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Whole cell screen based identification of spiropiperidines with potent antitubercular properties

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Abstract:

Whole cell based screens to identify hits against *Mycobacterium tuberculosis* (Mtb), carried out under replicating and non-replicating (NRP) conditions, resulted in the identification of multiple, novel but structurally related spiropiperidines with potent antitubercular properties. These compounds could be further classified into three classes namely 3-(3-aryl-1,2,4-oxadiazol-5-yl)-1'-alkylspiro[indene-1,4'-piperidine] (abbr. spiroindenes), 4-(3-aryl-1,2,4-oxadiazol-5-yl)-1'-alkylspiro[chromene-2,4'-piperidine] (abbr. spirochromenes) and 1'-benzylspiro[indole-1,4'-piperidin]-2(1H)-one (abbr. spiroindolones). Spiroindenes showed $\geq$4 $\log_{10}$ kill (at 2-12 μM) on replicating Mtb, but were moderately active under non replicating conditions. Whole genome sequencing efforts of spiroindene resistant mutants resulted in the identification of I292L mutation in MmpL3 (Mycobacterial membrane protein Large), required for the assembly of mycolic acid into the cell wall core of Mtb. MIC modulation studies demonstrated that the mutants were cross-resistant to spirochromenes but not to spiroindolones. This report describes lead identification efforts to improve potency while reducing the lipophilicity and hERG liabilities of spiroindenes. Additionally, as deduced from the SAR studies, we provide insights regarding the new chemical opportunities that the spiroindolones can offer to the TB drug discovery initiatives.

Tuberculosis continues to be one of the major killer diseases affecting the world today. It is increasingly becoming a hard-to-cure disease with the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of the causative agent, *Mycobacterium tuberculosis* (Mtb). Adding to the complication is the ability of the pathogen to remain as non-replicating persistors (NRP) that could contribute towards establishment of a chronic and latent infection. Reactivation of the disease may occur under immuno-compromised conditions.[1,2,3] All of these pathologically complex events lead to difficulties in both diagnosis and treatment of tuberculosis. The current 6-month short-course chemotherapy comprises of a 2 month-intensive treatment with a 4 drug combination (Isoniazid, Rifampicin, Pyrazinamide and Ethambutol), followed by a 4-month continuation therapy with Isoniazid and Rifampicin.[4] Non-compliance with this regimen due to its long duration and the side-effects of the medicines are major challenges to cure tuberculosis.[5,6] Hence, there is a desperate need for the discovery of new drugs that can shorten the treatment duration and be effective against MDR, XDR as well as latent tuberculosis.

Compounds with cidal properties on replicating and NRP Mtb would be considered attractive for TB drug discovery. Although, simulating the exact conditions that trigger NRP in latent TB is hard to achieve under *in vitro* conditions, various models have been developed that induce actively replicating Mtb to slow down growth and enter NRP.[7,8] The Wayne model mimics the hypoxic conditions observed in the necrotic lesions, to induce NRP in Mtb under *in vitro* conditions. In this model, two stages of NRP are observed, NRP1 is the first stage, where dissolved O_2 levels approach 1% saturation and is characterized by a slow rate of increase in turbidity without a corresponding increase in the number of CFU or synthesis of DNA. When dissolved O_2 content drops below 0.006% saturation, the bacilli shift to an anaerobic stage, NRP2, where there is no further increase in turbidity. It is hypothesised that the ability of tubercle bacilli to shift down into one or both of the two NRP stages, is responsible for the ability of the pathogen to remain dormant in the host for long periods of time.[8]

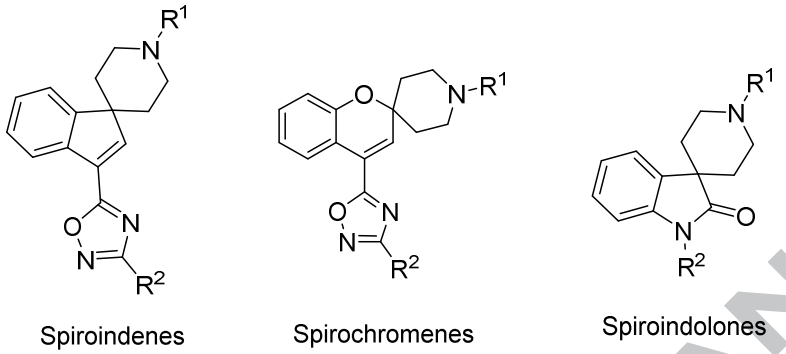
Another model for NRP is the streptomycin starved 18b (ss18b) strain, which is a clinical isolate of Beijing lineage, that is dependent on streptomycin for growth.[9] This strain grows actively when the media is supplemented with streptomycin (18b+) and is comparable to the replicating phase of WT H37Rv. In the absence of streptomycin, this strain slows down growth and enters an NRP phase, remaining viable for over 6 months and is referred to as the ss18b. Since the ss18b strain remains non-replicating under normal aerobic conditions, it is easy to handle and more amenable to use as an NRP model for High Throughput Screening (HTS).[9,10]

In this work, we describe a whole cell screen carried out on both replicating and ss18b Mtb, leading to the identification of novel spiropiperidine class of compounds with potent antitubercular properties (Figure 1A). Spiropiperdines include three structurally close chemical classes viz. 3-(3-aryl-1,2,4-oxadiazol-5-yl)-1'-alkylspiro[indene-1,4'-piperidine] (abbreviated as spiroindenes), 4-(3-aryl-1,2,4-oxadiazol-5-yl)-1'-alkylspiro[chromene-2,4'-piperidine] (abbr. spirochromenes) and 1'-benzylspiro[indole-1,4'-piperidin]-2(1H)-one (abbr. spiroindolones). Resistant mutant generation followed by whole genome sequencing (WGS) identified a mutation in MmpL3 that led to resistance against spiroindene compounds. Previous work in the TB drug discovery area has identified a variety of scaffolds that target MmpL3, including SQ109,[11] AU1235,[12] C215,[13] BM212,[14] THPP,[15] Spiros[15] and indolecarboxamides (NITD-349)[16] (Figure 1B). SQ109, a 1,2-diamine, is currently in clinical trials for the treatment of tuberculosis and was recently shown to target MmpL3 (Mycobacterial membrane protein Large) and thereby interfere with the transport of mycobacterial trehalose monomycolate (TMM), required for the assembly of mycolic acids into the cell wall core of Mtb.[11,17]

The mutants raised against spiroindenes were cross-resistant with spirochromene compounds but not the spiroindolones, suggesting that the latter could bind at a different site or may have a completely diverse mode of action. Preliminary SAR efforts led to improvement of solubility and reduced the cytotoxicity liabilities of the spiroindene series. While spirochromenes exhibited similar SAR trends to

spiroindenes, spiroindolone match-pairs of spiroindenes showed better physicochemical properties like lower logD, improved solubility etc. indicating an opportunity for scaffold-morphing to obtain more lead-like compounds suitable for antimycobacterial drug discovery.

A.



