1 Identification of novel chemical scaffolds that inhibit the growth of *Mycobacterium tuberculosis* in 2 macrophages

4 Authors

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Abstract

Mycobacterium tuberculosis is an important global pathogen for which new drugs are urgently required. The ability of the organism to survive and multiply within macrophages may contribute to the lengthy treatment regimen with multiple drugs that are required to cure the infection. We screened the MyriaScreen II diversity library of 10,000 compounds to identify novel inhibitors of *M. tuberculosis* growth within macrophage-like cells using high content analysis. Hits were selected which inhibited the intramacrophage growth of *M. tuberculosis* without significant cytotoxicity to infected macrophages. We selected and prioritized compound series based on their biological and physicochemical properties and the novelty of the chemotypes. We identified five chemical classes of interest and conducted limited catalog structure-activity relationship studies to determine their tractability. We tested activity against intracellular and extracellular *M. tuberculosis*, as well as cytotoxicity against murine RAW264.7 and human HepG2 cells. Benzene amide ethers, thiophene carboxamides and thienopyridines were only active against intracellular bacteria, whereas the phenylthiourea series was also active against extracellular bacteria. One member of a phenyl pyrazole series was moderately active against extracellular bacteria. We identified the benzene amide ethers as an interesting series for further work. These new compound classes serve as starting points for the development of novel drugs to target intracellular *M. tuberculosis*.

Keywords

High throughput screening; mycobacteria; anti-tubercular, drug discovery; high content microscopy; macrophage infection; intracellular activity.

37

38 INTRODUCTION

39 Tuberculosis (TB) is the leading cause of death from a bacterial infection worldwide. Despite the
40 availability of chemotherapy, TB is responsible for >10 million infections annually and >1 million deaths
41 (1,2). The increasing incidence of drug failure for TB due to widespread drug-resistant infections
42 underscores the urgent need for new drugs and new bacterial targets for *M. tuberculosis*.

43 *M. tuberculosis* is an intracellular pathogen which can survive and replicate within macrophages; this
44 property relies on the ability to block normal bactericidal activities and turns the macrophage into a niche
45 for replication (3–6). The physiology and metabolism of *M. tuberculosis* within macrophages is very
46 different from extracellular bacteria and so the complement of essential genes differs (7–9). From a drug
47 discovery perspective, this means that intracellular bacteria have additional essential processes that can be
48 targeted by chemical inhibition.

49 Whole cell screening has been broadly used to identify new drug candidates for tuberculosis (10–12). In
50 contrast to target-based drug discovery, in phenotypic screens every essential pathway is a potential drug
51 target. This greatly increases the likelihood of finding inhibitors and overcomes the barrier of obtaining
52 cell-penetrant molecules, which is often a limitation of biochemical screens (13). However, conditionally
53 essential genes that are required for *M. tuberculosis* growth within macrophages or *in vivo*, may be
54 overlooked in phenotypic screens against axenically-cultured *M. tuberculosis*. High-throughput screening
55 in conditions that closely resemble the intracellular environment have the potential to uncover novel
56 antimicrobials that target these conditionally essential genes, in addition to discovering compounds
57 targeting the host cells to increase bacterial elimination (14,15).

58 High-content imaging screening is a powerful technology to identify active compounds by monitoring
59 both bacterial and macrophage cell numbers simultaneously and in the same wells, thus leading to more
60 reliable data and a quicker assessment of compound attractiveness. We previously developed a
61 fluorescence-based, live-cell, high-content analysis (HCA) assay to examine drug efficacy against
62 intracellular *M. tuberculosis* (15).

63 In this study, we screened 10,000 diverse small molecules by high throughput HCA and identified active
64 compounds with low cytotoxicity. We selected a number of series for follow up based on their
65 physicochemical properties and the novelty of the chemotypes and identified five chemical classes of
66 interest. We tested available analogs for each series to complete a limited structure-activity relationship

study. These new compound classes serve as starting points for the development of new series for antitubercular drug discovery.

Methods

Cell culture

Murine RAW 264.7 macrophages (ATCC TIB-71) were grown at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI 1640 medium supplemented with 5% fetal bovine serum, 1 mM sodium pyruvate solution, and 2 mM GlutaGro supplement (Corning). HepG2 cells (ATCC HB-8065) were cultured in Dulbecco-modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), and 1× penicillin streptomycin solution (100 U/ml). *M. tuberculosis* H37Rv-LP (ATCC 25618) constitutively expressing codon-optimized DsRed from plasmid pBlazeC8 (DREAM8) (16) was cultured at 37 °C in Middlebrook 7H9 medium containing 10% v/v OADC (oleic acid, albumin, dextrose, catalase) supplement (Becton Dickinson) and 0.05% w/v Tween 80 (7H9-Tw-OADC) plus 100 µg/mL hygromycin B.

