A novel 6-benzyl ether benzoxaborole is active against *Mycobacterium tuberculosis in vitro*

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21 **Abstract**

22 We identified a novel 6-benzyl ether benzoxaborole with potent activity against
23 *Mycobacterium tuberculosis*. The compound had a minimum inhibitory concentration of
24 2 µM in liquid medium. The compound was also able to prevent growth on solid medium
25 at 0.8 µM and was active against intracellular bacteria (IC_{50} = 3.6 µM) without
26 cytotoxicity against eukaryotic cells (IC_{50} >100 µM). We isolated resistant mutants (MIC
27 ≥100 µM), which had mutations in Rv1683, Rv3068c and Rv0047c.

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30 Tuberculosis (TB) remains a serious global health problem, with an increase in
31 the reported incidence of new infections combined with increasing levels of drug
32 resistance (1). We are interested in finding both new molecules with anti-tubercular
33 activity and also to determine the mode of resistance to new agents, and/or their
34 molecular targets.

35 In screening the Anacor boron library we identified a member of the 6-
36 benzylether benzoxaborole class, 6-(benzyloxy)-4,7-dimethylbenzo[c][1,2]oxaborol-
37 1(3H)-ol (Figure 1 and Supplementary Information), with good *in vitro* activity against
38 *Mycobacterium tuberculosis* under aerobic conditions. Briefly, we tested the compound
39 in DMSO as two-fold serial dilutions against *M. tuberculosis* H37Rv (ATCC 25618) for 5
40 days in Middlebrook 7H9 medium supplemented with 10% OADC (oleic acid, albumin,
41 dextrose, catalase) and 0.05% w/v Tween-80. Growth was monitored by OD_{590}; the
42 minimum inhibitory concentration (MIC) was determined by fitting the growth inhibition
43 curve using the Levenberg Marquardt algorithm . MIC was defined as the concentration
44 required to inhibit growth by 90% (2). The compound had an MIC of 2.0 $\pm$ 0.24 µM
45 (n=6).

46 The cytotoxicity of the compound was determined in HepG2 cells were cultured
47 in DMEM, 10% fetal bovine serum (FBS), and 1X penicillin-streptomycin solution (100
48 U/mL). Cells were exposed to compounds for 2 days at 37°C, 5% CO_2 (final DMSO
49 concentration of 1%). Cell viability was measured using CellTiter-Glo® Reagent
50 (Promega) and determining relative luminescent units (RLU). Inhibition curves were
51 fitted using the Levenberg–Marquardt algorithm and used to calculate IC_{50} as the

concentration required to reduce cell viability by 50%. We tested the compound using either glucose or galactose as the carbon source and the IC_{50} was >100 µM (n=2) under both conditions. Therefore, we tested the compound for activity against intracellular bacteria using a luminescent strain of *M. tuberculosis* (3). THP-1 cells were infected overnight with *M. tuberculosis* at an MOI of 1 in complete RPMI (RPMI-1640, 10% FBS, 2 mM Corning glutagro, 1 mM sodium pyruvate). Extracellular bacteria were removed by washing and infected cells were seeded at 4 x 10^4 cells per well in 96-well plates containing compounds. Compounds were tested as a 10-point, 3-fold dilution series (0.5% DMSO). Infected cells were incubated for 3 days in a humidified atmosphere of 37°C, 5% CO_2. Relative luminescent units (RLU) were used as a measure of bacterial viability. Growth inhibition curves were fitted using the Levenberg–Marquardt algorithm; the IC_{50} and IC_{90} were defined as the compound concentrations that produced 50% and 90% inhibition of intracellular growth respectively. The IC_{50} and IC_{90} were 3.6 ± 0.07 and 22 ± 12 µM respectively (n=2).

We tested the ability of the compound to prevent growth on solid medium. We plated aerobically-cultured *M. tuberculosis* onto Middlebrook 7H10 plus 10% OADC containing compounds (4). Plates were incubated for 3-4 weeks at 37°C and growth recorded. The MIC_{99} under these conditions was 5 µM; we plated *M. tuberculosis* H37Rv onto solid medium containing 5X or 10X the MIC and isolated colonies isolated after 3-6 weeks. Clones were tested for resistance in liquid and solid medium. Four isolates with high-level resistance were confirmed with an MIC ≥100 µM. DNA isolated from these mutants was subjected to whole genome sequencing (5). Several single

nucleotide polymorphisms were identified (Table 1) and confirmed by PCR amplification and sequencing.

