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Evaluation of *N*-(phenylmethyl)-4-[5-(phenylmethyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-4-yl]benzamide inhibitors of *Mycobacterium tuberculosis* growth

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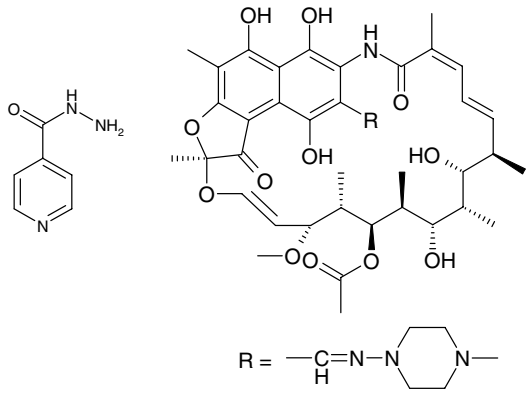
Abstract—The biological evaluation of imidazopiperidines as FAS II inhibitors of *Mycobacterium tuberculosis* growth has been carried out with a view to assessment of potential as lead compounds for the development of a new TB drug. A summary of the hit evaluation and current challenges is described herein.

The World Health Organisation (WHO) has prioritized tuberculosis (TB) as one of the top three diseases requiring attention by the international community.[1] The majority of TB cases occur in the developing world, although TB is fast re-emerging as a killer in developed world populations.[2] Conservative WHO estimates currently attribute around two million deaths worldwide per year to TB with the true figure probably masked by the rising mortality of HIV/TB patients.[3]

Non-compliance with the six-month, multi-drug chemotherapeutic regimen[4] is a major factor in the development of multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis* (*M.tb*) despite more widespread use of the WHO recommended DOTS (directly observed therapy, short course) treatment. MDR-TB is defined as strains of the *M.tb* resistant to at least two of the front-line TB drugs (Fig. 1), namely isoniazid **1** (shown in its pro-drug form) and rifampicin **2**.[5]

We were interested in exploring inhibitors which exhibit an alternative mechanism of inhibition to that of **1** thus maintaining activity in MDR-TB strains.



1 **2**

Figure 1. Two of the DOTS regime recommended drugs; isoniazid **1** and rifampicin **2**.

InhA, the biological target for **1**, is an enoyl-ACP reductase, part of the FASII system, which is involved in fatty acid chain elongation.[7] These chains contribute to the biosynthesis of mycolic acids which are key components of the mycobacterial cell wall. It has been suggested that **1** is activated by a catalase-peroxidase enzyme, the product of the *kat*G gene, allowing radical formation of a covalent bond to give isonicotinyl-NAD$^+$, prior to insertion into the protein structure.[6e]

Keywords: *Mycobacterium tuberculosis*; Imidazopiperidines; InhA.
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This has also been supported by crystallographic evidence.[6a] Once within the protein, the modified co-factor inhibits fatty acid chain saturation by preventing hydride delivery to the enoyl-ACP fatty acid chains.[7b] MDR-TB strains often contain mutations in the *kat*G gene,[8] thus activation of **1** via the catalase-peroxidase mechanism is prevented resulting in a significant reduction of its efficacy.[9]

Our investigation focused on identifying non-covalent inhibitors of InhA which are independent of upstream biochemical processes, in an attempt to provide novel chemotherapeutic agents for MDR-TB.

From a screen for inhibitors of InhA, we discovered the imidazopiperidine series **3**. Substructure searches led to the discovery of analogues within this series, which supported our original hit. Confirmation of activity was achieved from a resynthesised sample. We were interested in addressing the initial medicinal chemistry questions associated with the series, summarised in Figure 2. It was recognized that the original scaffold **3** provided a tractable hit which facilitated rapid expansions at both the carbonyl and piperidine positions using solid phase chemistry. In addition, it was thought that the imidazole ring could be replaced in order to evaluate its capacity as a hydrogen bond donor/acceptor. Since the original hits were previously tested as racemates, it was necessary to establish the activity of the individual enantiomers. With these considerations, our initial synthesis programme was prompted towards a suitable starting point from which to evaluate the activity of this series.