High content screening

Compounds were obtained in 384-well plates as 10 mM DMSO stock solutions and tested as described (15). Briefly, assay plates were prepared in clear bottom, black 384-well plates with 30 µL cRPMI and 0.6 µL compound in columns 3–22 (final concentration of 10 µM compound, 1% DMSO). 1% DMSO was used as the negative control, 10 mM isoniazid (INH) and 100 µM staurosporine (STA) were included as positive controls for anti-tubercular activity and cytotoxicity respectively. RAW267.4 cells were infected with *M. tuberculosis* DREAM8 as described (15) at an MOI of 1 for 24 h and extracellular bacteria removed by washing. Cells were recovered using Accumax, harvested, washed and resuspended in serum-free RPMI; 30 µL of infected cells were dispensed into each well at 3300 cells/well. Plates were incubated for 72 hours, 10 µL of 5X SYBR Green I was added and plates were imaged with an ImageXpress Micro High Content Screening System (Molecular Devices) using a 4x objective and FITC and Texas Red channels. MetaXpress was used to analyze images. The integrated intensity of *M. tuberculosis* or macrophages was calculated for each well. Growth inhibition was calculated for each test well by normalizing to the average integrated intensity of the DMSO control wells. IC₅₀ was calculated as the compound concentration required to reduce bacterial growth by 50%. TC₅₀ was calculated as the compound concentration required to reduce macrophage viability by 50%.

Cytotoxicity

Cytotoxicity against HepG2 cells was measured after 72 hours. Cells were seeded in 384-well plates at 1800 cells per well. Compounds were added as a 10-point three-fold serial dilution after 24 h (final assay

concentration of 1% DMSO). CellTiter-Glo® reagent (Promega) was added and relative luminescence units (RLU) measured. Data were normalized to the DMSO controls. Curves were fitted using the Levenberg–Marquardt algorithm; TC₅₀ was calculated as the compound concentration required to reduce cell viability by 50%.

Minimum inhibitory concentration

Minimum inhibitory concentrations (IC₉₀) against *M. tuberculosis* were determined in liquid medium as described (17). Bacterial growth was measured after 5 days by OD₅₉₀. IC₉₀ was defined as the concentration of compound required to inhibit growth of *M. tuberculosis* by 90% and was determined using the Levenberg–Marquardt least-squares plot.

RESULTS

High throughput screening of a library of small molecules

We were interested in identifying new anti-tubercular molecules with activity against intracellular bacteria. We used our previously validated high throughput high content screen to test the MyriaScreen II library of 10,000 diverse molecules which is comprised of ~60% singletons with diverse structural groups (TimTec/Sigma Aldrich). Molecules were tested at a single concentration of 10 μM (figure 1

A). We monitored cytotoxicity to the macrophages in the same wells to exclude false positives (since the bacteria are unable to replicate if the macrophages are dead). We identified 308 compounds with >70% bacterial growth inhibition; of these we discarded 134 with significant macrophage toxicity, defined as <70% macrophage survival (Figure 1B). This left 174 hit compounds that were non-cytotoxic but restricted the growth of *M. tuberculosis* in macrophages, a hit rate of 1.7%.

Figure 1. High content, high throughput screen against intracellular *M. tuberculosis*.

(A). Compounds were tested for activity against RAW264.7 cells infected with *M. tuberculosis* at an MOI of 1. The primary screen was conducted using a single concentration of each molecule at 10 μM. Inhibition of bacterial growth and macrophage viability were determined for each compound after 72 hours. (A). Anti-tubercular activity measured as % inhibition of bacterial growth. (B) Cytotoxicity (% viability) was plotted compared to inhibition of bacterial growth (% Inhibition). Compounds in the top right quadrant were eliminated from the hit list due to cytotoxicity. (C) Selected hit compounds were purchased and tested in dose response to determine IC₅₀ (concentration required to inhibit bacterial growth by 50%). Number of compounds in each group are noted.

Hit confirmation

We analyzed the 174 resulting hits and prioritized hit compounds based on their physicochemical properties including molecular weight (MW), calculated partition coefficient (cLogP), novelty, structural diversity, chemical tractability, and commercial availability. We selected 43 compounds for further analysis (Figure 2); we purchased new batches of compounds and determined the activity using a dose response format (Figure 1C). Seven compounds exhibited cytotoxicity against eukaryotic cells that hampered our ability to determine their anti-bacterial activity and were excluded from further study (Table 1). The IC₅₀ of the remaining 36 hits was determined (Figure 1C); of these all but one (35/36) had confirmed activity with IC₅₀ <50 μM against intracellular *M. tuberculosis* (Table 1). The majority of the hits (26) had an IC₅₀ <5 μM, and 6 compounds were potent with an IC₅₀ <1 μM (Figure 1C). The majority of the compounds had no cytotoxicity (TC₅₀ >50 μM); a few compounds were cytotoxic at higher concentrations, but retained good selectivity (Table 1).

Figure 2. Structure of primary hits selected for follow up.

