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Identification of Novel Chemical Scaffolds that Inhibit the Growth of *Mycobacterium tuberculosis* in Macrophages

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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 cytoxicity 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

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INTRODUCTION

Tuberculosis (TB) is the leading cause of death from a bacterial infection worldwide. Despite the availability of chemotherapy, TB is responsible for >10 million infections annually and >1 million deaths (Gordon and Parish, 2018; Global Tuberculosis Report, 2020). The increasing incidence of drug failure for TB due to widespread drug-resistant infections underscores the urgent need for new drugs and new bacterial targets for *M. tuberculosis*.

M. tuberculosis is an intracellular pathogen which can survive and replicate within macrophages; this property relies on the ability to block normal bactericidal activities and turns the macrophage into a niche for replication (Vandal et al., 2009; Deretic, 2014; VanderVen et al., 2015; Weiss and

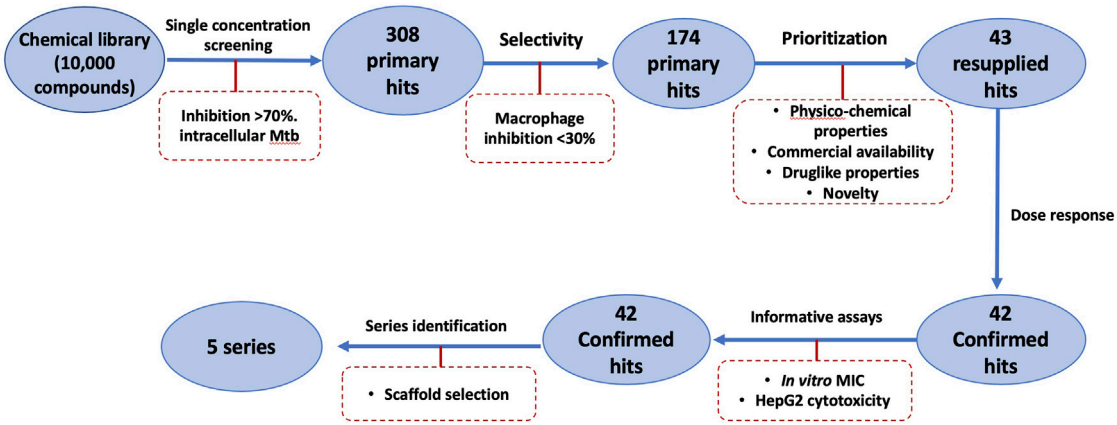


FIGURE 1 | Schematic of High Throughput Screening (HTS) of a library of small molecules.

Schaible, 2015). The physiology and metabolism of *M. tuberculosis* within macrophages is very different from extracellular bacteria and so the complement of essential genes differs (Sassetti et al., 2001; Sassetti et al., 2003; Sassetti and Rubin, 2003). From a drug discovery perspective, this means that intracellular bacteria have additional essential processes that can be targeted by chemical inhibition (Manjunatha and Smith, 2015; Parish, 2019; Parish, 2020).

Whole cell screening has been broadly used to identify new drug candidates for *tuberculosis*. In contrast to target-based drug discovery, in phenotypic screens every essential pathway is a potential drug target. This greatly increases the likelihood of finding inhibitors and overcomes the barrier of obtaining cell-penetrant molecules, which is often a limitation of biochemical screens (Payne et al., 2007). However, conditionally essential genes that are required for *M. tuberculosis* growth within macrophages or *in vivo*, may be overlooked in phenotypic screens against axenically-cultured *M. tuberculosis*. High-throughput screening in conditions that closely resemble the intracellular environment have the potential to uncover novel antimicrobials that target these conditionally essential genes, in addition to discovering compounds targeting the host cells to increase bacterial elimination (Manning et al., 2017; Njikan et al., 2018).

High-content imaging screening is a powerful technology to identify active compounds by monitoring both bacterial and macrophage cell numbers simultaneously and in the same wells, thus leading to more reliable data and a quicker assessment of compound attractiveness. We previously developed a fluorescence-based, live-cell, high-content analysis (HCA) assay to examine drug efficacy against intracellular *M. tuberculosis* (Manning et al., 2017).

In this study, we screened 10,000 diverse small molecules by high throughput HCA and identified active compounds with low cytotoxicity. We selected a number of series for follow up based on their physicochemical properties and the novelty of the chemotypes and identified five chemical classes of 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% CO_2 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 *Ds*Red from plasmid pBlazeC8 (DREAM8) (Carroll et al., 2018) was cultured at 37°C in Middlebrook 7H9 medium containing 10% v/v OADC (oleic acid, 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 (Manning et al., 2017). 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 (maximum inhibition) for anti-tubercular activity and cytotoxicity respectively. INH and STA were also included in dose response on each plate. RAW264.7 cells were infected with *M. tuberculosis* DREAM8 as described (Manning et al., 2017) 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 3,300 cells/well. Plates were incubated