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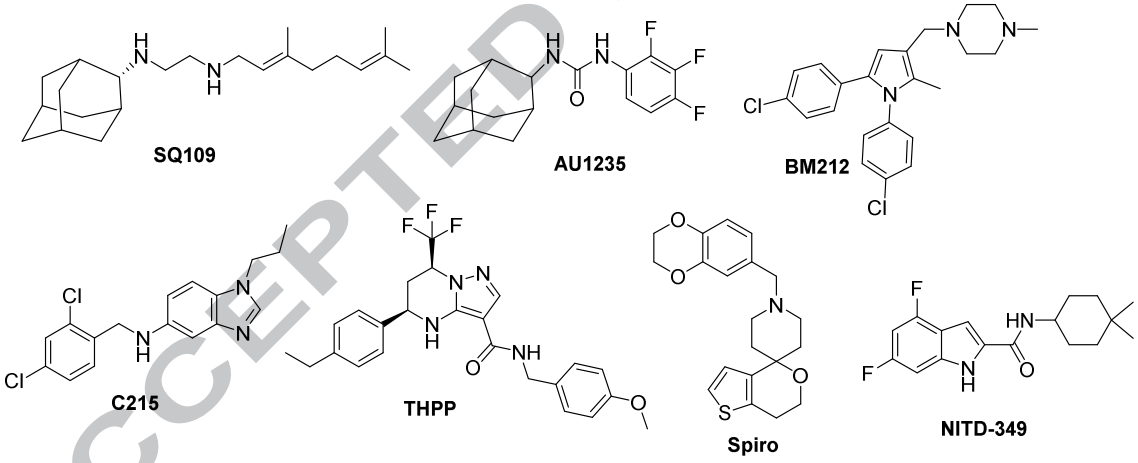


Figure 1. (A) Spiroindenes, Spirochromenes and Spiroindolones; (B) Known MmpL3 inhibitors

A collection of 320,000 compounds from AstraZeneca corporate library was screened at a single point concentration of 20 µM against replicating Mtb (H37Rv). At this concentration, 1600 compounds exhibited at least 80% inhibition of growth. These compounds were further screened in parallel to

determine the inhibitory concentrations against both replicating Mtb (H37Rv) and the non-replicating ss18b strains, as a 10 point concentration response with two-fold dilution, starting at 50 μM. A cut off of 10 μM (MIC) was used to select potent compounds for Mtb H37Rv. A higher cut off of 50 μM (IC_{80}) was used to identify compounds active against the non-replicating strain ss18b, based on the observation that several fold higher concentrations of compounds was required to inhibit non-replicating Mtb when compared to their MIC on replicating Mtb.[9] Compounds with potent activity on replicating Mtb were chosen for further analysis, irrespective of their activity on NRP Mtb. This resulted in two categories of compounds, one set that was active only on replicating Mtb and the other active on both replicating and NRP Mtb.

Hit evaluation based on potency, chemical structure and lead-like properties resulted in the identification of 14 clusters prioritized for confirmation of activity. In order to confirm the activity, solid stocks or resynthesized compounds of representatives from these clusters along with 758 near neighbours were profiled for MICs. This was followed by determination of Minimum Bactericidal concentration (MBC) on replicating Mtb H37Rv as well as non-replicating models (hypoxic H37Rv and ss18b). Cytotoxicity was also evaluated by measuring IC_{50} against A549 mammalian cells and reported as the Mammalian MIC (MMIC) (Figure 2). Spiropiperidines consisting of spiroindenes, spirochromenes and spiroindolones emerged as an attractive series from this screening cascade since they were cidal against replicating and NRP Mtb, while retaining a good window (>30 fold) between MMIC and MIC on replicating Mtb (Table 1). Additionally, a second series, 4-aminoquinolone piperidine amides was also identified as a result of this screening cascade and is discussed separately.[18] Since spiroindene was the largest subseries with good potency distribution in the spiropiperidine class; more detailed evaluation of this series was carried out.

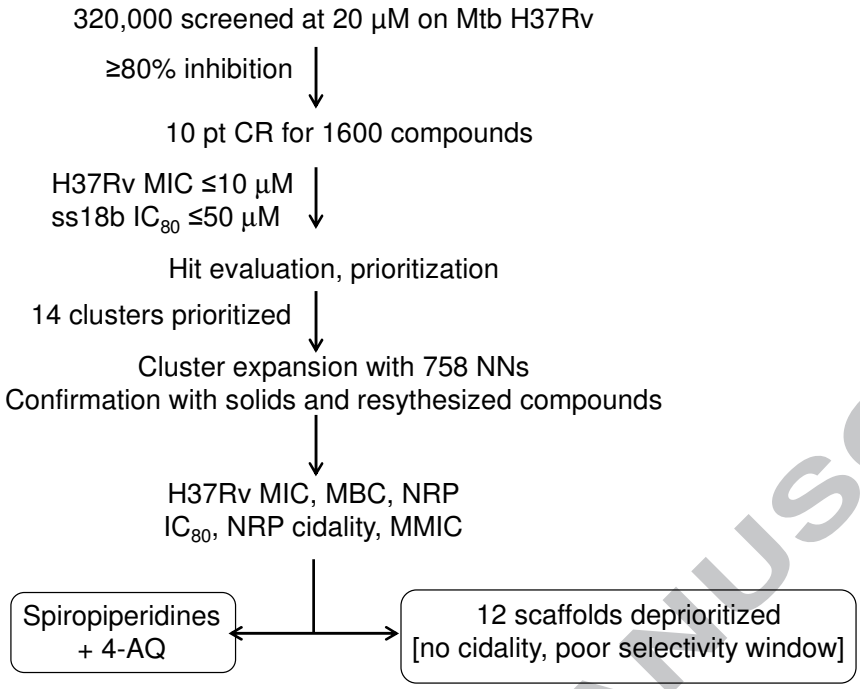
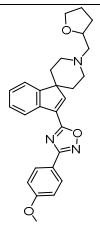
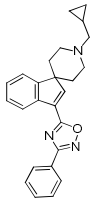
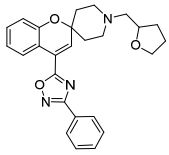
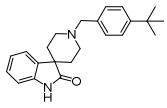


Figure 2. Whole cell Screening Cascade resulting in prioritization of spiropiperidines. NNs, Near Neighbours; CR, Concentration response; MMIC, IC_{50} against A549 mammalian cells 4-AQ, 4-Aminoquinolone amides

Table 1. *In vitro* properties of representative hits from spiropiperidine class

Compound	Structure	Class	Mtb MIC µM	Mtb MBC µM	ss18b IC_{80} µM	Hyp oxia IC_{80} µM	MMIC[a] µM	Human PPB[b] (% free)	Solubility[d] µM	ClogP	LogD