Two of the four strains had mutations in *Rv1683*, while the other two had mutations in *Rv0047c* and *Rv3068c*. The mutations in *Rv0047c* would result in a premature stop codon, while the mutations in *Rv3068c* would result in a threonine to alanine change. The *Rv0047c* gene is located upstream of *ino1*, which is involved in phosphatidylinositol metabolism and is required for growth on inositol (6). Rv0047c is proposed to be co-transcribed with *ino1* suggesting a link with inositol metabolism. Therefore, we determined if additional of inositol had any effect on the compound activity, but we saw no shift in MIC (range 5.4-5.9 μM with 6.25-100 μM inositol). We also tested L-histidine supplementation, but saw no difference (range 3.2-3.8 μM with 10-100 μM inositol). Since the mutation in *Rv0047c* was linked to a mutation in *Rv3068c* in both strains with the same nonsynonymous substitution it is possible the two strains are siblings. The *Rv3068c* gene encodes a non-essential enzyme, PgmA, a putative phosphoglucomutase involved in glucose metabolism.

Rv1638 encodes a possible bifunctional protein involved in catabolism and anabolism of triglycerides (7). In *Mycobacterium bovis*, BCG1721 (homolog of Rv1683) is responsible for accumulation and breakdown of triglycerides (TGs) stored as lipid droplets (LDs) (7). Several studies have shown TGs as carbon source utilized by *M. tuberculosis* in non-replicating persistence phase (8) and the buildup of TGs has been correlated with drug tolerance (9). It is not clear if the mutations we see would affect the enzymatic activity of the protein, or if the mutations might be in an enzyme binding site. However, it is of note that Rv1683 is one of three esterases active in the normoxia,

hypoxia and resuscitation phases of growth underlining its importance (10). Future work should help to elucidate if one of these is the true target, or if there are physiological changes which result in resistance.

In summary, we have identified a novel compound with efficacy against *M. tuberculosis* in both solid and liquid medium, as well as active against intracellular bacteria, but with no cytotoxicity, thus the profile of this compound is encouraging for future development. We have identified two routes to resistance to this compound in Rv1683, or Rv0047c and Rv3068c.

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Mutant isolate	MIC_{99} (μM)	Rv0047c	Rv3068c	Rv1683
RM1	100	wt	wt	L341P
RM2	>100	E128*	T351A	wt
RM3	>100	wt	wt	M200I A201T
RM4	100	E128*	T351A	wt

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150 **Table 1. Profile of resistant mutants**

151 Resistant mutants were isolated on solid medium. MIC_{99} was calculated on solid
152 medium (4). The SNPs listed in the table were identified by whole genome sequencing
153 and confirmatory PCR/sequence in each strain.

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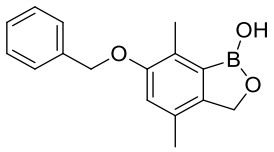
155 **(A)**



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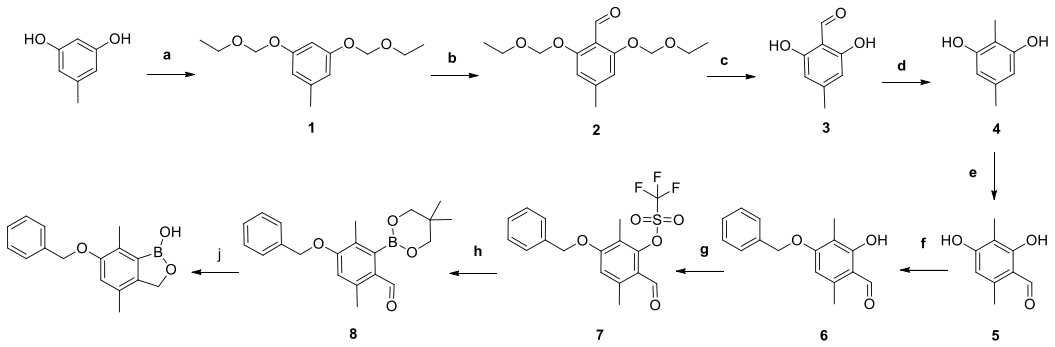
157 **(B)**



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160 **Figure 1. (A) Structure of 6-benzyl ether (B) Synthetic pathway for compound:** a)
161 chloromethyl ethyl ether, DIPEA, DCM, rt, overnight ; b) n-butyl lithium, DMF, THF,
162 18^{o}C, 1.5 h; c) HCl, THF, rt, overnight; d) sodium cyanoborohydride, THF, rt, 3 h; e)
163 Phosphorus oxychloride, DMF, rt, overnight; f) benzyl bromide, $NaHCO_3$, KI, AcCN,
164 80^{o}C, overnight; g) Triflic anhydride, triethylamine, DCM, rt, 3 H; h) 5,5,5',5'-tetramethyl-
165 2,2'-bi(1,3,2-dioxaborinane), $PdCl_2(dppf)_2$, potassium acetate, 1,4-dioxane, 90^{o}C,
166 overnight; j) sodium borohydride, THF, rt, 3 h, and then HCl, water, overnight.

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