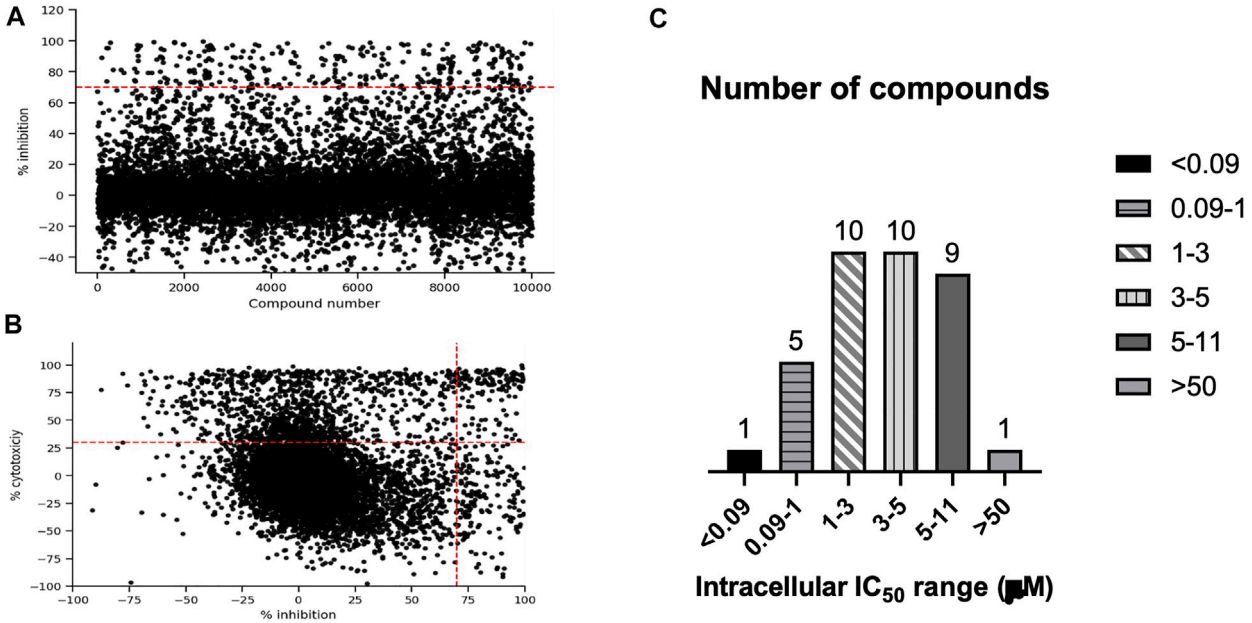


FIGURE 2 | High content, high throughput screen against intracellular M. *tuberculosis*. (A). Compounds were tested for activity against RAW264.7 cells infected with *M. tuberculosis* at an MOI of 1. The primary screen was conducted using a single concentration of each molecule at 10 1.1 M. Inhibition of bacterial growth and macrophage viability were determined for each compound after 72 h **(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.

for 72 h, 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 ×4 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. IC_{50} was calculated as the compound concentration required to reduce bacterial growth by 50%. TC_{50} was calculated as the compound concentration required to reduce macrophage viability by 50%.

Cytotoxicity

Cytotoxicity against HepG2 cells was measured after 72 h. Cells were seeded in 384-well plates at 1,800 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; TC_{50} was calculated as the compound concentration required to reduce cell viability by 50%.

Minimum Inhibitory Concentration

Minimum inhibitory concentrations (IC_{90}) against *M. tuberculosis* were determined in liquid medium as described (Ollinger et al., 2013). Bacterial growth was measured after 5 days by OD_{590}. IC_{90} 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 2A**). 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 1**, **Figure 2B**). 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**).

We saw some negative inhibition values in the screen, which is not unusual. Compound fluorescence will lead to negative inhibition and is seen more often in the green end of the spectrum than the red. Cytotoxicity can lead to increased