Molecule ID	MW	clogP	Intracellular activity	
			Anti-tubercular activity IC ₅₀ (μM)	Cytotoxicity TC ₅₀ (μM)
TPN-0006204	311.34	3.24	11 ± 0.5 (n=2)	>100 (n=2)
TPN-0006205	322.22	3.97	1.4 ± 0.64 (n=4)	57 ± 23 (n=3)
TPN-0006206	426.99	6.41	NC	57 ± 20 (n=3)
TPN-0006207	350.27	5.00	6.3 ± 0.3 (n=2)	>100 (n=2)
TPN-0006209	379.28	4.89	NC	68 ± 0.50 (n=2)
TPN-0006211	285.39	2.80	7.6 ± 3.5 (n=2)	38 ± 6.0 (n=2)
TPN-0006216	330.20	4.55	2.8 ± 0.78 (n=4)	>100 (n=4)
TPN-0006217	276.35	3.32	4.9 ± 2.1 (n=4)	75 ± 16 (n=4)
TPN-0073027	323.41	3.69	5.8 ± 2.1 (n=5)	>100 (n=5)
TPN-0073061	320.19	2.92	1.3 ± 0.15 (n=4)	18 ± 4.3 (n=4)
TPN-0073062	272.37	4.21	0.39 ± 0.13 (n=4)	>100 (n=4)

TPN-0073063	311.40	4.58	0.25 ± 0.23 (n=6)	47 ± 28 (n=6)
TPN-0073559	332.42	4.29	NC	50 ± 24 (n=4)
TPN-0073560	384.88	5.85	NC	23 ± 4.7 (n=4)
TPN-0073562	319.19	4.53	2.9 ± 0.52 (n=3)	16 ± 11 (n=3)
TPN-0073563	329.20	4.09	4.7 ± 1.1 (n=5)	28 ± 7.0 (n=5)
TPN-0073565	355.17	4.82	7.0 ± 1.8 (n=4)	>100 (n=4)
TPN-0073571	399.29	5.15	NC	15 ± 5.0 (n=2)
TPN-0073576	332.42	4.39	1.1 ± 0.36 (n=4)	>100 (n=4)
TPN-0073578	272.14	4.19	1.7 ± 0.85 (n=4)	11 ± 2.2 (n=4)
TPN-0073584	478.58	2.61	5.1 ± 1.7 (n=3)	>100 (n=2)
TPN-0073585	424.94	6.81	0.4 ± 0.11 (n=3)	9.4 ± 1.9 (n=3)
TPN-0073588	456.53	4.67	6.5 ± 1.7 (n=3)	67 ± 8.5 (n=2)
TPN-0089695	356.80	3.76	4.7 ± 1.2 (n=2)	45 ± 17 (n=2)
TPN-0089788	367.19	3.25	2.5 ± 0.57 (n=5)	>100 (n=5)
TPN-0095025	292.80	5.17	4.4 ± 1.7 (n=3)	26 ± 7.1 (n=3)
TPN-0095026	387.67	5.21	NC	19 ± 12 (n=2)
TPN-0095027	295.34	1.45	0.10 ± 0.010 (n=4)	72 ± 16 (n=4)
TPN-0095028	248.30	2.49	4.4 ± 1.3 (n=2)	54 ± 20 (n=2)
TPN-0095029	472.93	5.54	>50 (n=3)	>50 (n=2)
TPN-0095030	228.68	3.11	6.9 ± 1.9 (n=3)	>100 (n=2)
TPN-0095031	361.31	2.41	0.036 ± 0.007 (n=5)	40 ± 25 (n=5)
TPN-0095032	335.25	6.51	3.5 ± 0.83 (n=3)	9.9 ± 0.98 (n=3)
TPN-0095033	370.42	1.15	3.4 ± 2.6 (n=3)	>100 (n=3)
TPN-0095034	431.27	4.32	8.4 ± 2.3 (n=4)	>100 (n=4)
TPN-0095035	462.47	4.78	1.0 ± 0.02 (n=3)	>50 (n=3)
TPN-0095036	379.30	5.22	3.5 ± 0.97 (n=3)	27 ± 2.8 (n=3)
TPN-0095037	357.88	4.20	2.0 ± 0.53 (n=3)	54 ± 26 (n=3)
TPN-0095038	273.13	3.26	2.5 ± 0 (n=2)	72 ± 15 (n=2)
TPN-0095039	363.22	4.68	2.1 ± 0.74 (n=3)	24 ± 6.1 (n=3)
TPN-0095040	434.50	4.74	NC	31 ± 18 (n=2)
TPN-0095041	339.41	4.36	3.6 ± 0.50 (n=3)	13 ± 5.5 (n=3)
TPN-0095042	343.86	3.80	3.7 ± 0.62 (n=3)	21 ± 9.3 (n=3)

134

135 **Table 1. Activity of primary hits.** NC – not calculated due to cytotoxicity. ND – not determined. cLogP
136 was calculated using Collaborative Drug Discovery (<https://www.collaborativedrug.com/>).

137

138 **Cytotoxicity**

139 We further evaluated cytotoxicity using the HepG2 cell line cultured with either glucose or galactose in
 140 the medium (Table 2). HepG2 cells cultured with galactose rely on mitochondrial oxidative
 141 phosphorylation rather than glycolysis which increases their susceptibility to mitochondrial toxicants
 142 allowing us to identify compounds with mitochondrial toxicity (18). The majority of the compounds
 143 showed no difference in cytotoxicity between the two conditions with the exception of compound TPN-
 144 0073571 which had increased cytotoxicity against HepG2 cells in galactose (ratio of TC_{50glu}/TC_{50gal} of ~4)
 145 suggesting that this compound may impair mitochondrial function. Interestingly the compound showed
 146 similar cytotoxicity against the murine macrophages ($TC_{50} = 15 \pm 5 \mu M$). Overall, cytotoxicity against the
 147 two cell lines (murine RAW and human HepG2 cells) was comparable (Table 2).