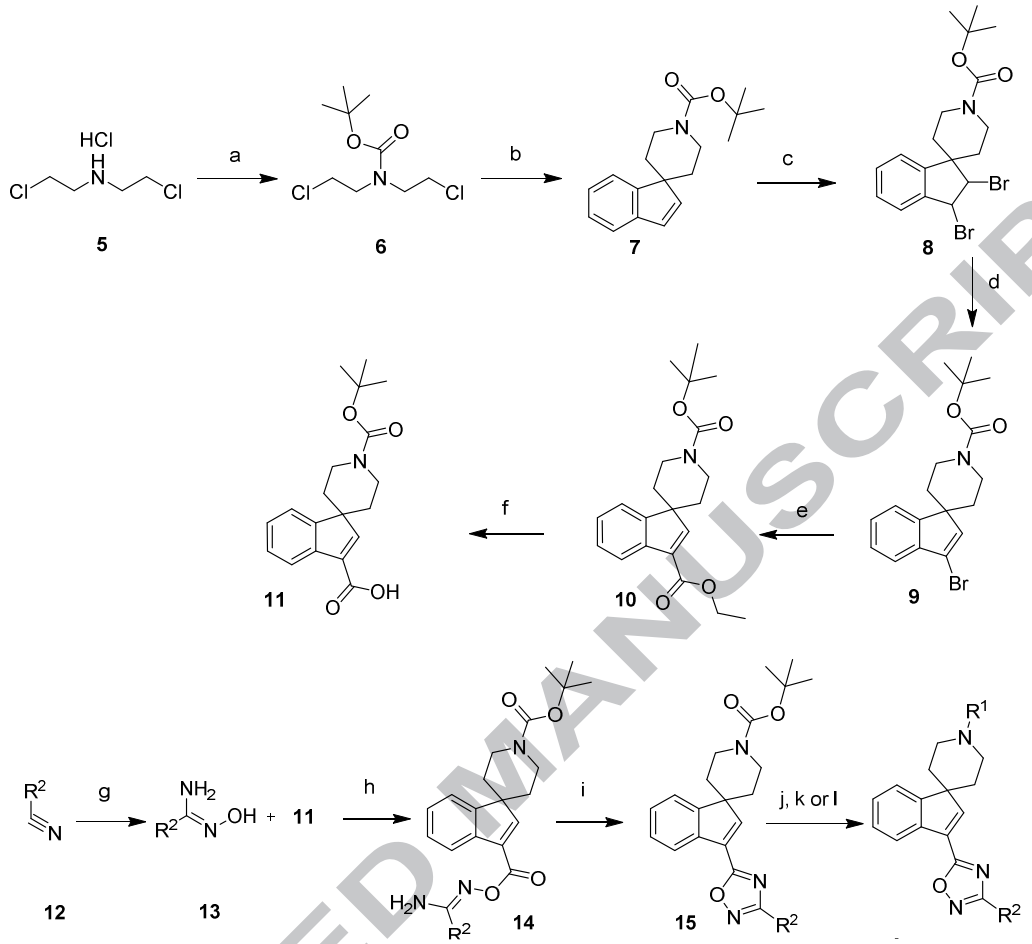
1		Spiro indene	0.3	0.8	>200	>200	68	<1	<1	4.19	>5.2
2		Spiro indene	0.4	0.4	50	100	21	<1	1	4.85	>4.8
3		Spiro chromene	0.8	1.3	50	200	5	<1	3	3.94	>4.8
4		Spiro indolone	3	12	50	200	>50	ND [c]	4	4.34	4.3

[a]MMIC, IC_{50} against A549 mammalian cells; [b]Plasma protein binding; [c]not determined; [d] high-throughput aqueous solubility generated at pH7.4 using solids from dried DMSO stocks.

Spiroindene compounds were synthesized as described in the scheme 1. The key intermediate 1'-(Boc) spiro[indene-1,4'-piperidine]-3-carboxylic acid **11** was synthesized in multigram scale using a 8-step synthesis starting from commercially available bis(2-chloroethyl)amine hydrochloride **5** (Scheme 1). As a first step towards this synthesis, bis(2-chloroethyl)amine hydrochloride **5** was protected with acid labile Boc-group. A rapid stirring of **5** with 10% NaOH solution, followed by addition of di-*tert*-butyl dicarbonate resulted in *tert*-butyl bis(2-chloroethyl)carbamate **6** in about 88% yield. Intermediate, *tert*-butyl spiro[indene-1,4'-piperidine]-1'-carboxylate **7** was prepared starting from 1*H*-indene. Addition of LHMDS onto a cooled solution of 1*H*-indene in THF resulted in a carbanion which was quenched with

carbamate **6** to give the required spiropiperidine **7** at about 46% yield. Bromination of spiropiperidene **7** at 5ºC resulted in *tert*-butyl 2,3-dibromo-2,3-dihydrospiro[indene-1,4'-piperidine]-1'-carboxylate **8** in quantitative yield. The dibromide **8** was subjected to dehydrohalogenation with potassium hydroxide and the resulting monobromide intermediate **9** was carbonylated with carbon monoxide in ethanol to give 1'-*tert*-butyl 3-ethyl spiro[indene-1,4'-piperidine]-1',3-dicarboxylate **10** in about 46% yield. Carbonylation was carried out using $Pd(OAc)_2$ as catalyst. Optimum results were obtained when the reaction was carried out in a steel bomb at 100 psi pressure and 110ºC temperature for 12 h. Subsequent hydrolysis of ester **10** using NaOH in methanol-water led to the desired compound **11** in quantitative yields.

Refluxing nitrile **12** with hydroxylamine hydrochloride in ethanol resulted in corresponding amidoximes **13** in quantitative yields. The general applicability of a varied set of aliphatic and aromatic nitrites was useful in bringing diversity at R^2-position. Amidoxime **13** was coupled with 1'-(Boc) spiro[indene-1,4'-piperidine]-3-carboxylic acid **11** using a simple EDCI/HOBt protocol and the resulting amido ester **14** was cyclized with potassium acetate in DMF to give corresponding 1,2,4-oxadiazole substituted spiroindenes **15** in moderate yields. Intermediate **15** was deprotected with HCl in 1,4-dioxan and was either alkylated with alkyl bromides using potassium carbonate or could be coupled with corresponding acid employing HATU/DIEA protocol to result in title compounds.

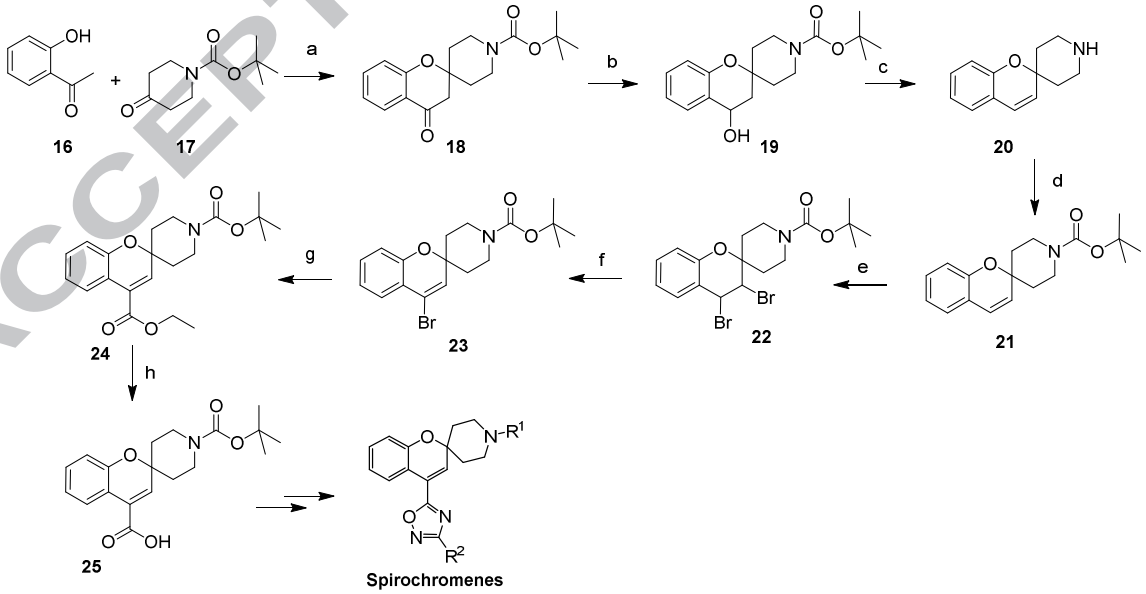


Scheme 1. Synthesis of spiroindenes

Reagents and conditions: (a) Boc anhydride, 10% aq. NaOH, DCM, RT, 6 h.; (b) LHMDS, 1*H*-indene, THF, <10°C, 2.5 h.; (c) Br_2, THF, <5ºC, 2 h.; (d) KOH, EtOH, RT, 5 h.; (e) 1,3-bis(diphenylphosphino)propane, $Pd(OAc)_2$, NEt_3, EtOH, carbon monoxide (gas), 100 psi, 110ºC, 12 h.; (f) NaOH, water, 60ºC, 2 h. (g) $NH_2OH.HCl$, EtOH, K_2CO_3, reflux, 18 h; (h) EDCI.HCl, HOBt, DCM, RT 3h.; (i) KOAc, DMF, MW, 120ºC, 25 min.; (j) 1,4-dioxan, HCl (conc) RT, 1h.; (k) R^1-Br, K_2CO_3, DMF, 120°C, 15 h.; (l) R^1-COOH, HATU, DIEA, DMF, RT 2 h.