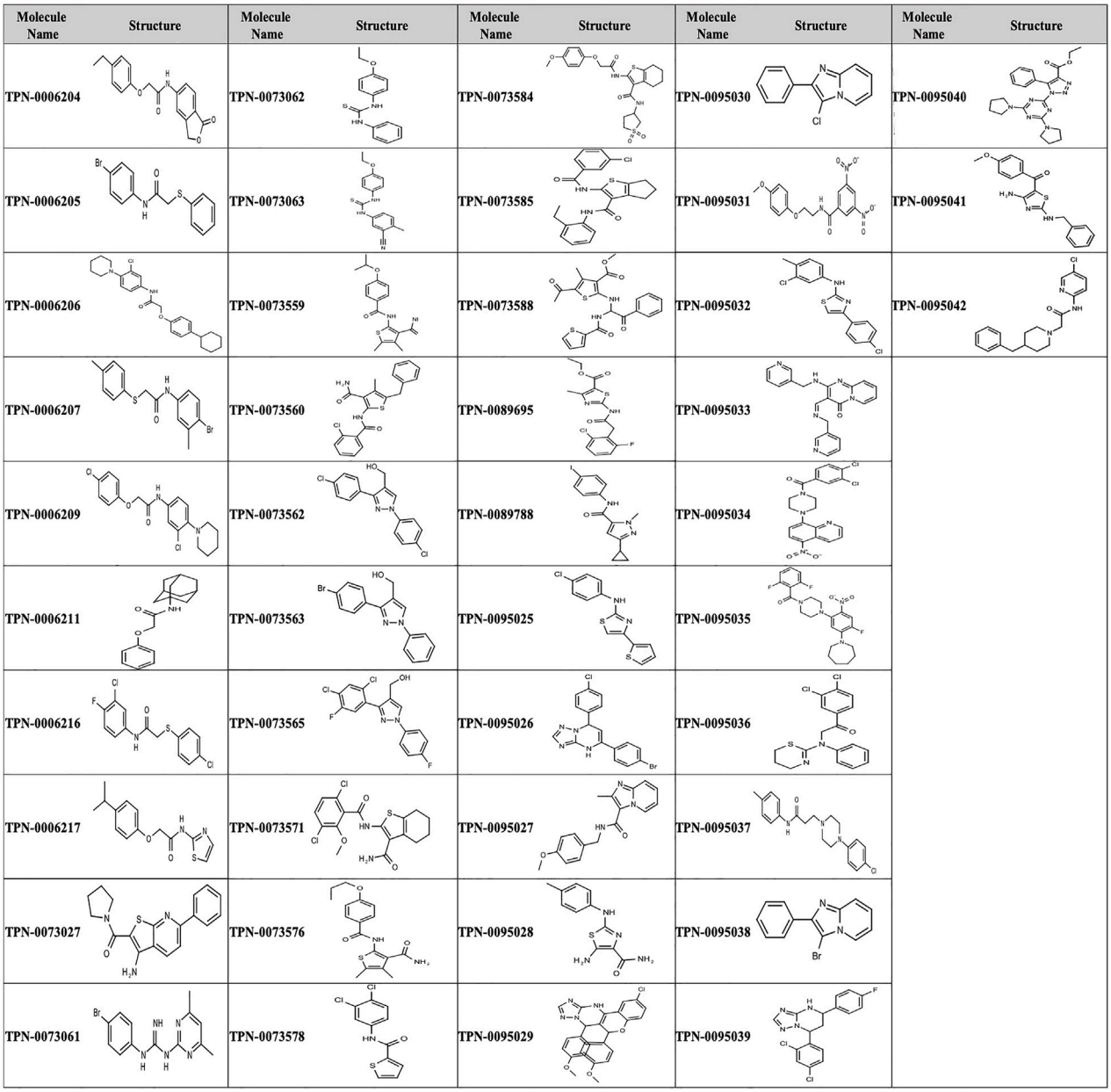


FIGURE 3 | Structure of primary hits selected for follow up.

signal from the bacterial populaton, presumably because the bacteria are released into the medium and no longer intracellular; we see this effect with staurosporine. Changes in growth of RAW264.7 cells could also be a factor, since uninfected cells will grow, whereas the infected cells do not tend to grow; if compounds are very active, this could lead to increased macrophage growth.

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 1**, **Figure 3**); we purchased new batches of compounds and determined the activity using a dose response format (**Figure 2C**). 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 IC_{50} of the remaining 36 hits was determined (**Figure 3C**); of these all but one (35/36) had confirmed activity with IC_{50} < 50 μM against intracellular *M. tuberculosis* (**Table 1**). The majority of the hits (Mori et al., 2015) had an IC_{50} <5 μM, and 6 compounds were potent with an IC_{50} < 1 μM (**Figure 3C**).

TABLE 1 | Activity of primary hits.

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[b]	>100[b]
TPN-0006205	322.22	3.97	1.4 ± 0.64[d]	57 ± 23[c]
TPN-0006206	426.99	6.41	NC	57 ± 20[b]
TPN-0006207	350.27	5	6.3 ± 0.3[b]	>100[b]
TPN-0006209	379.28	4.89	NC	68 ± 0.50[b]
TPN-0006211	285.39	2.8	7.6 ± 3.5[b]	38 ± 6.0[b]
TPN-0006216	330.2	4.55	2.8 ± 0.78[d]	>100[d]
TPN-0006217	276.35	3.32	4.9 ± 2.1[d]	75 ± 16[d]
TPN-0073027	323.41	3.69	5.8 ± 2.1[e]	>100[e]
TPN-0073061	320.19	2.92	1.3 ± 0.15[d]	18 ± 4.3[d]
TPN-0073062	272.37	4.21	0.39 ± 0.13[d]	>100[d]
TPN-0073063	311.4	4.58	0.25 ± 0.23[f]	47 ± 28[f]
TPN-0073559	332.42	4.29	NC	50 ± 24[d]
TPN-0073560	384.88	5.85	NC	23 ± 4.7[d]
TPN-0073562	319.19	4.53	2.9 ± 0.52[c]	16 ± 11[c]
TPN-0073563	329.2	4.09	4.7 ± 1.1[e]	28 ± 7.0[e]
TPN-0073565	355.17	4.82	7.0 ± 1.8[d]	>100[d]
TPN-0073571	399.29	5.15	NC	15 ± 5.0[b]
TPN-0073576	332.42	4.39	1.1 ± 0.36[d]	>100[d]
TPN-0073578	272.14	4.19	1.7 ± 0.85[d]	11 ± 2.2[d]
TPN-0073584	478.58	2.61	5.1 ± 1.7[c]	>100[b]
TPN-0073585	424.94	6.81	0.4 ± 0.11[c]	9.4 ± 1.9[c]
TPN-0073588	456.53	4.67	6.5 ± 1.7[c]	67 ± 8.5[b]
TPN-0089695	356.8	3.76	4.7 ± 1.2[b]	45 ± 17[b]
TPN-0089788	367.19	3.25	2.5 ± 0.57[e]	>100[e]
TPN-0095025	292.8	5.17	4.4 ± 1.7[c]	26 ± 7.1[c]
TPN-0095026	387.67	5.21	NC	19 ± 12[b]
TPN-0095027	295.34	1.45	0.10 ± 0.01[d]	72 ± 16[d]
TPN-0095028	248.3	2.49	4.4 ± 1.3[b]	54 ± 20[b]
TPN-0095029	472.93	5.54	>50[c]	>50[b]
TPN-0095030	228.68	3.11	6.9 ± 1.9[c]	>100[b]
TPN-0095031	361.31	2.41	0.036 ± 0.007[e]	40 ± 25[e]
TPN-0095032	335.25	6.51	3.5 ± 0.83[c]	9.9 ± 0.98[c]
TPN-0095033	370.42	1.15	3.4 ± 2.6[c]	>100[c]
TPN-0095034	431.27	4.32	8.4 ± 2.3[d]	>100[d]
TPN-0095035	462.47	4.78	1.0 ± 0.02[c]	>50[c]
TPN-0095036	379.3	5.22	3.5 ± 0.97[c]	27 ± 2.8[c]
TPN-0095037	357.88	4.2	2.0 ± 0.53[c]	54 ± 26[c]
TPN-0095038	273.13	3.26	2.5 ± 0[b]	72 ± 15[b]
TPN-0095039	363.22	4.68	2.1 ± 0.74[c]	24 ± 6.1[c]
TPN-0095040	434.5	4.74	NC	31 ± 18[b]
TPN-0095041	339.41	4.36	3.6 ± 0.50[c]	13 ± 5.5[c]
TPN-0095042	343.86	3.8	3.7 ± 0.62[c]	21 ± 9.3[c]