148 Activity against *M. tuberculosis* in axenic culture

149 We wanted to determine if our compounds were selective for intracellular bacteria, so we determined the
 150 IC_{90} against *M. tuberculosis* in axenic culture. Only 12 compounds had $IC_{50} > 20 \mu M$ against extracellular
 151 *M. tuberculosis*, with six of these having an IC_{90} of $> 20 \mu M$ (Table 2). None of the compounds were
 152 more active against *M. tuberculosis* growing in axenic culture, with the extracellular/intracellular
 153 IC_{50} ratios ranging from 4 to 28.

Molecule ID	HepG2 cytotoxicity TC_{50} (μM)		Anti-tubercular activity in axenic culture (μM)	
	Glucose	Galactose	IC_{50}	IC_{90}
TPN-0006204	59 ± 10 (n=5)	47 ± 17 (n=4)	>20 (n=2)	>20 (n=2)
TPN-0006205	>100 (n=4)	>100 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0006206	>100 (n=2)	>100 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0006207	>100 (n=2)	>100 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0006209	>100 (n=2)	>100 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0006211	53 ± 1.5 (n=2)	43 ± 1 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0006216	44 ± 2 (n=2)	33 ± 4 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0006217	91 ± 3 (n=2)	64 ± 1.5 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0073027	82 ± 25 (n=5)	64 ± 10 (n=4)	>20 (n=3)	>20 (n=3)
TPN-0073061	22 ± 4.0 (n=3)	22 ± 3.1 (n=3)	6.3 ± 0.15 (n=2)	8.2 ± 0.1 (n=2)
TPN-0073062	>100 (n=4)	>100 (n=4)	11 ± 5.3 (n=4)	>20 (n=4)

TPN-0073063	40 ± 11 (n=4)	34 ± 6.5 (n=4)	5 ± 1.5 (n=3)	9.5 ± 3.2 (n=3)
TPN-0073559	49 ± 8.5 (n=3)	41 ± 11 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0073560	>100 (n=2)	42 ± 3.3 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0073562	31 ± 2.1 (n=3)	31 ± 1.9 (n=4)	12 ± 0.5 (n=2)	19 ± 1.5 (n=2)
TPN-0073563	34 ± 0 (n=3)	34 ± 1.9 (n=3)	ND	ND
TPN-0073565	46 ± 2.8 (n=4)	43 ± 2.1 (n=5)	>20 (n=2)	>20 (n=2)
TPN-0073571	>100 (n=2)	26 ± 5.0 (n=2)	ND	ND
TPN-0073576	83 ± 2.9 (n=3)	92 ± 7 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0073578	3.9 ± 1.1 (n=3)	4.7 ± 0.54 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0073584	>100 (n=3)	>100 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0073585	>25 (n=3)	>25 (n=3)	>5 (n=2)	>5 (n=2)
TPN-0073588	>100 (n=2)	>100 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0089695	>100 (n=5)	>100 (n=4)	19 ± 0 (n=2)	>20 (n=2)
TPN-0089788	52 ± 18 (n=4)	30 ± 12 (n=4)	>20 (n=2)	>20 (n=2)
TPN-0095025	35 ± 1.0 (n=2)	28 ± 1.5 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0095026	19 ± 3.5 (n=2)	18 ± 5 (n=2)	19 ± 1 (n=2)	>20 (n=2)
TPN-0095027	76 ± 12 (n=3)	22 ± 2.2 (n=3)	0.17 ± 0.02 (n=3)	0.60 ± 0.20 (n=3)
TPN-0095028	54 ± 9.4 (n=3)	62 ± 6.2 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0095029	>50 (n=2)	>50 (n=2)	>10 (n=2)	>10 (n=2)
TPN-0095030	>100 (n=2)	>100 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0095031	57 ± 6.2 (n=3)	52 ± 0.47 (n=3)	0.20 ± 0.07 (n=3)	0.40 ± 0.14 (n=3)
TPN-0095032	23 ± 1.5 (n=2)	19 ± 0.5 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0095033	>100 (n=3)	>100 (n=3)	>20 (n=1)	>20 (n=1)
TPN-0095034	>100 (n=3)	>100 (n=3)	>20 (n=2)	>20 (n=2)
TPN-0095035	27 ± 0 (n=2)	21 ± 2.0 (n=2)	9.6 ± 0.1 (n=2)	>10 (n=2)
TPN-0095036	21 ± 3.4 (n=3)	20 ± 0.47 (n=3)	>20 (n=1)	>20 (n=1)
TPN-0095037	84 ± 2.5 (n=2)	56 ± 4 (n=2)	>20 (n=2)	>20 (n=2)
TPN-0095038	>100 (n=2)	>100 (n=3)	9.2 ± 1.0 (n=2)	>20 (n=2)
TPN-0095039	29 ± 7.7 (n=4)	30 ± 3.2 (n=5)	14 ± 2.1 (n=3)	18 ± 1.4 (n=3)
TPN-0095040	>50 (n=3)	>50 (n=3)	>10 (n=2)	>10 (n=2)
TPN-0095041	52 ± 5.5 (n=2)	24 ± 0.5 (n=2)	14 ± 1 (n=2)	>20 (n=2)
TPN-0095042	26 ± 3.0 (n=2)	19 ± 1.0 (n=2)	>10 (n=2)	>10 (n=2)

Table 2. Cytotoxicity and activity against extracellular *M. tuberculosis*.