The key intermediate 1'-(Boc) spiro[chromene-2,4'-piperidine]-4-carboxylic acid **25** was synthesized starting with Aldol condensation of *o*-hydroxyacetophenone **16** with 1-Boc-piperidinone **17** in presence

of pyrrolidine (Scheme 2). The resulting α,β-unsaturated ketone intermediate generated *in-situ* undergoes intramolecular Michael type addition to result in spirochromone **18** in about 80% yield.[19] Reduction of spirochromone **18** with sodium borohydride gave intermediate **19** which on dehydration with *p*-toluene sulfonic acid gave spiro[chromene-2,4'-piperidine] **20**. During the dehydration stage the Boc-group on piperidine became de-protected and was protected again with Boc anhydride to yield intermediate **21**. Bromination of spirochromene **21** at 0ºC resulted in *tert*-butyl 3,4-dibromospiro[chroman-2,4'-piperidine]-1'-carboxylate **22** in about 88% yield. The dibromide **22** was subjected to dehydrohalogenation with potassium hydroxide and the resulting monobromide **23** intermediate was carbonylated with carbon monoxide in ethanol to give 1'-*tert*-butyl 4-ethyl spiro[chromene-2,4'-piperidine]-1',4-dicarboxylate **24** in about 69% yield. Optimum results were obtained at 75 psi carbon monoxide pressure and 60ºC temperature in presence of $Pd(OAc)_2$ as catalyst. Hydrolysis of ester **24** using NaOH in methanol-water provided the desired key intermediate 1'-(*tert*-butoxycarbonyl)spiro[chromene-2,4'-piperidine]-4-carboxylic acid **25** which was subjected to protocol similar to that of spiroindene synthesis (Scheme 1) to obtain the required spirochromenes.

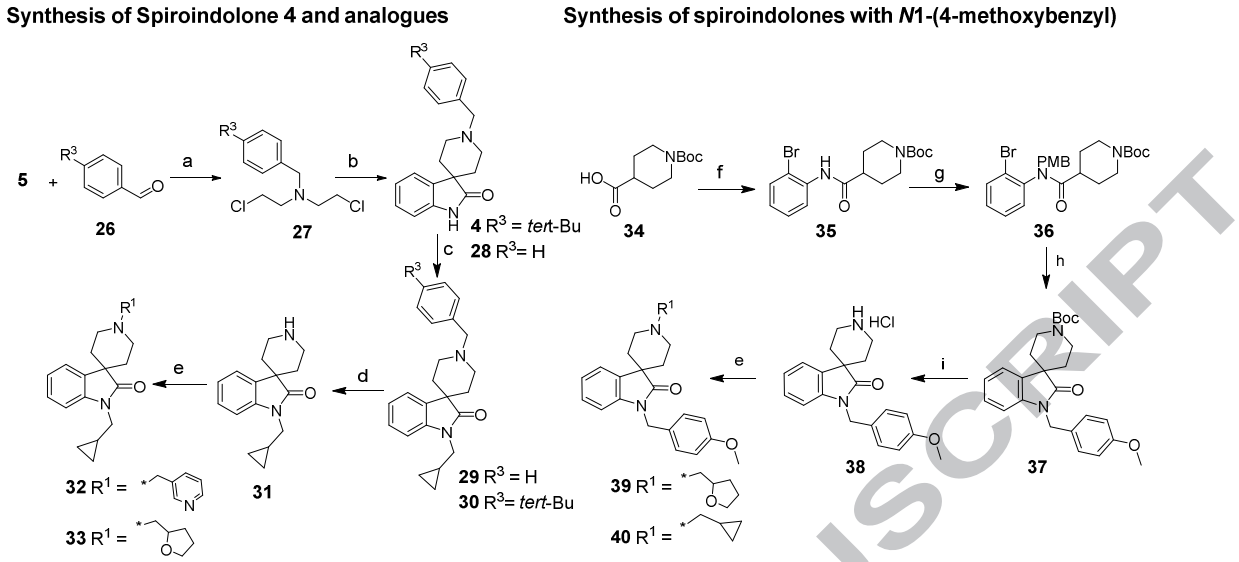


Scheme 2. Synthesis of spirochromenes

Reagents and conditions: (a) Pyrrolidine, EtOH, 0-25^{o}C, 20 h.; (b) $NaBH_4$, MeOH, 0-25^{o}C, 3 h.; (c) PTSA, Toluene, 130^{o}C, 16 h.; (d) Boc anhydride, 1,4-dioxane-H_2O (1:1), NaOH, 0-25^{o}C, 3 h.; (e) Br_2, CCl_4, 0^{o}C, 4 h.; (f) KOH, EtOH, 85^{o}C, 16 h.; (g) BINAP, $Pd(OAc)_2$, NEt_3, EtOH, carbon monoxide (gas), 75 psi, 60ºC, 12h.; (h) MeOH, 5% aq. NaOH, 60ºC, 3 h.

Synthesis of spiroindolone **4** and *N*1-cyclopropylmethyl analogues began with reductive amination of bis(2-chloroethyl)amine **5** with substituted/unsubstituted benzaldehydes **26** to give tertiary amines **27** (scheme 3). The spiroindolone ring was constructed by the treatment of 2-oxindole with amine **27** using excess of NaH to give required compounds **4** and **28**. Compound **29** was obtained by base mediated alkylation of compound **4** with cyclopropylmethyl bromide. Similar alkylation of compound **28** gave intermediate **30** which was used for further transformations. Pd/C catalysed debenzylation of intermediate **30** to intermediate **31** followed by alkylation of **31** using corresponding alkyl halides in presence of Hunig's base gave compounds **32** and **33**.

Synthesis of *N*1-(4-methoxybenzyl) analogues of spiroindolone was achieved following a different protocol as shown in scheme 3. The commercially available acid **34** was coupled with the 2-bromoaniline by using 1-propanephosphonic acid cyclic anhydride (T_3P) as a promoter in the presence of a base to give amide **35**. The secondary amide **35** was converted into tertiary amide **36** by alkylation with *p*-methoxybenzyl chloride using NaH as a base. The required spiropiperidine ring was then constructed by using intramolecular Buchwald-Hartwig reaction of amide **36** to give intermediate **37** in good yield. The required alkyl groups were introduced by Boc deprotection of intermediate **37** by treating with HCl followed by alkylation of intermediate **38** to give compounds **39** and **40**.