Number of runs.
[a]*n = 1.*
[b]*n = 2.*
[c]*n = 3.*
[d]*n = 4.*
[e]*n = 5.*
[f]*n = 6.*
[g]*n = 7.*
NC–not calculated due to cytotoxicity. ND–not determined. cLogP was calculated using Collaborative Drug Discovery (https://www.collaborativedrug.com/).

The majority of the compounds had no cytotoxicity (TC_{50} > 50 μM); a few compounds were cytotoxic at higher concentrations, but retained good selectivity (**Table 1**). The IC_{50} for INH against *M. tuberculosis* was 0.23 ± 0.08 μM (n = 192) and for STA against the RAW264.7 cells was 0.0034 ± 0.0013 μM (n = 112).

Cytotoxicity

We further evaluated cytotoxicity using the HepG2 cell line cultured with either glucose or galactose in the medium (**Table 2**). HepG2 cells cultured with galactose rely on mitochondrial oxidative phosphorylation rather than glycolysis which increases their susceptibility to mitochondrial toxicants

TABLE 2 | Cytotoxicity and activity against extracellular *M. tuberculosis*.

Molecule ID	HepG2 cytotoxicity TC_{50} (μM)		Anti-tubercular activity in axenic culture (μM)	
	Glucose	Galactose	IC_{50}	IC_{90}
TPN-0006204	59 ± 10[d]	47 ± 17[e]	>20[b]	>20[b]
TPN-0006205	>100[e]	>100[c]	>20[b]	>20[b]
TPN-0006206	>100[b]	>100[b]	>20[b]	>20[b]
TPN-0006207	>100[b]	>100[b]	>20[b]	>20[b]
TPN-0006209	>100[b]	>100[b]	>20[b]	>20[b]
TPN-0006211	53 ± 1.5[b]	43 ± 1[b]	>20[b]	>20[b]
TPN-0006216	44 ± 2[b]	33 ± 4[b]	>20[b]	>20[b]
TPN-0006217	91 ± 3[b]	64 ± 1.5[b]	>20[b]	>20[b]
TPN-0073027	82 ± 25[d]	64 ± 10[e]	>20[b]	>20[b]
TPN-0073061	22 ± 4.0[c]	22 ± 3.1[c]	6.3 ± 0.15[b]	8.2 ± 0.1[b]
TPN-0073062	>100[e]	>100[e]	11 ± 5.3[e]	>20[e]
TPN-0073063	40 ± 11[e]	34 ± 6.5[e]	5 ± 1.5[c]	9.5 ± 3.2[c]
TPN-0073559	49 ± 8.5[c]	41 ± 11[c]	>20[b]	>20[b]
TPN-0073560	>100[b]	42 ± 3.3[c]	>20[b]	>20[b]
TPN-0073562	31 ± 2.1[c]	31 ± 1.9[e]	12 ± 0.5[b]	19 ± 1.5[b]
TPN-0073563	34 ± 0[c]	34 ± 1.9[c]	ND	ND
TPN-0073565	46 ± 2.8[e]	43 ± 2.1[d]	>20[b]	>20[b]
TPN-0073571	>100[b]	26 ± 5.0[b]	ND	ND
TPN-0073576	83 ± 2.9[c]	92 ± 7[c]	>20[b]	>20[b]
TPN-0073578	3.9 ± 1.1[c]	4.7 ± 0.54[c]	>20[b]	>20[b]
TPN-0073584	>100[c]	>100[c]	>20[b]	>20[b]
TPN-0073585	>25[c]	>25[c]	>5[b]	>5[b]
TPN-0073588	>100[b]	>100[c]	>20[b]	>20[b]
TPN-0089695	>100[d]	>100[e]	19 ± 0[b]	>20[b]
TPN-0089788	52 ± 18[e]	30 ± 12[e]	>20[b]	>20[b]
TPN-0095025	35 ± 1.0[a]	28 ± 1.5[b]	>20[b]	>20[b]
TPN-0095026	19 ± 3.5[b]	18 ± 5[b]	19 ± 1[b]	>20[b]
TPN-0095027	76 ± 12[c]	22 ± 2.2[c]	0.17 ± 0.02[c]	0.60 ± 0.20[c]
TPN-0095028	54 ± 9.4[c]	62 ± 6.2[c]	>20[b]	>20[b]
TPN-0095029	>50[b]	>50[b]	>10[b]	>10[b]
TPN-0095030	>100[b]	>100[b]	>20[b]	>20[b]
TPN-0095031	57 ± 6.2[c]	52 ± 0.47[c]	0.20 ± 0.07[c]	0.40 ± 0.14[c]
TPN-0095032	23 ± 1.5[b]	19 ± 0.5[b]	>20[b]	>20[b]
TPN-0095033	>100[c]	>100[c]	>20[a]	>20[a]
TPN-0095034	>100[c]	>100[c]	>20[b]	>20[b]
TPN-0095035	27 ± 0[b]	21 ± 2.0[b]	9.6 ± 0.1[b]	>10[b]
TPN-0095036	21 ± 3.4[c]	20 ± 0.47[c]	>20[a]	>20[a]
TPN-0095037	84 ± 2.5[b]	56 ± 4[b]	>20[b]	>20[b]
TPN-0095038	>100[b]	>100[c]	9.2 ± 1.0[b]	>20[b]
TPN-0095039	29 ± 7.7[e]	30 ± 3.2[d]	14 ± 2.1[c]	18 ± 1.4[c]
TPN-0095040	>50[c]	>50[c]	>10[b]	>10[b]
TPN-0095041	52 ± 5.5[b]	24 ± 0.5[b]	14 ± 1[b]	>20[b]
TPN-0095042	26 ± 3.0[b]	19 ± 1.0[b]	>10[b]	>10[b]