Identification of novel chemotypes

We performed structural clustering on the set of 174 hits in order to identify common and potentially privileged scaffolds. Compounds were grouped into series sharing the same scaffold/chemotype, each featuring 2 to 8 compounds with 8 singletons. Scaffolds used in the substructure searching, along with their frequency in the hit list and the entire library are depicted in Table 3.

Series	Abbreviation	# Screened ¹	# Primary hits ²	Active compounds ³
Benzene amide ether	BAE	15	10	8
Phenyl thiourea	PTU	6	4	3
Thienopyridine	TIP	8	2	1
Phenyl pyrazole	PPL	6	5	3
Thiophene carboxamide	TPC	8	8	8

Table 3. Series selected for follow-up.

¹Number of compounds screened in the library. ²Number of compounds with activity in the single point screen. ³Number of confirmed hits (IC₅₀ < 20 μM).

The prioritization of compounds was not based solely on biological potency, but focus was placed on the quality/structural novelty of each chemotype, along with their physicochemical properties. We highlighted five chemotypes that were of interest for further development. A representative compound from each series is shown in Figure 3

Figure 3. Representative molecules from selected series

Benzene-amide ether (BAE) series:

We identified 15 compounds with a benzene amide ether scaffold in the primary screen, of which 10 were active (defined as >70% inhibition of bacterial replication) (Table 3). The BAE series has not previously been identified in phenotypic screens against replicating *M. tuberculosis*, although one compound from this series (**6**) had activity against non-replicating *M. tuberculosis* (19). In addition, compound **12** was identified as an inhibitor of *M. tuberculosis* with an IC_{90} of 6.9 μ g/mL(20). We purchased 13 commercially-available compounds and determined intracellular activity against *M. tuberculosis*, although we did not see activity up to 20 μ M. Eleven compounds had activity against intracellular *M. tuberculosis* (Table 4). Two compounds (**4** and **7**) were cytotoxic to macrophages and we could not calculate activity.

Benzene amide ether
Compound #	X	R ₁	R ₂	cLogP	Extracellular (μM)	Intracellular (μM)		HepG2 Cytotoxicity IC ₅₀ (μM)	
					IC ₉₀	Mtb IC ₅₀	RAW TC ₅₀	Galactose	Glucose
1	S			2.86	>20	7.8 ± 1.6	>100	>100	>100
					n=2	n=2	n=2	n=2	n=2
2	O			3.24	>20	10 ± 0.5	>100	47 ± 17	59 ± 10
					n=2	n=3	n=1	n=4	n=5
3	S	Ph		3.97	>20	1.4 ± 0.6	57 ± 24	>100	>100
					n=2	n=4	n=3	n=3	n=4
4	O			6.41	>20	NC	57 ± 20	>100	>100
					n=2		n=3	n=2	n=2
5	S			5.00	>20	6.3 ± 0.3	>100	>100	>100
					n=2	n=2	n=2	n=2	n=2
6	O			3.59	>20	13 ± 0	86 ± 1.0	>100	>100
					n=2	n=2	n=2	n=2	n=2
7	O			4.89	>20	NC	68 ± 0.5	>100	>100
					n=2		n=2	n=2	n=2
8	O	Ph		2.80	>20	7.6 ± 3.5	38 ± 6.0	43 ± 1.0	53 ± 1.5
					n=2	n=2	n=2	n=2	n=2
9	O			2.50	>20	8.2 ± 1.7	>100	61 ± 4.0	>100
					n=2	n=2	n=2	n=2	n=2
10	O			3.35	>20	6.6 ± 1.6	75 ± 16	55 ± 5.0	93 ± 3.5
					n=2	n=2	n=2	n=2	n=2
11	O			3.58	>20	12 ± 1.6	60 ± 27	57 ± 3.0	96 ± 2.5
					n=2	n=2	n=2	n=2	n=2
12	S			4.55	>20	2.4 ± 0.5	>100	33 ± 4.0	44 ± 2.0
					n=2	n=2	n=2	n=2	n=2
13	O			3.32	>20	4.9 ± 2.1	70 ± 1.6	64 ± 1.5	91 ± 3.0
					n=2	n=4	n=4	n=2	n=2

Table 4. Catalog structure-activity relationship of the BAE series.

NC – not calculated due to cytotoxicity. cLogP was calculated using Collaborative Drug Discovery (<https://www.collaboratedrug.com/>).