The 4-(4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridin-4-yl)benzoic acid (imidazopiperidine) scaffold was prepared using literature procedures.[10] Treatment of this imidazopiperidine with activated Fmoc–succinimide in aqueous 1,4-dioxane in the presence of sodium hydrogen carbonate gave the deprotected racemic imidazopiperidine key intermediate **3** (R^1 = Fmoc, R^2 = OH, R^3 = Fmoc) in 76% yield. The scaffold was used without further purification for modification at the carboxy and piperidine positions using solid phase chemistry[11] described in Scheme 1.

Tentagel™ was allowed to react with the formyl methoxybutanoic acid under HATU coupling conditions to give the linker resin **4**.[11] Treatment of **4** with selected amines under reductive amination conditions gave the secondary amines **5** which were then subjected to HATU coupling conditions with **3** (R^2 = OH) to give the Fmoc protected imidazopiperidine loaded resins **6**. Deprotection of the Fmoc group followed by a second round of reductive aminations with a number of pre-selected aldehydes[12] resulted in the desired imidazopiperidines attached to the solid phase resin **7**. Cleavage of the products from the resin was achieved using trifluoroacetic acid to give the desired crude compounds in Table 1 in good yields.

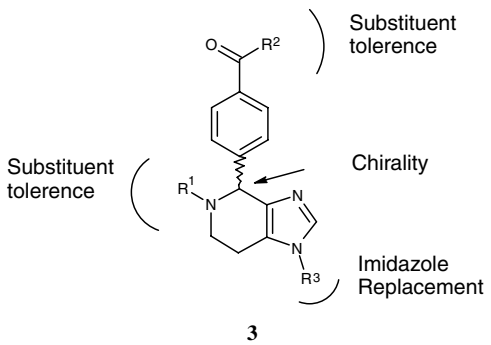

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Figure 2. The proposed medicinal chemistry issues requiring investigation within the imidazopiperidine series **3**.

Imidazole replacement was carried out in solution using a modification of the original route, Scheme 2. Phenylethylamine **10** was reacted under coupling conditions with monomethyltetraphthalate to give **11** in good yield. Treatment of **11** with $POCl_3$ in the presence of a dehydrating agent at reflux gave the imine intermediate which when subjected to hydride reduction afforded the racemic scaffold **12** in 82% yield over two steps. Structural diversity was attributed to the scaffold at this point under the conditions of reductive amination using pre-selected aldehydes to give the alkylated piperidine analogues **13a**–**c** in good yields. Treatment of the methyl ester under mild conditions of hydrolysis afforded the carboxylic acid which was coupled to a number of preselected benzylamines to afford the final compounds **14a**–**c**.

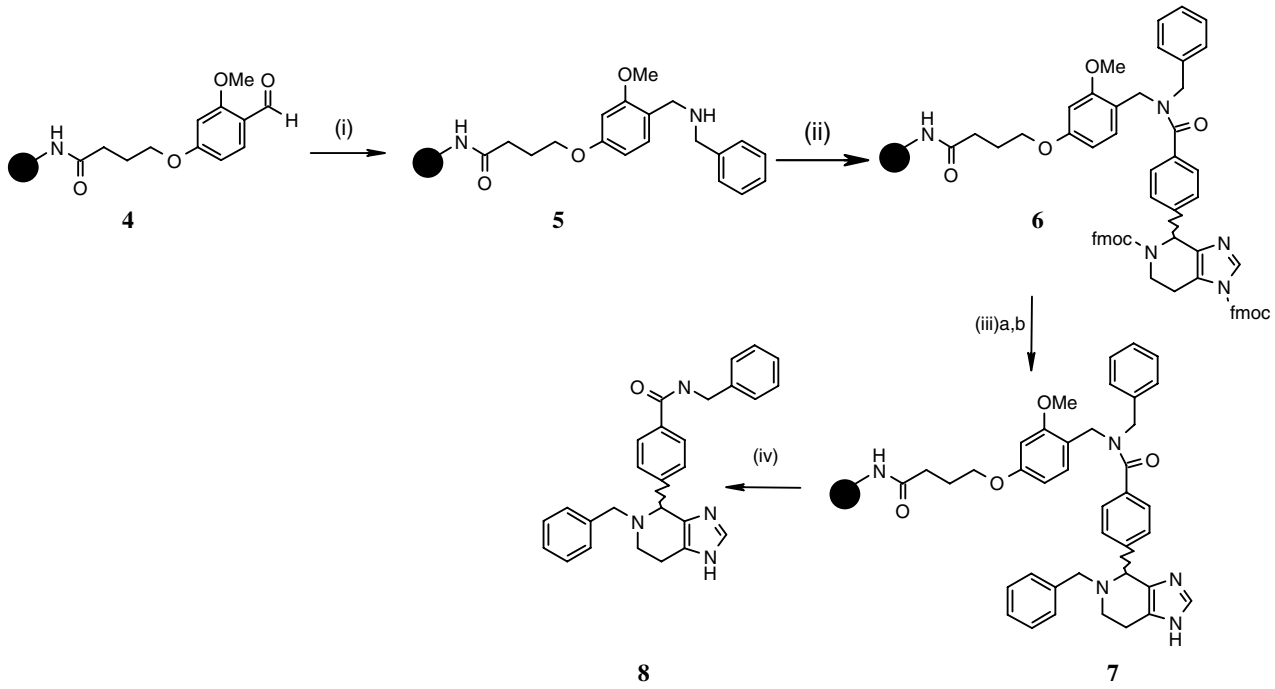
The high-throughput screen was carried out using a 384-well microplate assay in which reduction of 2-*trans* dodecenoyl co-enzyme A and concomitant oxidation of NADH by InhA was monitored indirectly by reacting remaining NADH with the dye MTS (Promega), coupled via the electron carrier PMS (Sigma), to produce a coloured derivative[14] (abs at 492 nm).