Scheme 3. Synthesis of spiroindolones

Reagents and Conditions: (a) $NaBH(OAc)_3$, NEt_3, DCM; (b) 2-Oxindole, NaH; (c) K_2CO_3, cyclopropylmethyl bromide; (d) H_2, Pd/C, EtOAc; (e) R^1X, DIPEA, CH_3CN. (f) 2-bromoaniline, DIPEA, T_3P; (g) NaH, PMBCl, DMF; (h) NaOBut, $Pd(dba)_3$, BINAP, 1,4-dioxane; (i) HCl, Et_2O

Out of 83 compounds tested from the spiroindene class, 32 compounds had an MIC ≤2 µM against replicating Mtb. These were profiled for cidality on replicating Mtb as well as on non-replicating Mtb using hypoxia and ss18b models (Figure 3). The MBC/MIC ratio was ≤ 4 for all compounds tested under replicating conditions. IC_{80} values matched well (less than 4 fold variation) between the two models for NRP Mtb. The majority of the compounds tested in NRP models had IC_{80} greater than 10 µM (Figure 3). Most compounds that were active on the NRP models had NRP cidality (MBC) in the range of 25-200 µM (2 log kill, data not shown) on both ss18b and hypoxic Mtb. Further optimization of the compounds could result in more potent compounds. Two compounds with IC_{80} values of 50 µM and 100 µM respectively, were not cidal on hypoxic or ss18b Mtb up to 200 µM. It is possible that they are cidal at higher concentrations, but this could not be tested due to their limiting solubility. No visible precipitation of the compound was observed up to the reported IC_{80} concentrations. Isoniazid and

clofazimine were used as controls in all the assays with NRP models. Clofazimine had MBC of 0.6 µM against ss18b and 2.6 µM against hypoxic Mtb H37Rv and 0.6 µM against replicating Mtb H37Rv. Isoniazid did not have MBC against the non-replicating Mtb (ss18b and hypoxic models), but had an MBC of 0.5 µM on replicating Mtb H37Rv.

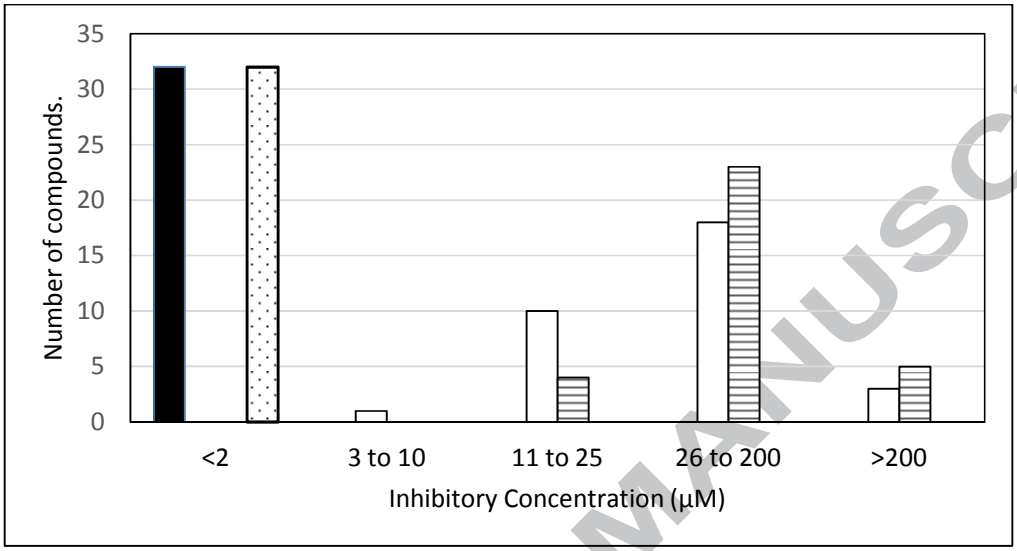


Figure 3. Mtb cidality and NRP activity of spiroindene compounds with MIC ≤ 2µM. ■ MBC in Replicating Mtb, ⊡ MIC in replicating Mtb, □ Hypoxia IC_{80}, ▤ ss18b IC_{80}.

Compound **1**, which was one of the most potent compounds from the initial hits, was selected for further profiling in *in vitro* cidality assays. Killing kinetics study under *in vitro* conditions for compound **1** indicated >4 $\log_{10}$ kill, in 7 days when compared to the start cfu of 4.8 x10^6 cfu/mL. The study showed that the activity of spiroindenes was dependent on time of exposure; although some concentration dependence was observed, it was not significant (Figure 4A). To determine if spiroindenes were active on intracellular Mtb, compound **1** was tested against macrophage cells infected with Mtb as described in the methods. The compound was cidal on intracellular Mtb with ~1.3 $\log_{10}$ cfu/ml reduction in cell number (Figure 4B). The potent cidality on replicating Mtb in addition to the ability to kill intracellular Mtb suggests that spiroindene series has cidal properties suitable for further lead optimization.

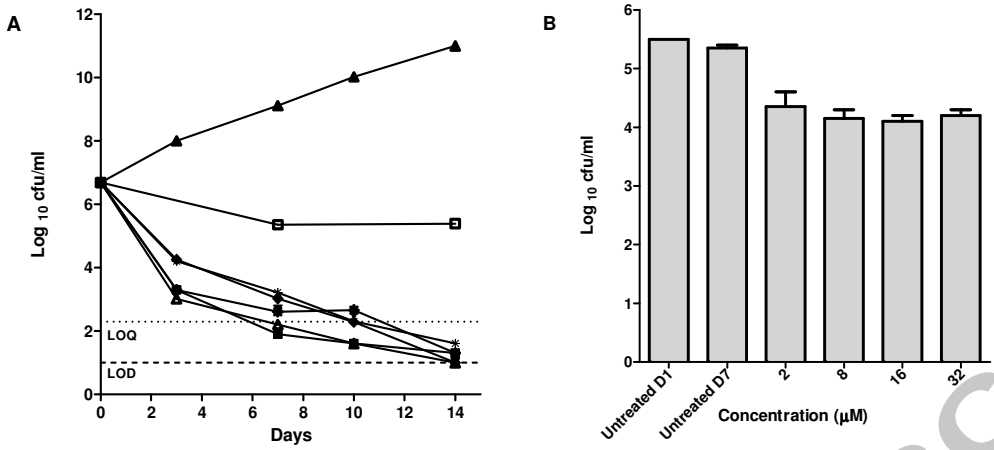


Figure 4. Bactericidal properties of Compound **1**. (A) Kill kinetics of H37Rv at various concentrations (µM). Δ50,■25, ✱12.5, ▼6.25, ◆3.12, ✳ 1.56, □ 0.78, ▲ No drug. (B) Activity on intracellular H37Rv. LOQ is the limit of quantification. LOD is the limit of detection. D1 and D7 are Day1 and Day7 post infection respectively.

The spiroindene compounds were tested against A549 cells, which are adenocarcinomic human alveolar basal epithelial cells. Cytotoxicity of the compounds was measured as their IC_{50} on the A549 cells (MMIC) to determine the safety margin compared to the MIC on replicating Mtb. Although majority of the compounds showed <5 fold window between MMIC and Mtb MIC, a few compounds (**47**, **57** and **59**, Table 2) with potent MIC had MMIC >100 µM (pMMIC < 4) giving a 500-1000 fold safety window (Figure S1 in the supporting information). This suggests that there is sufficient scope to reduce the safety risk against mammalian cells without compromising on the potency against Mtb.

The series had potent antimycobacterial properties (<2 µM MBC). However, most of the compounds (Mtb MIC ≤ 1 µM, pMIC ≥ 6) were highly lipophilic (ClogP >4) resulting in very low aqueous solubility (<1 µM) and poor plasma free fractions (<1% free) (Table 1 and 2, Figure S2 in the supporting information). Also, several of the compounds exhibited potent inhibitory activity on mammalian cells which would potentially pose a safety risk. Hence, chemistry attempts were focused on reducing the lipophilicity of the compounds, with the hypothesis that this would result in an improvement of

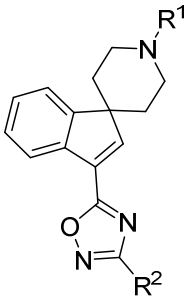
physicochemical properties including solubility, plasma protein binding and reduced toxicity on mammalian cells (Table 2).