Number of runs.
[a]*n = 1.*
[b]*n = 2.*
[c]*n = 3.*
[d]*n = 4.*
[e]*n = 5.*
[f]*n = 6.*
[g]*n = 7.*

allowing us to identify compounds with mitochondrial toxicity (Kamalian et al., 2015). The majority of the compounds showed no difference in cytotoxicity between the two conditions with the exception of compound TPN-0073571 which had increased cytotoxicity against HepG2 cells in galactose (ratio of TC_{50glu}/TC_{50gal} of ~4) suggesting that this compound may impair mitochondrial function. Interestingly the compound showed similar cytotoxicity against the murine macrophages (TC_{50} = 15 ± 5 μM). Overall, cytotoxicity against the two cell lines (murine RAW 264.7 and human HepG2 cells) was comparable (**Table 2**).

Activity Against *M. tuberculosis* in Axenic Culture

We wanted to determine if our compounds were selective for intracellular bacteria, so we determined the IC_{90} against *M.*

TABLE 3 | Series selected for follow-up.

Series	Abbreviation	# screened[a]	# Primary hits[b]	Active compounds[c]
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

[a]*Number of compounds screened in the library.*
[b]*Number of compounds with activity in the single point screen.*
[c]*Number of confirmed hits (IC_{50} < 20 μM).*

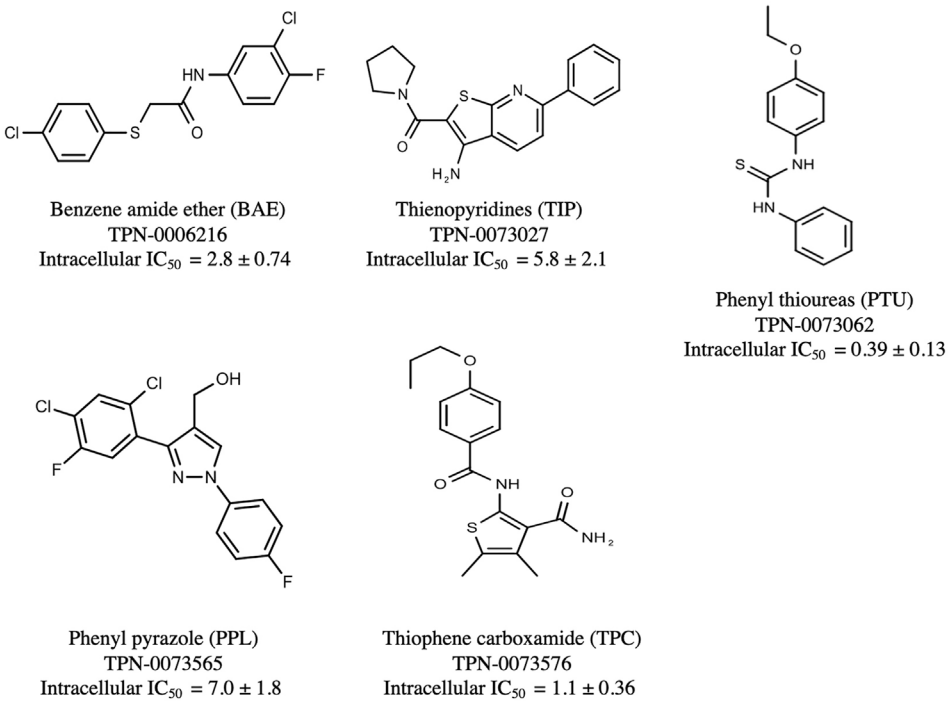


FIGURE 4 | Representative molecules from selected series.

tuberculosis in axenic culture. Only 12 compounds had IC_{50} > 20 μM against extracellular *M. tuberculosis*, with six of these having an IC_{90} of >20 μM (**Table 2**). None of the compounds were more active against *M. tuberculosis* growing in axenic culture, with the extracellular/intracellular IC_{50} ratios ranging from 4 to 28.

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**.

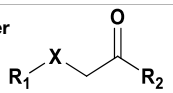
The prioritization of compounds was not based solely on biological potency, but focus was placed on the quality/structural novelty of each chemotype, along with their physicochemical properties. We highlighted five chemotypes that were of interest for further development. A representative compound from each series is shown in **Figure 4**.

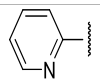
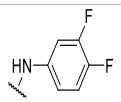
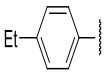
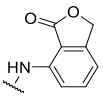
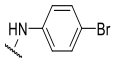
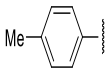
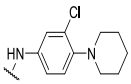
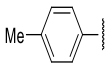
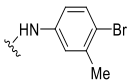
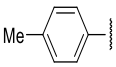
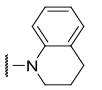
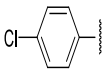
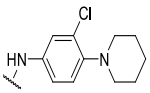
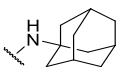
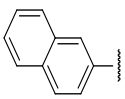
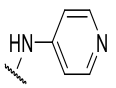
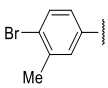
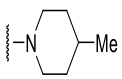
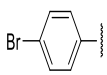
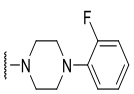
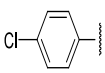
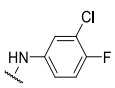
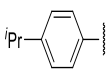
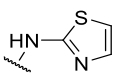
Benzene-Amide Ether Series

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* (Christophe et al., 2009). In addition, compound **12** was identified as an inhibitor of *M. tuberculosis* with an IC_{90} of 6.9 μg/ml (20). We purchased 13 commercially-available compounds and determined intracellular activity against *M. tuberculosis*, although we did not see activity up to 20 μM. Eleven compounds had activity against intracellular *M.*

TABLE 4 | Catalog structure-activity relationship of the BAE series.