Determination of whole cell activity was carried out by measuring the minimum inhibitory concentration (MIC) required to inhibit growth of broth cultures of *M.tb* incubated in 96-well microtitre plates. Growth was measured indirectly via metabolic reduction of the fluorescent dye Alamar blue.

All prepared compounds were screened in the InhA biochemical assay with the broad-spectrum antibacterial triclosan (IC_{50} = 2.1 μM), a known InhA inhibitor, as the standard and compounds displaying significant activity were evaluated in the *M.tb* whole cell assay. A selection of these results is presented in Table 1.

Preliminary observations for this series showed that substitution was required at both R^1 and R^2 to achieve activity. The scope of SAR at R^1 and R^2 was evaluated from an array of imidazopiperidine racemic analogues, examples of which are presented in Table 1 (compounds **8e**–**l**).

The most active compounds contained the electron donating *para*-methoxybenzylamine group in the R^2 position while complemented by the presence of either the mono or dichloro benzyl analogue at the R^1 position

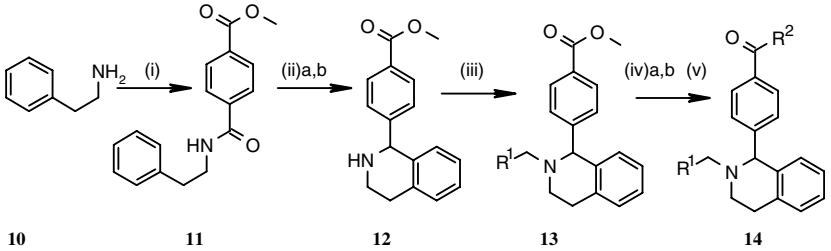


Scheme 1. Reagents and conditions: (i) Benzylamine, CH_3COOH, $NH_4HB(OAc)_3$, DCM, rt, 1 h; (ii) **3**, HATU, DMF, rt, 1 h; (iii) a—piperidine, DMF, rt, 1 h; b—benzaldehyde, CH_3COOH, $NH_4HB(OAc)_3$, DCM, rt, 1 h; (iv) CF_3COOH, DMF, rt, 0.5 h.

Table 1. InhA enzyme and whole cell activity (vs *M.tb*) for a selection of imidazopiperidine inhibitors

Compound	R^1	R^2	$IC_{50}/\mu M$[a]	MIC/μM[b]
8e	2,3-Di chlorobenzyl	4-Methoxy benzyl	0.24	32
8f	3-Chloro benzyl	4-Methoxy benzyl	0.41	63
8g	2,3-Di chlorobenzyl	Benzyl	1.39	32
8h	3-Chloro benzyl	Benzyl	3.02	32
8i	Naphthyl	4-Methoxy benzyl	3.5	32
8j	2,3-Di chlorobenzyl	2-Trifluoro benzyl	9.81	32
8k	2,4,5-Tri methoxy benzyl	4-Methoxy benzyl	10.18	63
8l	Naphthyl	Benzyl	13.38	16
9a[c]	2,3-Di chlorobenzyl	4-Methoxy benzyl	>10	63
9b[d]	2,3-Di chlorobenzyl	4-Methoxy benzyl	0.08	63
9c[c]	3-Chloro benzyl	4-Methoxy benzyl	>10	32
9d[d]	3-Chloro benzyl	4-Methoxy benzyl	0.2	63

[a] Drug concentration at 50% reduction in enzymatic activity.
[b] Minimum drug concentration at 50% inhibition of cellular growth.
[c] Enantiomer 1 with retention time < 7 min.
[d] Enantiomer 2 with retention time > 7 min.