187
188 Eight compounds showed good intracellular activity ($IC_{50} < 10 \mu M$). There was a ten-fold difference
189 between the most potent compound (**3**, $IC_{50} = 1.4 \mu M$) and the least potent compound (**13**, $IC_{50} = 13 \mu M$).
190 Compounds generally showed low cytotoxicity against both RAW264.7 cells and HepG2 cells with either
191 $TC_{50} > 100 \mu M$ or a selectivity index of > 50 (TC_{50}/IC_{50}). Compound **3** and compound **12** were the most
192 potent ($IC_{50} = 1.4 \mu M$ and $IC_{50} = 2.4 \mu M$ respectively). Although, compound **3** had some cytotoxicity, the
193 selectivity index was 40. Compound **12** was not cytotoxic, with $TC_{50} > 100 \mu M$ and a selectivity index of
194 > 42 . None of the compounds were active against replicating *M. tuberculosis* in axenic medium ($IC_{90} > 20$
195 μM) demonstrating clear selectivity for intracellular bacteria.

196
197 We were able to determine some elements of the SAR for this series using the limited compound set
198 available. Compounds with a sulfur atom as a linker were generally more potent than those with an
199 oxygen as a linker, with a range of 1.4 -6.3 μM compared to 4.9-13 μM . The closest comparison was
200 between **13** with the oxygen linker ($IC_{50} = 4.9 \mu M$) and compounds **3** and **12** ($IC_{50} = 1.4 \mu M$ and 2.4 μM
201 respectively). In addition, the two molecules with significant toxicity to macrophage cells contained the
202 oxygen linker (**4** and **7**). Four compounds (**1**, **3**, **5** and **12**) with one or two substitution of halogen atoms
203 on the amide phenyl ring (R_2) exhibited good intracellular activity.

204
205 It seems that lipophilicity did not correlate with anti-tubercular activity. Although the most active
206 compounds (**3** and **12**) had cLogP of 3.97 and 4.55 respectively, compound **13** with cLogP of 3.32 was
207 only two-fold less active and compounds with cLogP < 3 still retained good activity ($IC_{50} < 10 \mu M$). An
208 electron-withdrawing moiety on the para position of the amide phenyl group (R_2) appears to be an
209 essential requirement for good anti-mycobacterial activity. The amide phenyl ring (R_2) is not critical to
210 maintain antimycobacterial activity, since compounds **8** and **12** with saturated rings. Both compounds **8**
211 and **10** with adamantane and piperidine groups respectively, had good activity ($IC_{50} = 7.6 \mu M$ and $IC_{50} =$
212 $6.6 \mu M$). Further exploration of the length of the carbon chain or inclusion of other heteroatoms would
213 expand the SAR, but such molecules are not available commercially and would require chemical
214 synthesis.

215

216 **The phenylthiourea (PTU) series**

We identified 6 compounds with a phenyl thiourea scaffold in the primary screen, of which 4 were active (Table 3). We purchased 5 commercially-available compounds and determined intracellular activity against *M. tuberculosis*. Four compounds had potent activity against intracellular *M. tuberculosis* (Table 5). One compound (**18**) was cytotoxic and we could not calculate activity. One compound (**17**) had significant cytotoxicity against macrophages (TC₅₀ of 1.8 μM) and was also toxic for HepG2 cells (TC₅₀ of 5.5 μM). The remaining three compounds had excellent potency (0.30 to 1.3 μM) and good selectivity. Compound **14** with a guanidine functionality as the core had higher cytotoxicity (SI = 14) than the two potent molecules (**15** and **16**) with the thiourea core (SI = >250 and >150 respectively).

Phenyl thiourea series
Compound #	Compound #	cLogP	X	R ₁	R ₂	Extracellular (μM)	Intracellular (μM)		HepG2 Cytotoxicity IC ₅₀ (μM)	
						IC ₉₀	Mtb IC ₅₀	RAW TC ₅₀	Galactose	Glucose
TPN-0073061	14	2.92	NH			8.2 ± 0.1 (n=2)	1.3 ± 0.1 (n=4)	18 ± 4.3 (n=4)	22 ± 3.1 (n=3)	212 ± 4 (n=3)
TPN-0073062	15	4.21	S	Ph		>20 (n=4)	0.40 ± 0.1 (n=4)	>100 (n=4)	>100 (n=3)	>100 (n=4)
TPN-0073063	16	4.58	S			9.5 ± 3.2 (n=3)	0.30 ± 0.2 (n=6)	47 ± 28 (n=7)	35 ± 6.6 (n=3)	64 ± 40 (n=4)
TPN-0073070	17	4.60	O			15 (n=1)	0.70 ± 0.2 (n=2)	1.8 ± 0.5 (n=2)	5.5 ± 0 (n=2)	5.6 ± 0 (n=2)
TPN-0073077	18	5.16	O			>20 (n=1)	NC	68 ± 23 (n=2)	>100 (n=1)	>100 (n=1)

Table 5. Catalog structure-activity relationship of the PTU series.

NC – not calculated due to cytotoxicity. cLogP was calculated using Collaborative Drug Discovery (<https://www.collaboratedrug.com/>).

In contrast to the BAE series, the PTU molecules were also active against extracellular bacteria; 3 of the 5 molecules had IC₉₀ <20 μM. In general, PTUs were 5-10-fold more active against intracellular bacteria. To the best of our knowledge, this is the first report of these compounds as antitubercular agents although other thiourea derivatives have antitubercular activity(21–24).