Benzene amide ether

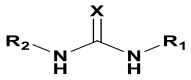


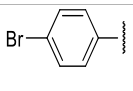
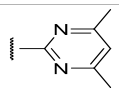
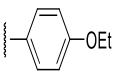
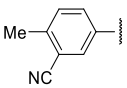
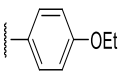
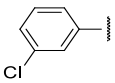
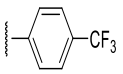
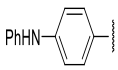
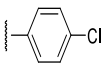
Compound		cLogP	R1	R2	R3	Extracellular (μM)	Intracellular (μM)		HepG2 cytotoxicity IC50 (μM)	
ID	#					IC_{90}	Mtb IC_{50}	RAW TC_{50}	Galactose	Glucose
TPN-0006203	1	2.86	S			>20[b]	7.8 ± 1.6[b]	>100[b]	>100[b]	>100[b]
TPN-0006204	**2**	3.24	O			>20[b]	10 ± 0.5[c]	>100[a]	47 ± 17[d]	59 ± 10[e]
TPN-0006205	**3**	3.97	S	Ph		>20[b]	1.4 ± 0.6[d]	57 ± 24[c]	>100[c]	>100[d]
TPN-0006206	**4**	6.41	O			>20[b]	NC	57 ± 20[c]	>100[b]	>100[b]
TPN-0006207	**5**	5	S			>20[b]	6.3 ± 0.3[b]	>100[b]	>100[b]	>100[b]
TPN-0006208	**6**	3.59	O			>20[b]	13 ± 0[b]	86 ± 1.0[b]	>100[b]	>100[b]
TPN-0006209	**7**	4.89	O			>20[b]	NC	68 ± 0.5[b]	>100[b]	>100[b]
TPN-0006211	**8**	2.8	O	Ph		>20[b]	7.6 ± 3.5[b]	38 ± 6.0[b]	43 ± 1.0[b]	53 ± 1.5[b]
TPN-0006212	**9**	2.5	O			>20[b]	8.2 ± 1.7[b]	>100[b]	61 ± 4.0[b]	>100[b]
TPN-0006213	**10**	3.35	O			>20[b]	6.6 ± 1.6[b]	75 ± 16[b]	55 ± 5.0[b]	93 ± 3.5[b]
TPN-0006215	**11**	3.58	O			>20[b]	12 ± 1.6[b]	60 ± 27[b]	57 ± 3.0[b]	96 ± 2.5[b]
TPN-0006216	**12**	4.55	S			>20[b]	2.4 ± 0.5[b]	>100[b]	33 ± 4.0[b]	44 ± 2.0[b]
TPN-0006217	**13**	3.32	O			>20[b]	4.9 ± 2.1[d]	70 ± 1 6[d]	64 ± 1.5[b]	91 ± 3.0[b]

Number of runs.
[a]*n = 1.*
[b]*n = 2.*
[c]*n = 3.*
[d]*n = 4.*
[e]*n = 5.*
[f]*n = 6.*
[g]*n = 7.*
NC – not calculated due to cytotoxicity. cLogP was calculated using Collaborative Drug Discovery (https://www.collaborativedrug.com/).

TABLE 5 | Catalog structure-activity relationship of the PTU series.

Phenyl thiourea series

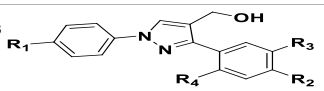


Compound		cLogP	X	R1	R2	Extracellular (μM)	Intracellular (μM)		HepG2 cytotoxicity IC50 (μM)	
ID	#					IC_{90}	Mtb IC_{50}	RAW TC_{50}	Galactose	Glucose
TPN-0073061	**14**	2.92	NH			8.2 ± 0.1[b]	1.3 ± 0.1[d]	18 ± 4.3[d]	22 ± 3.1[c]	212 ± 4[c]
TPN-0073062	**15**	4.21	S	Ph		>20 days	0.40 ± 0.1 d	>100[d]	>100[c]	>100 days
TPN-0073063	**16**	4.58	S			9.5 ± 3.2[c]	0.30 ± 0.2[f]	47 ± 28[g]	35 ± 6.6[c]	64 ± 40 days
TPN-0073070	**17**	4.6	O			15[a]	0.70 ± 0.2[b]	1.8 ± 0.5[b]	5.5 ± 0[b]	5.6 ± 0[b]
TPN-0073077	**18**	5.16	O			>20[a]	NC	68 ± 23[b]	>100[a]	>100[a]

Number of runs.
[a]*n = 1.*
[b]*n = 2.*
[c]*n = 3.*
[d]*n = 4.*
[e]*n = 5.*
[f]*n = 6.*
[g]*n = 7.*
NC – not calculated due to cytotoxicity. cLogP was calculated using Collaborative Drug Discovery (https://www.collaborativedrug.com/).

TABLE 6 | Catalog structure-activity relationship of the PPL series.

