and conserved hypotheticals of unknown function. Since MceR3 is a regulatory protein, we propose that a regulon member might be involved in compound degradation or metabolism. It is interesting to note that the addition of 6-methyl group to AN6291 yielded a more potent compound, while the mutations all mapped to ndh not mce3R, which suggest that this methyl group might interfere with this resistance mechanism.

In conclusion, we identified a new benzoxaborole series, the 7-phenyl benzoxaboroles, that had activity against M. tuberculosis in vitro and may target Ndh rather than LeuRS. The 7-phenyl benzoxaboroles are effective against M. tuberculosis in axenic culture and in macrophage culture, with no cytoxicity. We characterized two routes to resistance, one of which is via gene regulation (Mce3R) and the other in Ndh. It is possible that Ndh represents the target of the 7-phenyl benzoxaborole compounds while Mce3R has some effect on the target or on the compounds through activity of a gene within its regulon. We propose that this is a series with potential for further development and target validation.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at [dx.doi.org/10.1016/j.tube.2017.11.003](https://dx.doi.org/10.1016/j.tube.2017.11.003).

**References**

[1] WHO. Global tuberculosis report. 2015 [http://www.who.int/tb/publications/global_report/en/].
[2] Duarte CD, Barreiro EJ, Fraga CA. Privileged structures: a useful concept for the rational design of new lead drug candidates. Mini Rev Med Chem 2007;7:1108–19.
[3] Ollinger J, Bailey MA, Moraski GC, Casey A, Florio S, Alling T, et al. A dual read-out assay to evaluate the potency of compounds active against Mycobacterium tuberculosis. PLoS One 2013;8:e60531.
[4] Loerger TR, Feng Y, Ganesula K, Chen X, Dobos KM, Fortune S, et al. Variation among genome sequences of H37Rv strains of Mycobacterium tuberculosis from multiple laboratories. J Bacteriol 2010;192:3645–53.
[5] Andreu N, Zehner A, Fletcher T, Elkington PT, Ward TH, Ripoll J, et al. Optimization of bioluminescent reporters for use with mycobacteria. PLoS One 2010;5:e10777.
[6] Palencia A, Li X, Bu W, Choi W, Ding CZ, Easom EE, et al. Discovery of novel oral protein synthesis inhibitors of Mycobacterium tuberculosis that target leucyl-tRNA synthetase. Antimicrob Agents Chemother 2016;60:6271–80.
[7] Loerger TR, O’Malley T, Liao R, Guzin KM, Hickey MJ, Mohaiden N, et al. Identification of new drug targets and resistance mechanisms in Mycobacterium tuberculosis. PLoS One 2013;8:e75245.
[8] Yano T, Rahman M, Anaja KK, Schechter NM, Rubin H, Scott CP. Mycobacterium tuberculosis type II NADH:menaquinone oxidoreductase catalyzes electron transfer through a two-site ping-pong mechanism and has two quinone-binding sites. Biochemistry 2014;53:1179–90.
[9] Weinstein EA, Yano T, Li LS, Avarbock D, Avarbock A, Helm D, et al. Inhibitors of type II NADH:menaquinone oxidoreductase act as a class of antitubercular drugs. Proc Natl Acad Sci U. S. A 2005;102:4548–53.
[10] Shirude PS, Paul B, Roy Choudhury N, Kedari C, Bandodkar B, Ugarkar BG. Quinolinyl pyrimidines: potent inhibitors of NDH-2 as a Novel class of anti-TB agents. ACS Med Chem Lett 2012;3:736–40.
[11] Lee AS, Teo AS, Wong SY. Novel mutations in ndh in isoniazid-resistant Mycobacterium tuberculosis isolates. Antimicrob Agents Chemother 2001;45:2157–9.
[12] Rueda J, Relfast P, Mejia GI, Zapata E, Rozo JC, Ferro BE, et al. Genotypic analysis of genes associated with independent resistance and cross-resistance to isoniazid and ethionamide in Mycobacterium tuberculosis clinical isolates. Antimicrob Agents Chemother 2015;59:7805–10.
[13] Vilchez C, Jacobs JR, WR. Resistance to isoniazid and ethionamide in Mycobacterium tuberculosis: genes, mutations, and causalities. Microbiol Spectr 2014;2. Mgm2-0014-2013.
[14] Heikal A, Nakatani Y, Dunn E, Weimar MR, Day CL, Baker EN, et al. Structure of the bacterial type II NADH dehydrogenase: a monotopic membrane protein with an essential role in energy generation. Mol Microbiol 2014;91:950–64.
[15] Sena FV, Batista AP, Catarino T, Brito JA, Archer M, Vierltler M, et al. Type II NADH:quinoine oxidoreductase from Staphylococcus aureus has two distinct binding sites and is rate limited by quinone reduction. Mol Microbiol 2015;98:272–88.
[16] de la Paz Santamango M, Klepp L, Nuñez-Garcia J, Blanco FC, Soria M, Garcia-Pelayo MC, et al. Mce3R, a TetR-type transcriptional repressor, controls the expression of a regulon involved in lipid metabolism in Mycobacterium tuberculosis. Microbiology 2009;155:2249–55.