The phenyl pyrazole (PPL) series:

We identified 6 compounds with a phenyl pyrazole scaffold in the primary screen, of which 5 were active (Table 3). We purchased 4 commercially-available compounds and determined intracellular activity against *M. tuberculosis*. All four compounds (**19-22**) had activity against intracellular *M. tuberculosis* (Table 6). Although the compounds demonstrated some cytotoxicity against HepG2 cells and/or macrophages, the selectivity index was good (>5). These sets of compounds have 1,3,4-trisubstituted pyrazoles where C-4 bears a hydroxymethyl group. Compound **19** was the most potent with good intracellular activity (IC₅₀ = 2.9 μM) and > 30-fold selectivity against HepG2 cells. Compound **21** also showed weak activity (IC₅₀ = 18.5 μM) against axenically-grown *M. tuberculosis*. Previous work has identified similar pyrazole-containing compounds with anti-tubercular activity that target MmpL3. In these studies, a 1,3,5-trisubstituted pyrazole as a central core had good activity against axenically-cultured *M. tuberculosis* (25). Our molecules differ in that they only have weak activity against extracellular bacteria. The molecules we identify here are phenyl pyrazoles which have not been reported previously to have activity against either intracellular or extracellular bacteria.

Phenyl pyrazole series
Compound #	Compound #	cLogP	R ₁	R ₂	R ₃	R ₄	Extracellular (μM)	Intracellular (μM)		HepG2 Cytotoxicity IC ₅₀ (μM)	
							IC ₉₀	Mtb IC ₅₀	RAW TC ₅₀	Galactose	Glucose
TPN-0073562	19	4.53	Cl	Cl	H	H	19 ± 1.5	2.9 ± 0.5	16 ± 11	31 ± 2.2	31 ± 2.1
							(n=2)	(n=3)	(n=3)	(n=3)	(n=3)
TPN-0073563	20	4.09	H	Br	H	H	ND	4.3 ± 1.5	28 ± 7.0	35 ± 3.7	34 ± 0.0
								(n=3)	(n=5)	(n=3)	(n=3)
TPN-00773565	21	4.82	F	Cl	F	Cl	>20	7 ± 1.8	>100	43 ± 2.3	46 ± 2.8
							(n=2)	(n=4)	(n=4)	(n=4)	(n=4)
TPN-0073583	22	4.07	F	Cl	H	H	>20	5.8 ± 0.4	>100	57 ± 28	66 ± 29
							(n=2)	(n=3)	(n=4)	(n=3)	(n=3)

Table 6. Catalog structure-activity relationship of the PPL series.

ND – not determined. cLogP was calculated using Collaborative Drug Discovery (<https://www.collaboratedrug.com/>).

The thiophene carboxamide series (TPC)

We identified 8 compounds with a thiophene carboxamide scaffold in the primary screen, of which all 8 were active (Table 3). We purchased 8 commercially-available compounds (**23-30**) and determined intracellular activity against *M. tuberculosis*. Five compounds had activity against intracellular *M. tuberculosis* with good activity of IC₅₀ <10 μM (Table 7). Three compounds (**23-25**) were cytotoxic and we could not calculate activity.

Thiophene carboxamide series										
Compound #	Compound #	cLogP	R ₁	R ₂	R ₃	R ₄	Extracellular (μM)	Intracellular (μM)		HepG2 Cytotoxicity IC ₅₀
							IC ₉₀	Mtb IC ₅₀	RAW TC ₅₀	Galactose Glucose
TPN-0073559	23	4.29			Me	Me	>20 n=2	NC	50 ± 24 n=4	41 ± 11 n=3 49 ± 8.5 n=3
TPN-0073560	24	5.85			Me	Bn	>20 n=2	NC	23 ± 4.7 n=4	42 ± 3.3 n=3 >100 n=2
TPN-0073571	25	5.15			-(CH ₂) ₂ -	-(CH ₂) ₂ -	ND	NC	24 ± 6.5 n=2	26 ± 5.0 n=2 >100 n=2
TPN-0073576	26	4.39			Me	Me	>20 n=2	1.0 ± 0.4 n=3	>100 n=4	89 ± 5.5 n=2 83 ± 2.9 n=3
TPN-0073578	27	4.19			H	H	>20 n=2	1.7 ± 0.8 n=3	11 ± 2.2 n=3	4.7 ± 0.5 n=3 3.9 ± 1.1 n=3
TPN-0073584	28	2.61			-(CH ₂) ₂ -	-(CH ₂) ₂ -	>20 n=2	5.1 ± 1.7 n=3	>100 n=3	>100 n=3 >100 n=3
TPN-0073585	29	6.81			-(CH ₂) ₂ -	-(CH ₂) ₂ -	>5 n=2	0.40 ± 0.1 n=3	9.4 ± 1.9 n=3	>25 n=3 >25 n=3
TPN-0073588	30	4.67			Me	COMe	>20 n=2	6.5 ± 1.7 n=3	67 ± 8.5 n=2	>100 n=3 93 ± 5.5 n=2

Table 7. Catalog structure-activity relationship of the TPC series.

ND – not determined. NC – not calculated due to cytotoxicity. cLogP was calculated using Collaborative Drug Discovery (<https://www.collaborativedrug.com/>).