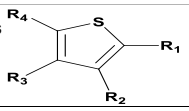
Phenyl pyrazole series

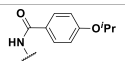
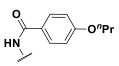
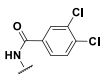
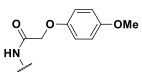
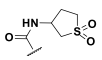
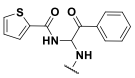


Compound		cLogP	R1	R2	R3	R4	Extracellular (μM)	Intracellular (μM)		HepG2 cytotoxicity IC50 (μM)	
ID	#						IC_{90}	Mtb IC_{50}	RAW TC_{50}	Galactose	Glucose
TPN-0073562	19	4.53	Cl	Cl	H	H	19 ± 1.5[b]	2.9 ± 0.5[c]	16 ± 11[c]	31 ± 2.2[c]	31 ± 2.1[c]
TPN-0073563	20	4.09	H	Br	H	H	ND	4.3 ± 1.5[c]	28 ± 7.0[e]	35 ± 3.7[c]	34 ± 0.0[c]
TPN-0073565	21	4.82	F	Cl	F	Cl	>20[b]	7 ± 1.8[d]	>100[d]	43 ± 2.3[d]	46 ± 2.8[d]
TPN-0073583	22	4.07	F	Cl	H	H	>20[b]	5.8 ± 0.4[c]	>100[d]	57 ± 28[c]	66 ± 29[c]

Number of runs.
[a]*n = 1.*
[b]*n = 2.*
[c]*n = 3.*
[d]*n = 4.*
[e]*n = 5.*
[f]*n = 6.*
[g]*n = 7.*
ND–not determined. cLogP was calculated using Collaborative Drug Discovery (https://www.collaborativedrug.com/).

TABLE 7 | Catalog structure-activity relationship of the TPC series.

Thiophene carboxamide series 

Compound		cLogP	R1	R2	R3	R4	Extracellular (μM)	Intracellular (μM)		HepG2 cytotoxicity IC50 (μM)	
ID	#						IC_{90}	Mtb IC_{50}	RAW TC_{50}	Galactose	Glucose
TPN-0073559	**23**	4.29			Me	Me	>20[b]	NC	50 ± 24[d]	41 ± 11[c]	49 ± 8.5[c]
TPN-0073560	**24**	5.85			Me	Bn	>20[b]	NC	23 ± 4.7[d]	42 ± 3.3[c]	>100[b]
TPN-0073571	**25**	5.15			-(CH2)2-	-(CH2)2-	ND	NC	24 ± 6.5[b]	26 ± 5.0[b]	>100[b]
TPN-0073576	**26**	4.39			Me	Me	>20[b]	1.0 ± 0.4[c]	>100[d]	89 ± 5.5[b]	83 ± 2.9[c]
TPN-0073578	**27**	4.19			H	H	>20[b]	1.7 ± 0.8[c]	11 ± 2.2[c]	4.7 ± 0.5[c]	3.9 ± 1.1[c]
TPN-0073584	**28**	2.61			-(CH2)2-	-(CH2)2-	>20[b]	5.1 ± 1.7[c]	>100[c]	>100[c]	>100[c]
TPN-0073585	**29**	6.81			-(CH2)2-	-(CH2)-	>5[b]	0.40 ± 0.1[c]	9.4 ± 1.9[c]	>25[c]	>25[c]
TPN-0073588	**30**	4.67			Me	COMe	>20[b]	6.5 ± 1.7[c]	67 ± 8.5[b]	>100[c]	93 ± 5.5[c]

Number of runs.
[a]*n = 1.*
[b]*n = 2.*
[c]*n = 3.*
[d]*n = 4.*
[e]*n = 5.*
[f]*n = 6.*
[g]*n = 7.*
ND – not determined. NC – not calculated due to cytotoxicity. cLogP was calculated using Collaborative Drug Discovery (https://www.collaborativedrug.com/).

tuberculosis (**Table 4**). Two compounds (**4** and **7**) were cytotoxic to macrophages and we could not calculate activity.

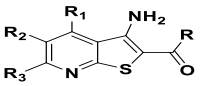
NC–not calculated due to cytotoxicity. cLogP was calculated using Collaborative Drug Discovery (https://www.collaborativedrug.com/).

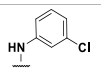
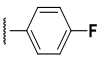
Eight compounds showed good intracellular activity (IC_{50} <10 μM). There was a ten-fold difference between the most potent compound (**3**, IC_{50} = 1.4 μM) and the least potent compound (**13**, IC_{50} = 13 μM). Compounds generally showed low cytotoxicity against both RAW264.7 cells and HepG2 cells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

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

It seems that lipophilicity did not correlate with anti-tubercular activity. Although the most active compounds (**3** and **12**) had cLogP of 3.97 and 4.55 respectively, compound **13** with cLogP of 3.32 was only two-fold less active and compounds with cLogP <3 still retained good activity (IC_{50} <10 μM). An electron-withdrawing moiety on the para

TABLE 8 | Catalog structure-ctivity relationship of the TIP series.