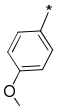
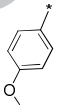
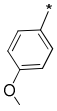
Polar side-chains were added at the R^1 position (Table 2) to increase the polarity of the compounds. Hydroxylated side-chains at R^1 position resulted in potent MICs with reduced lipophilicity, but unfortunately these compounds had potent MMICs (Table 2, compounds **41**and **42**) and hence, were unsuitable for further progression. Compounds **44** and **45** with carboxylic acid and sulphonamide in side-chain at R^1 position respectively, were inactive on Mtb whereas compound **46** with a primary amide in the side-chain had a weak MIC against Mtb. Methylation of the primary amide to a secondary or tertiary amide in the side-chain led to better MICs without any improvement in either solubility or plasma protein binding (compounds **47** and **48** respectively). Compound **49** with isosteric replacement of amide in the side-chain with 1,2,4-oxadiazole showed weaker MIC. Direct attachment of an amide to the piperidine *N* led to compounds with much weaker MICs on Mtb (compounds **50** and **51**), hence these were not useful.

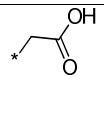
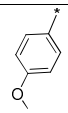
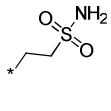
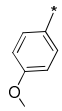
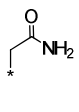
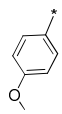
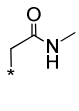
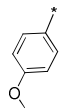
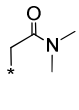
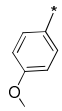
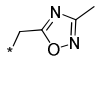
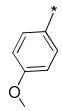
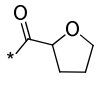
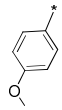
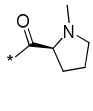
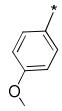
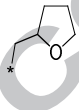
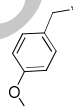
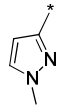
Modifications were made at the R^2 position to reduce lipophilicity. Replacement of the aryl group with methyl resulted in a completely inactive compound **52**, indicating essentiality of an aryl group at this position. Replacing aryl with benzyl led to compound **53** with modest potency without significant improvement in the properties. Isosteric replacement of the aryl group with thiophene resulted in a potent compound **54**, with similar physicochemical properties; a more polar and smaller pyrazole group as in compound **55** was not tolerated at this position. Pyridines were well tolerated at the R^2 position (compounds **56**-**61**) with a modest improvement in solubility and plasma protein binding. Compound **60** with 4-methoxy-3-pyridyl group showed potent Mtb MIC of 0.4 μM, but was highly insoluble due to its crystalline nature, and could not be progressed further. Other variation like replacing oxadiazole with amide groups or direct attachment of a pyridine ring to the indene led to loss of Mtb MICs.

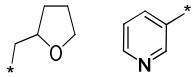
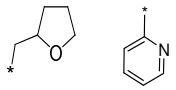
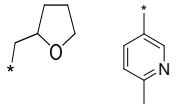
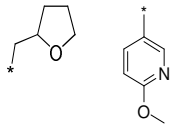
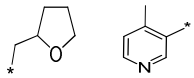
Among all the pyridines tested, compound **57** with 3-pyridyl and compound **59** with 4-methyl-3-pyridyl group showed Mtb MICs of 3.1 and 1.6 µM respectively and both the compounds were not cytotoxic on the A549 cells (MMIC >100 µM).

Table 2. SAR to reduce lipophilicity to improve solubility and PPB



Compound	R^1	R^2	Mtb MIC (µM)	Hypoxia IC_{80} (µM)	MMIC[a] (µM)	ClogP	LogD	Human_PPB[b] (% free)	Solubility[d] (µM)	Predicted pKa	hERG IC_{50} µM
1			0.3	>200	68	4.19	>5.2	<1	<1	8.6	65
41			12	25	2	3.21	3.2	<1	3	8.6	ND[c]
42			0.8	100	3	3.52	ND[c]	ND[c]	ND[c]	9.2	ND[c]
43			0.4	50	19	4.18	ND[c]	ND[c]	ND[c]	8.9	ND[c]

44			>100	>200	>100	1.24	3.9	1.6	1	2.4, 10.4	>33.3
45			>100	>200	ND[c]	2.65	>4.7	<1	<1	10.7, 8.2	>33.3
46			50	25	60.5	2.81	>5	<1	<1	7.3	>33.3
47			12	>200	>100	3.19	>5.3	<1	3	6.7	>33.3
48			6.3	50	12	3.50	>5.3	<1	8	7.3	>33.3
49			50	>200	64	3.33	>5.5	ND[c]	<1	6.2	>33.3
50			100	>200	37	3.43	>5	<1	<1	-	27
51			50	50	4	3.76	3.7	<1	6	9.3	24
52			>200	>200	>200	2.32	2.9	9.2	895	8.6	1.6
53			25	50	10.2	4.04	>4.5	<1	3	8.6	1.4
54			1.1	100	12	3.98	ND[c]	<1	2	8.6	62
55			>100	>200	68	2.56	3.3	11	578	8.6	5.0

56			6.3	>200	75.7	2.89	3.8	2.3	16	8.6	3.2
57			3.1	200	>100	2.89	3.8	3.2	18	8.6	6.7
58			12.5	>200	56.3	3.10	3.5	5.1	123	8.6	5.5
59			1.6	100	>100	3.24	>4.5	1.5	11	8.6	4.3
60			0.4	>200	ND[c]	3.56	ND[c]	ND[c]	ND[c]	8.6	ND
61			6.3	200	31.5	2.94	>4.2	1.4	11	8.6	8.8

[a]MMIC, IC_{50} against A549 mammalian cells; [b]plasma protein binding; [c]Not determined; [d] high-throughput aqueous solubility generated at pH7.4 using solids from dried DMSO stocks.

Most of the spiroindenes showed potent inhibition of hERG (human ether-à-go-go-related channel), presumably due to the basic, lipophilic nature of the scaffold (Table 2). But, compounds with a less basic piperidine nitrogen (e.g. Compounds **46**, **47**, **48**, **49** with a basic pka of 6.3 -7.2 compared to pka 8.6 for compound **1**) showed much lower inhibition on hERG channels (hERG IC_{50} >33 μM, Table 2). This observation may be useful during lead optimization phase to allay cardiac risk due to hERG inhibition.

Compounds **1**, **57** and **59** were screened for MIC against single drug resistant clinical isolates of Mtb as well as a set of non-mycobacterial strains including *Staphylococcus aureus* ARC517, *Streptococcus pneumoniae* ARC548, *Haemophilus influenzae* ARC446, *Escherichia coli* ARC523, *Pseudomonas*

aeruginosa ARC545 and *Klebsiella pneumoniae* ARC1865. The spiroindene compounds were inactive (MIC >100 μM) on all of the non-mycobacterial strains tested. The compounds retained activity on all the clinical strains tested suggesting that this series has the potential to act on resistant strains of Mtb (Table 3).

Table 3. Activity of spiroindenes on drug resistant strains of Mtb.