Three compounds (**26**, **27** and **29**) showed good intracellular potency with IC₅₀ <5 μM. All except **26** and **28** were cytotoxic against macrophages with IC₅₀ ranging from 9-67 μM; compound **27** was the most toxic and also showed cytotoxicity against HepG2 cells (IC₅₀ = 4.7 μM). However, despite this, compounds **26**, **28**, **29**, and **30** all had good selectivity (SI>10) – values for HepG2 were >80, 20, >60 and

15 respectively. Two compounds (**24** and **25**) were more cytotoxic against HepG2 cells in galactose media (ratio ~4) suggesting that they may impair mitochondrial function. However, these were also toxic against macrophages and were not considered active. None of the compounds had any activity against extracellular *M. tuberculosis*. To the best of our knowledge, this is the first report of these compounds as antitubercular agents.

The thienopyridines series (TIP)

We identified 8 compounds with a thiophene carboxamide scaffold in the primary screen, of which 2 were active (Table 3).

Thienopyridines series

Compound #	Compound #	cLogP	R ₁	R ₂	R ₃	R ₄	Extracellular (μM)	Intracellular (μM)		HepG2 Cytotoxicity IC ₅₀
							IC ₉₀	Mtb IC ₅₀	RAW TC ₅₀	Galactose Glucose
TPN-0073022	31	3.66		(methoxy methyl)	H	Me	>20	>100	15 ± 9	64 51
								n=3	n=2	n=1 n=1
TPN-0073023	32	3.42	Ph	(methoxy methyl)	H	Me	>20	17 ± 6.5	67 ± 18	49 68 ± 1.0
								n=5	n=4	n=1 n=2
TPN-0073024	33	3.43		H	-(CH ₂) ₂ -	-(CH ₂) ₂ -	>20	62 ± 31	>100	>100 >100
								n=3	n=2	n=1 n=1
TPN-0073025	34	4.00		H	H	Ph	>20	9.0 ± 6.6	30 ± 6.6	42 61 ± 48
								n=5	n=6	n=1 n=2
TPN-0073026	35	7.23		Ph	H	Ph	>20	>100	>100	>100 >100
								n=3	n=1	n=1 n=1
TPN-0073027	36	3.69		H	H	Ph	>20	6.8 ± 3.3	>100	64 ± 12 72 ± 22
								n=6	n=7	n=4 n=3
TPN-0073030	37	3.27		H	-(CH ₂) ₂ -	-(CH ₂) ₂ -	>20	1.1	>100	>100 >100
								n=1	n=3	n=1 n=1

Table 8. Catalog structure-ctivity relationship of the TIP series. cLogP was calculated using

Collaborative Drug Discovery (<https://www.collaboratedrug.com/>).

We purchased 7 commercially-available compounds (**31-37**) and determined intracellular activity against *M. tuberculosis* Analogs varied in terms of substitution on the pyridine ring but all possessed the amino

group at the C-3 position and a carboxamide substitution on the thiophene ring. Four compounds had activity against intracellular *M. tuberculosis* (Table 8), but only two had activity <10 μ M (compounds **34** and **36**). Compound **34** bearing a ketone moiety had weak activity. Two compounds (**31** and **35**) were not active. Compounds were selective, but had no activity against axenically cultured bacteria.

DISCUSSION

We screened a diversity in order to find novel chemical matter that targets intracellular *M. tuberculosis*. We identified a large number of hits with a hit rate of 1.7%. This is comparable to the hit rate we see with other whole cell screens and in other groups. We were able to follow up on several series to conduct limited catalog SAR as a first evaluation of tractability. The majority of the hit series we followed up from in this screen were more active against intracellular bacteria and in some cases lacked any extracellular activity. Since we focused on identifying new scaffolds, this was largely expected, since scaffolds with known anti-tubercular activity from previous phenotypic screens would have been deprioritized in our selection process. For the five series we prioritized, this is the first report of antimycobacterial activity of the majority of these compounds. This highlights the power of the screen assay in identifying novel compounds that are conditionally active against intracellular *M. tuberculosis*. Compounds that are only active against intracellular bacteria could be targeting pathways that are only essential for growth within the macrophages. Alternatively, they could target host pathways that enhance bacterial clearance or could be accumulated and/or metabolized within the macrophage. Further work to identify targets and mode of action is warranted on series with attractive properties.

Early identification of potentially toxic compounds during library screening has a significant impact on both the cost and the success rate of the drug discovery process. We were able to identify cytotoxic compounds rapidly and to exclude compounds that may look active due to killing the macrophages rather than anti-bacterial activity.. Out of the 308 hits that we identified as mycobacterial growth inhibitors, we excluded 134 molecules with significant toxicity to the macrophages representing almost a third of the hits. We also saw that compounds had comparable cytotoxicity against both macrophage and HepG2 cell lines, demonstrating the power of this assay in predicting potential cytotoxicity of the compounds early in the screening process.

CONCLUSION

We have identified five novel and tractable chemical scaffolds with promising activity against intracellular *M. tuberculosis* using a high throughput, high content screen. The mechanism of action and targets remains to be characterized for each series. However, these series represent attractive starting points for lead generation and full structure-activity relationship studies.

ACKNOWLEDGEMENTS

We thank Yulia Ovechkina and Sultan Chowdhury for technical assistance and useful discussion.

Research reported in this publication was supported by NIAID of the National Institutes of Health under award number R01AI132634 .The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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