Scheme 2. Reagents and conditions: (i) $C_9H_8O_4$, EDCI, TEA, HOBT, EtOAc/THF (1:1), 0 °C—rt, 18 h, 95%; (ii) a—$POCl_3$,P_2O_5, reflux, 1.5 h, 95%; b—$NaBH_4$, MeOH/H_2O, rt, 16 h, 82%; (iii) R^1CHO CH_3COOH, $NaHB(OAc)_3$, DCM, rt, 16 h; (iv) a—NaOH, THF/H_2O (80:20), rt, 70 h; b—HCl; (v) $R^2CH_2NH_2$, EDCI, TEA, Hobt, EtOAc/THF (1:1), 0 °C—rt, 20 h.

of the piperidine nitrogen (**8e**, $IC_{50} = 0.24$ μM). There is a minor drop off in activity upon removal of one chloride group (**8f**, $IC_{50} = 0.41$ μM) however, in general, where R^2 was *para*-methoxybenzylamine a degree of activity was preserved for example, in the case where R^1 was the naphthyl (**8i**, $IC_{50} = 3.5$ μM) or 2,4,5-tri-

methoxybenzyl (**8k**, IC_{50} = 10.2 μM) when compared to the non-substituted benzyl group at R^2 (**8l**, IC_{50} = 13.38 μM) indicating a potential lipophilic requirement at this position.

Activity in the biochemical assay decreased significantly when R^1 was replaced with alkyl or *cyclo*-alkyl alternatives[13] concluding that the series displayed a narrow SAR in the biochemical assay and essentially flat SAR in the whole cell assay.

When the three most active compounds (**8e**, **f**, **g**) were separated into their enantiomers by chiral HPLC, a general gain in enantiospecific activity was observed when compared to the racemic mixtures (compound **9**). While the absolute stereochemistry of the active enantiomer was not determined, the specificity was highlighted by the most active enantiomer **9b** (IC_{50} = 80 nM vs >10 μM for **9a**) where the parent racemate **8e** had previously displayed an IC_{50} = 240 nM.

The requirement of the imidazole group was investigated by combining the features of activity for the active imidazopiperidine compounds (**8e**, **g**) but replacing the imidazole group with a phenyl group. This caused a complete loss of activity in the biochemical assay and no significant whole cell activity indicating that the imidazole group was indeed a necessary feature for activity against the enzyme.

No significant broad-spectrum antibacterial activity was seen for **8e**, **8f**, **8i**, **8j**, **8k**, **9b**, **9c** and **9d** when compared with triclosan, amoxicillin and ciprofloxacin[15] suggesting that this series may be acting by a non-specific mechanism.

In an attempt to address the disconnect between the in vitro and cellular activity, it was decided to profile a selection of these compounds through a high throughput artificial membrane assay[16] which relies on passive diffusion for the compound to permeate through a monolayer of phospho-lipid membrane. It was found that these compounds displayed a range of permeabilities with the separated enantiomers all displaying medium to high permeabilities when compared with the standard.

Potential aggregation or promiscuous inhibition within the series was assessed using an in-house assay.[17] Compounds screened were found not to have significant activities. This was reaffirmed with the enantiomers: **9a**, **b**, **c** and **d** where there was clear demonstration of enantiospecificity for one enantiomer over the physiochemically similar other. With the level of potency observed for specific enantiomers it was concluded that this series is highly unlikely to be acting in an aggregative fashion.

However, upon further examination of this series within alternative cell lines it was found that a degree of toxicity was prevalent. When incubated against liver (HEPG2) cells Table 2 at concentrations 10-fold less than the highest meaningful MIC, selectivity (vs MIC) was significantly less than the desired 10-fold requirement (typically 20–30% inhibition at ca. 12 μM) giving a strong indication of dual off-target activity.