Thienopyridines series 

Compound		cLogP	R1	R2	R3	R4	Extracellular (μM)	Intracellular (μM)		HepG2 cytotoxicity IC50 (μM)	
ID	#						IC_{90}	Mtb IC_{50}	RAW TC_{50}	Galactose	Glucose
TPN-0073022	31	3.66		(methoxy methyl)	H	Me	>20[a]	>100[c]	15 ± 9[b]	64[a]	51[a]
TPN-0073023	32	3.42		(methoxy methyl)	H	Me	>20[a]	17 ± 6.5[e]	67 ± 18[d]	49[a]	68 ± 1.0[b]
TPN-0073024	33	3.43		H	-(CH2)2-	-(CH2)2-	>20[a]	62 ± 31[c]	>100[b]	>100[a]	>100[a]
TPN-0073025	34	4		H	H	Ph	>20[a]	9.0 ± 6.6[e]	30 ± 6.6[f]	42[a]	61 ± 48[b]
TPN-0073026	35	7.23		Ph	H	Ph	>20[a]	>100[c]	>100[a]	>100[a]	>100[a]
TPN-0073027	36	3.69		H	H	Ph	>20[a]	6.8 ± 3.3[f]	>100[g]	64 ± 12[d]	72 ± 22[c]
TPN-0073030	37	3.27		H	-(CH2)2-	-(CH2)-	>20[a]	1.1[a]	>100[c]	>100[a]	>100[a]

Number of runs.
[a]*n = 1.*
[b]*n = 2.*
[c]*n = 3.*
[d]*n = 4.*
[e]*n = 5.*
[f]*n = 6.*
[g]*n = 7.*
cLogP was calculated using CollaborativeDrug Discovery (https://www.collaborativedrug.com/).

position of the amide phenyl group (R_2) appears to be an essential requirement for good anti-mycobacterial activity. The amide phenyl ring (R_2) is not critical to maintain antimycobacterial activity, since compounds **8** and **12** with saturated rings. Both compounds **8** and **10** with adamantane and piperidine groups respectively, had good activity (IC_{50} = 7.6 μM and IC_{50} = 6.6 μM). Further exploration of the length of the carbon chain or inclusion of other heteroatoms would expand the SAR, but such molecules are not available commerically and would require chemical synthesis.

The Phenylthiourea 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 (TC_{50} of 1.8 μM) and was also toxic for HepG2 cells (TC_{50} of 5.5 μM). The remaining three compounds had excellent potency (0.30–1.3 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).

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

In contrast to the BAE series, the PTU molecules were also active against extracellular bacteria; 3 of the 5 molecules had IC_{90} < 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 (Liav et al., 2008; Plutín et al., 2016; Ghorab et al., 2017; Doğan et al., 2020).

The Phenyl Pyrazole 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 (IC_{50} = 2.9 μM) and >30-fold selectivity against HepG2 cells. Compound **21** also showed weak activity (IC_{50} = 18.5 μM) against axenically-grown *M. tuberculosis*.

The Thiophene Carboxamide Series

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 acitivity of IC_{50} < 10 μM (**Table 7**). Three compounds (**23–25**) were cyotoxic and we could not calculate activity.

Three compounds (**26**, **27** and **29**) showed good intracellular potency with IC_{50} < 5 μM. All except **26** and **28** were cytotoxic against macropahges with IC_{50} ranging from 9 to 67 μM; compound **27** was the most toxic and also showed cytotoxicity against HepG2 cells (IC_{50} = 4.7 μM). However, despite this, compounds **26**, **28**, **29**, and **30** al l 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*.

The Thienopyridines Series (TIP)

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

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 μM (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

Identification of new anti-tubercular agents that are active against emerging MDR and XDR-TB is critical. The identification of drugs that inhibit new targets will contribute to developing effective drug regimens for drug-resistant strains. *M. tuberculosis* is an intracellular pathogen which can replicate within macrophages where the set of essential genes will differ from extracellular bacteria. Thus screening compounds against intracellular bacteria has the potential to identify compounds that inhibit novel targets.

We screened a diversity library of small molecules 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.

Previous studies had identified compounds that restrict *M. tuberculosis* growth within the context of macrophages and it was demonstrated that these compounds inhibited the novel target decaprenyl-phosphoribose epimerase (DprE1) which catalyses the synthesis of decaprenyl-phospho-arabinose (Christophe et al., 2009). In addition, compounds which selectively inhibit *M. tuberculsois* growth within the macrophages with unknown mechanism of action were found. Other studies identified host-targeted small molecules that restrict the intracellular growth of *M. tuberculosis* (Stanley et al., 2014). Our series do not overalp with those previously identified.

For the five series we prioritized, this is the first report of antimycobacterial activity of the majority of these compounds. Other works have reported compounds with similar substructures, but our series are structurally distinct overall. We identified a novel series (PTU) containing a thiourea group. To the best of our knowledge, this is the first report of this structural class of compounds as antitubercular agents although other thiourea derivatives have antitubercular activity (Liav et al., 2008; Plutín et al., 2016; Ghorab et al., 2017; Doğan et al., 2020). We identified a novel phenyl pyrazole series. 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* (Poce et al., 2019). 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. We identified a novel thiophene carboxamide series. To the best of our knowledge, this is the first report of these compounds as antitubercular agents. Our molecules are not structurally similar to the previously reported thiophene carboxamide prodrug (Mori et al., 2015), although both contain a thiophene group and an amide group, the linkages and other features of the molecules are different.

This highlights the power of the screen assay in identifying novel compounds that are conditionally active against intracellular *M. tuberculosis*. These conditionally-active compounds would be overlooked in other screens against extracellular bacteria. There is accumulating evidence that intracellular bacteria are both heterogenous and antibiotic tolerant (Vijay et al., 2017; Adams et al., 2019; Mandal et al., 2019; Mishra et al., 2019; Nair et al., 2019), and this may be one reason why drug treatment to achieve sterilization takes >4 months. The inclusion of compounds which specifically target intracellular bacteria in a drug regiment could be one approach to shorten therapy. In support of this, GSK286, a molecule identified from intracellular screens targeting cholesterol metabolism, is in phase II clinical trials.

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 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.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors contributed to conception and design of the study. SA, AM, LF, and YO conducted the experimental work. All authors analyzed the data. SA and TP wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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