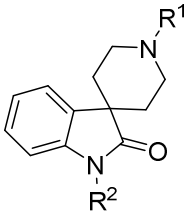
Strain Source	Origin	STR	INH	RIF	EMB	OFL	**1**	**57**	**59**
		MIC (μM)							
M. tuberculosis ATCC 2794	Reference strain	0.5	0.2	0.008	1.2	0.14	0.39	1.56	1.56
M. tuberculosis Beijing (E-47/94)	Reference strain	0.5	0.2	0.008	1.2	0.14	0.39	0.78	0.39
M. tuberculosis D-211	Sputum Isolate	0.5	0.2	0.008	1.2	0.14	0.39	1.56	1.56
M. tuberculosis 18b+	Streptomycin dependent	0.5	0.2	0.008	1.2	0.14	0.78	0.78	1.56
M. tuberculosis STR^R 136570	Sputum Isolate	>55	0.2	0.008	1.2	0.14	0.78	0.78	1.56
M. tuberculosis INH^R 912253	Sputum Isolate	0.5	>29	0.008	1.2	0.14	0.39	1.56	1.56
M. tuberculosis RIF^R 19000	Sputum Isolate	0.5	0.2	>4.8	1.2	0.14	0.39	0.78	0.78
M. tuberculosis EMB^R 17003	Sputum Isolate	0.5	0.2	0.008	>19.5	0.14	0.39	1.56	1.56
M. tuberculosis OFL^R	Sputum Isolate	0.5	0.2	0.008	1.2	>11	0.78	3.12	1.56

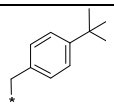
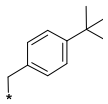
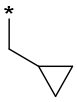
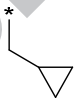
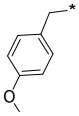
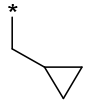
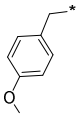
STR: streptomycin; INH: Isoniazid; RIF: Rifampicin; EMB: Ethambutol; OFL: Ofloxacin

Spirochromenes being structurally very similar to spiroindenes were found to follow SAR and SPR trends similar to spiroindenes. Hence, detailed SAR studies for the same are not presented here.

Spiroindolones differ in structure from spiroindenes and spirochromenes as these lack the aryloxadiazole substituents at R^2 position. Moreover, spiroindolone has more polar amide functionality in the core structure which in principle, allow obtaining more polar compounds with better physicochemical properties. Preliminary SAR exploration at R^1 position of spiroindolones (Table 4) indicated that bulky, lipophilic substituent like 4-*tert*-butylbenzyl group was needed to obtain potent MICs (compounds **4** and **28**) whereas, *N*-1 in indolone core (R^2 position) could be substituted with small alkyl groups like cyclopropylmethyl with some improvement in Mtb MIC (compounds **30**). Compounds **32** and **33** with *N*-cyclopropylmethyl substituents, but with more polar side-chains like pyridylmethyl and tetrahydrofurylmethyl at R^1 had much weaker Mtb MICs. Changing R^2 to 4-methoxybenzyl as in compounds **39** and **40** did not improve Mtb MICs further. But, these compounds **32** to **40** showed good physicochemical properties like logD <3.5, solubility >100 μM, improved plasma free fraction. Compound **39**, which is a spiroindolone analogue of spiroindene **53**, showed improved physicochemical properties such as lower logD, high solubility compared to compound **53** while retaining Mtb MIC (Table 5). Thus, there is a potential to obtain lead compounds with improved physicochemical properties using spiroindolone core and the antitubercular activity of these compounds may be optimized by adding substituted oxadiazoles or other heterocycles at R^2 position of indolone ring similar to spiroindene class.

Table 4. Preliminary SAR for spiroindolones



Compound	R^1	R^2	Mtb MIC (μM)	Hypoxia IC_{80} (μM)	MMIC[a] (μM)	ClogP	LogD	Human_PPB[b] (% free)	Solubility[d] (μM)
4		H	3.2	200	>50	4.34	4.2	2	3.6
28		H	>100	-	>50	2.51	2.8	29	>1000
30			0.8	>200	33	5.61	4.2	<1	<1
32			50	ND[c]	>50	2.29	3.2	ND[c]	673
33			200	ND[c]	>50	2.26	2.5	37	>1000
39			50	>200	>50	3.23	2.9	16	598
40			50	>200	>50	3.76	2.7	16	550

[a]MMIC, IC_{50} against A549 mammalian cells; [b]plasma protein binding; [c]Not determined; [d] high-throughput aqueous solubility generated at pH7.4 using solids from dried DMSO stocks.

Both spiroindene and spiroindolone compounds showed moderate to high rate of metabolism *in vitro* as measured in the presence of human liver microsomes and rat hepatocytes (Table 5). This could be due to high lipophilicity and metabolically labile soft spots in these compounds which would need further optimization. Compounds, in general, showed good permeability in a Caco-2 assay indicating suitability for oral absorption.

Table 5. *In vitro* DMPK properties

Compound	**1**	**57**	**59**	**4**	**30**	**39**
Human microsomal Clint (µL/min/mg)	22	37	37	83	28	65
Rat hepatocyte Clint (µL/min/1E6)	22 (78)	100	37	34	100	65

Compounds **57** and **59** were selected for PK studies as MMIC was >100 µM for both compounds. Both the compounds showed rapid metabolism in presence of human microsomes as well as rat hepatocytes, which is indicative of cytochrome P450 mediated oxidative metabolism (Table 5). Hence, these were profiled for plasma exposure *via* oral route at dose of 100 mg/Kg in mice in the presence of 1-aminobenzotriazole (ABT) (Figure 5, Table 6). ABT, which is a known non-specific cytochrome P450 inhibitor,[20] was used here to reduce the CYP mediated metabolism of the compounds so as to achieve plasma concentration above MIC for a longer duration. Both the compounds had a similar C_{max} and AUC. Total plasma concentrations of **57** and **59** were above the MIC for 6 and 20 h respectively. But

free plasma concentrations for both the compounds were below MIC for the entire dose duration. Further scaffold optimization could result in compounds with better free levels above MIC, which would be ideal for *in vivo* efficacy studies.

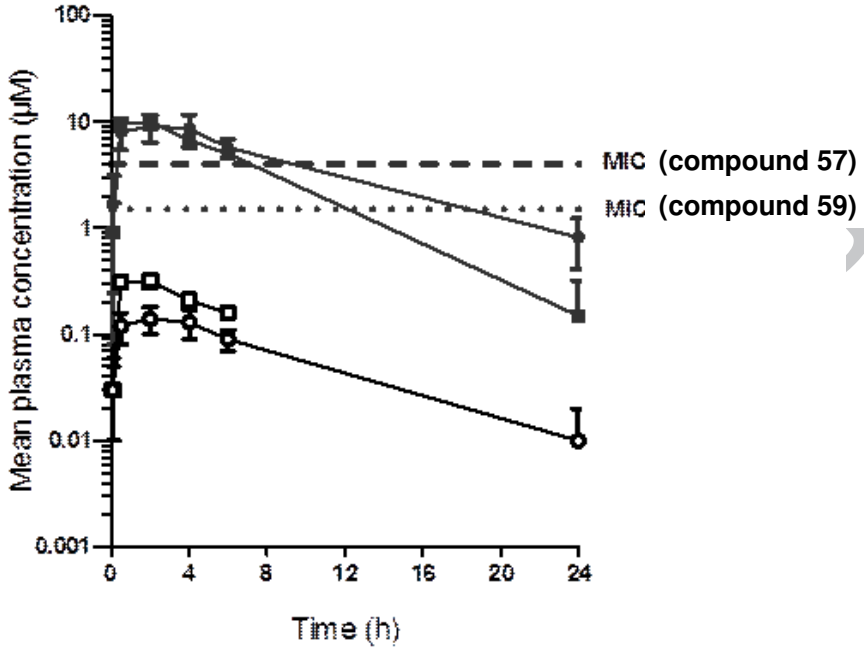


Figure 5. Mouse oral PK at 100 mg/Kg in the presence of ABT. ■ Compound **57**, ● Compound **59**, □ Compound **57**, ○Compound **59**. Filled symbols are total concentrations and open symbols are free plasma concentrations. Error bars indicated standard deviations from three mice.