Table 2. Whole cell activity (vs *M.tb*) and HEPG2 toxicity data for a selection of imidazopiperidine inhibitors

Compound	MIC[a] (*M.tb*)	Tox_{50}(HEPG2)[a](% Inh)
8k	63	>11.5 (20)
8l	16	>13.2 (20)
8i	32	>12.4 (25)
8f	63	>12.8 (25)

Whole cell activity (vs *M.tb*), HEPG2 toxicity data for a selection of imidazopiperidine inhibitors.

[a] Concentration, μM; HEP, hepatic cell line; % Inh, percentage inhibition at quoted concentration.

These data suggested that although potent compounds could be identified, the strategy was not likely to enhance *M.tb* cellular activity. Work is ongoing to increase the therapeutic index by identification of the secondary mechanism of action.

Acknowledgments

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11. General procedure for solid phase synthesis of imidazopiperidine analogues. The modified resin **4** (200 mg, 0.094 mmol) was washed with dichloromethane and then allowed to stir in DMF for 15 min. The amine was added and the suspension allowed to stir for a further 1 h prior to the addition of sodium triacetoxy borohydride (10 equiv, 0.94 mmol, 247 mg). The suspension was allowed to stir at room temperature for 18 h. after which it was filtered and washed with DCM (50 ml) DMF (50 ml), ethanolamine (10%, in DMF, 50 ml) DMF and finally methanol (50 mL) to give **5**. The resin was dried under vacuum at 40 °C.
    The acid **3** (323 mg, 5 equiv), HATU (5 equiv, 178.6 mg) andPEA (163 μL) were added to DMF (5 mL) and stirred for 1 h. The modified resin **5** as a suspension in DMF was added and the suspension allowed to stir at room temperature for 18 h.
    The resin was filtered, and washed with DMF, DCM, methanol and DCM (20 mL,respectively) to give **6** which was used immediately without drying. The resin **6** was added to a solution of piperidine in DMF (20% solution) and the suspension allowed to stir at room temperature for 2 h prior to filtration and washing with DCM, Methanol and DCM (20 mL). The loaded resin, aldehyde and acetic acid were stirred at room temperature for 2 h prior to the addition of the sodium triacetoxy borohydride in one portion. The suspension was allowed to stir at room temperature for 56 h prior to filtration and washing with DCM, MeOH and DCM (20 mL) to give **7**, which was used without further modification for the next step.
    To a solution of TFA/H_2O (80:20) was added **7** in one portion. The suspension was stirred at room temperature for 3 h, filtered and washed with DCM and Methanol (20 mL). The filtrate was concentrated to give **8** and analysed by HPLC-MS. All compounds which showed greater than 90% purity and a weight greater than 1 mg were selected for testing.
    Separation of enantiomers from the parent racemates **8e**, **f** and**g** was achieved using preparative chiral HPLC. On a Chiralpak AD column (2 cm) with a loading of 15 mg mL^{-1}, a flowrate of 15 mL min^{-1} and detection at $\lambda = 215$ nm, fractions were collected using an ethanol/heptane gradient up to 30% ethanol (over 15 min) to give the separation of the enantiomers **9a**–**d**.
12. A range of aldehydes were chosen from work carried out on docking within the InhA active site, pharmacophore generation and best fit models.
13. Data for these analogues have been omitted.
14. Assays were performed using an appropriate dilution of enzyme with 0.25 mM DDCoA and 0.2 mM NADH in 30 mM PIPES buffer (pH 6.8). The final reaction volume was 50 μL. The reaction was stopped and the signal developed by the addition of 25 μL of 0.4 mg mL^{-1} MTS, 4 μg mL^{-1} PMS and 0.3 M urea in PBS buffer.
15. The following strains of bacteria using the standards amoxicillin, ciprofloxacin and triclosan were used to profile the compounds cited vide supra: *Staphylococcus aureus* ATCC29213, *Streptococcus pneumoniae* 1629, *S. pneumoniae* Ery2, *Klebsiela pneumoniae* KP1, *K. pneumoniae* KP3, *Escherichia coli* EC2, *E. coli* ATCC25922, *Proteus mirabilis* PM1, *P. mirabilis* PM5, *Pseudomonas aeruginosa* PA1, *P. aeruginosa* ATCC 27853, *Stenotrophomonas maltophilia* T68214, *Haemophilus influenzae* 20001H, *H. influenzae* 07001H. Activity was only seen with **8K** at 2 μg mL^{-1} for *S. aureus* ATCC 29213.
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