Table 6. Oral PK in the presence of ABT for Spiroindenes in mice

PK Parameter	Compound **57**		Compound **59**	
	Mean	SD	Mean	SD
C_{max} (µM)	10.1	1.5	9.1	2.6
T_{max} (h)	1.5	0.9	2	0

T_{last} (h)	24	0	24	0
AUC_{inf} (h*µM)	69.5	3.2	99.7	20.7
$t_{1/2}$ (h)	3.5	1.2	6.2	1.7

Isolation and identification of mutants resistant to spiroindenes: Spontaneous resistant mutants to compound **1** were isolated by plating *M. tuberculosis* H37Rv cultures on 7H10 agar plates containing different concentrations of compounds (5 to 10 fold MIC). Spontaneous mutants appeared at a frequency of $5*10^{-9}$ for compound **1**.

Two H37Rv mutants were selected from different plates containing compound **1** at 10X MIC (6 µM). These two mutants, TB333 and TB334, showed 16 fold or higher MIC compared to WT H37Rv by RBMA. Both these mutants were analysed by whole genome sequencing followed by bioinformatic analysis to identify polymorphism that could be responsible for the observed resistance phenotype. A common single point mutation was identified in *mmpL3* gene at nucleotide position 874 (a874c), corresponding to the codon 292 in MmpL3 (I292L), in both TB333 and TB334 mutants. This mutation was confirmed by Sanger sequencing. Since both TB333 and TB334 were shown to have the same mutation, only one of the mutants (TB333) was used for further analysis of the mutation.

In order to further confirm that the a874c mutation in *mmpl3* was able to confer resistance to compound **1**, this mutation was introduced in Mtb H37Rv by recombineering. After electroporation with the primer containing the mutation, bacteria were plated on compound **1**. Several colonies were isolated and the presence of the SNP was confirmed by sequencing. Subsequently, their resistance profile was evaluated by RBMA. All of them showed the same level of resistance as of TB333 (3 µM). Taken together, these results provide evidence that the mutation in *mmpL3* has a key role in conferring resistance to compound **1**.

The *mmpL3* gene encodes the inner membrane transporter MmpL3, part of the RND (Resistance-Nodulation-Cell Division) superfamily, and is conserved in all sequenced mycobacterial genomes. Out of 12 MmpL-type proteins present in the H37Rv strain, MmpL3 was the only one predicted to be essential by insertional inactivation[21] and by transposon mutagenesis,[22] suggesting a possible fundamental role for this protein in Mtb. Moreover, in the last years, a plethora of papers showed that MmpL3 is the target of a series of small molecules with different chemical structures.[11,12,13,14,15,16,23,24] The mutation (I292L) identified in the resistant mutants isolated by us maps to the beginning of the predicted fifth membrane spanning domain and is a conservative mutation (a hydrophobic amino acid is substituted by another hydrophobic amino acid). This is not uncommon; indeed some SNPs observed in mutants resistant to BM212[14] or to pyrazolopyrimidine[15] are also conservative mutations. Remuinan MJ and collaborators[15] showed that mutants resistant to similar "spiro" compound have a point mutation at codon 292 leading to an amino acid change from I to S or T, providing additional evidence that the I at position 292 is an important residue in MmpL3 structure/function. These mutations could interfere with the function of MmpL3, avoiding the uptake or efflux of essential components or metabolites of the cell. It was demonstrated that MmpL3 is involved in the efflux of TMM, donor of mycolates to either arabinogalactan or another TMM molecule, yielding Trehalose Di Mycolate (TDM).[25] Moreover, it was shown that MmpL3 can bind heme *in vitro*, suggesting that it can act simultaneously as exporter and importer.[26]

Spontaneous mutants arose from compound **57** and compound **59** selection plates at frequency of $5*10^{-9}$ for compound **57** and $3.3*10^{-9}$ for compound **59** respectively. These isolates, named TB365 and TB366 (compound **57**) and TB375, TB376 and TB377 (compound **59**) showed an MIC value of >30 μM (>16X parental strain MIC) and 15.5 μM (33X parental strain MIC), respectively. All the mutant isolates showed a cross-resistance with compound **1** (MIC=3 μM), as well as TB333 showed cross-resistance with both compound **57** and compound **59** (Table 7). Sequencing of *mmpL3* in the mutants

TB365, TB366 and TB375 identified the same mutation as TB333 and TB334 (a874c). This suggests that a common mechanism of resistance and possibly a common target exists for all three compounds from this series. It is possible that MmpL3 is the target for this series, but further experiments to demonstrate the inhibition of MmpL3 function is required to confirm this hypothesis.

Spiroindene resistant mutant TB333 was found to be cross-resistant to spirochromene **3** (Table 7), suggesting similar mode of resistance for the series; whereas TB333 retained sensitivity towards spiroindolones **4** and **30**. This suggests that these spiroindolone compounds, due to large structural differences at the R^2 position have either a different mode of binding to MmpL3 or might interact with a different target in Mtb.

Table 7. MIC modulation against spiroindene resistant mutants

	Compound **1**	Compound **57**	Compound **59**	Compound **3**	Compound **4**	Compound **30**	Streptomycin
WT	0.2	1.5	0.5	3.1	3.1	0.8	1
TB333	3	>25	12.5	12.5	1.6	0.4	1
TB365	3	>31	15.5		-	-	0.5
TB366	3	>31	15.5		-	-	0.5
TB375	3	>31	15.5		-	-	1
TB376	3	>31	15.5		-	-	1
TB377	1.5	15.5	15.5		-	-	1

Since the mutants have been isolated under aerobic conditions, it is possible that an additional target exists that may be more relevant for the activity under hypoxia. We did not pursue target identification under hypoxic conditions, since most compounds had moderate potency on NRP Mtb along with poor solubility. Further optimization is needed to obtain better compounds which would be ideal for target identification studies in NRP Mtb.

In conclusion, spiropiperidines were identified as a novel series with potent antitubercular properties based on a whole cell screen on replicating Mtb followed by further evaluation carried out under multiple physiological conditions. The spiropiperidine series comprised of three closely related classes of compounds namely spiroindenes, spiroindolones and spirochromenes. Since spiroindenes were the largest subseries with good potency spread in the spiropiperidine series, a more detailed analysis of this class was carried out. The growth inhibitory effect of spiroindenes was restricted to mycobacterial species only, making them ideal for their specific use in long term therapy. In addition, these compounds retained their potency on different clinical isolates including single drug resistant strains, thereby demonstrating their value in a clinical setting. The spiroindenes were also bactericidal under replicating, non-replicating and intracellular conditions, suggesting that this class warrants further lead optimization efforts.

Attempts to yield lead-like compounds with reduced lipophilicity, basicity, cytotoxicity and hERG liabilities, have indicated routes useful for further improvisation. Preliminary SAR studies on spiroindolones indicated opportunity for scaffold-hopping to make more novel lead-like compounds.

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Supporting Information Available. Biology and DMPK methods, HERG method, Chemistry experimental procedures and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Graphical Abstract

Whole cell screen based identification of spiropiperidines with potent antitubercular properties

Leave this area blank for abstract info.

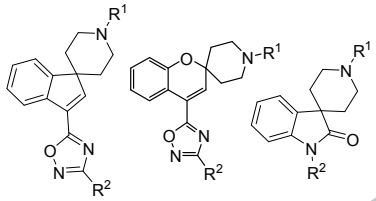
320,000 screened at 20 μM on Mtb H37Rv

≥80% inhibition

1600 compounds

H37Rv MIC ≤10 μM
ss18b IC_{80} ≤50 μM
Hit evaluation

Spiropiperidines



Spiropiperidines
Mtb MIC 0.3-50 μM
Hypoxia IC_{80} 25-200 μM