Aminopyrazolo[1,5-a]pyrimidines as potential inhibitors of *Mycobacterium tuberculosis*: Structure activity relationships and ADME characterization

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Whole-cell high-throughput screening of a diverse SoftFocus library against *Mycobacterium tuberculosis* (*Mtb*) generated a novel aminopyrazolo[1,5-a]pyrimidine hit series. The synthesis and structure activity relationship studies identified compounds with potent antimycobacterial activity. The SAR of over 140 compounds shows that the 2-pyridylmethylamine moiety at the C-7 position of the pyrazolopyrimidine scaffold was important for *Mtb* activity, whereas the C-3 position offered a higher degree of flexibility. The series was also profiled for *in vitro* cytotoxicity and microsomal metabolic stability as well as physicochemical properties. Consequently liabilities to be addressed in a future lead optimization campaign have been identified.

1. Introduction

Tuberculosis (TB) is the second leading cause of death from a single infectious agent after HIV. According to the World Health Organization 2014 report, it is estimated that there were 9.0 million new TB cases and 1.5 million lives were lost to the disease in 2013.¹ There is no universally effective vaccine against infection by *Mycobacterium tuberculosis* (*Mtb*), the causative pathogen,² and an estimated one-third of the world's population already harbours latent TB of which an estimated 10% will develop active disease during their lifetime, rendering eradication near impossible.³ First-line TB chemotherapy involves the use of four drugs (rifampicin, isoniazid, ethambutol, and pyrazinamide) administered over a 2 month intensive phase, followed by a further 4 month continuation phase using only rifampicin and isoniazid.⁴ Limitations to the current regimen due to a range of adverse side-effects and prolonged treatment time has led to non-compliance in patients, inevitably resulting in resistant forms of the disease and patient relapse. The severe challenges to TB control brought by the increasing prevalence of multidrug-resistant (MDR) and extensively drug-

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resistant (XDR) TB, further exacerbated by HIV co-infection, have culminated in recent reports of emergent strains that are resistant to all known TB drugs.⁴ Moreover, of the estimated 480 000 cases of MDR TB in 2013, a very small fraction were treated according to WHO standards, reflecting such diverse factors as low diagnosis rates, higher drug costs and longer treatment times, the use of less effective drugs that cause more toxic side effects leading to patient non-compliance, drug misuse through private sector availability in many high burden countries, and inadequate health-care oversight.^{1,2,5-8} There are a number of anti-TB drug candidates in pre-clinical and clinical development^{9,10,11} and, in 2012, the first new drug in more than 40 years, bedaquiline (TMC207), was approved by the FDA for treatment of MDR TB through an accelerated approval process.^{12a} However, there are major challenges to its wide-scale use for a variety of reasons. First, there is limited clinical data available. Second, its use is confined to carefully monitored settings in view of hepatotoxicity and cardiotoxicity safety concerns. Third, in many countries, patient eligibility is not predetermined since MDR patients typically commence treatment prior to receiving results of the often time consuming and expensive drug sensitivity tests.^{12b,c} Therefore, there is an immediate need both for more effective and faster acting new therapies with novel mechanisms of action to simplify or replace the existing drug cocktails required for TB treatment. Aiding this goal is a shift in the paradigm of TB drug discovery with many pharmaceutical companies and academia exploring collaborative models by adopting an open source philosophy of conducting early-stage TB drug discovery research in order to address the need for new TB drugs.¹³ In this regard a diverse range of heterocyclic cores, which provide starting points for medicinal optimization have been disclosed.¹³ In addition, there have been improvements in both the *in vitro* and *in vivo* strategies employed for lead optimization in order to maximise the probability of developing clinical candidates.¹⁴

In order to find new lead compounds active against *Mtb*, a 35,000 compound SoftFocusTM library was obtained from Biofocus and screened against the virulent *Mtb* H37Rv strain. High-throughput screening was carried out in liquid culture (7H9 medium) under aerobic conditions, using glucose as a carbon source. Whole-cell active compounds were selected from the screens using several criteria, including potency, intellectual property overlap, and chemical-structural properties. One of the libraries was designated SFK29 (SoftFocus Kinase) based on a 7-(aminomethyl)aryl pyrazolo[1,5-a]pyrimidine series, Figure 1. The diverse compound library represented more than 200 scaffolds that had primarily been designed as potential kinase inhibitors, GPCR antagonists, or ion channel modulators. The whole-cell screen confirmed that, of the 248 7-(aminomethyl)aryl pyrazolo[1,5-a]pyrimidines screened, six compounds showed Minimum Inhibitory Concentrations (MIC₉₉, representing 99% growth inhibition) less than 5 μ M.

Pyrazolo[1,5-a]pyrimidines are of chemical and pharmacological importance as purine analogues, and derivatives have been applied in various therapeutic areas such as antitumor,¹⁵⁻¹⁹ antidepressant,²⁰⁻²¹ antibacterial,²² antiviral,²³ antidiabetic²⁴ and antihypertensive agents.^{25,26} Representatives of the SFK29 series (**1-6**, Figure 1) were selected for resynthesis based on their activities (MIC₉₉ < 5 μ M) and confirmatory testing

against *Mtb* H37Rv in Middlebrook 7H9 broth, with MIC₉₉ values after 14 days reported within an acceptable range of activity.

Figure 1. Structure of 7-(aminomethyl)arylpyrazolo[1,5-a]pyrimidine hits identified by *Mtb* phenotypic screening.

Based on its structural novelty, broad activity, and acceptable potencies, the aminopyrazolo[1,5-a]pyrimidine series was selected for a medicinal chemistry optimisation campaign. Herein, we present the synthesis, structure activity relationship (SAR), and *in vitro* Absorption, Distribution, Metabolism, Excretion (ADME) studies. Preliminary mode-of-action studies are also presented.

2. Chemistry

SAR studies focused around substitution with a variety of amino groups in the 7-position, and aryl groups in the 3-position. Using **1** as the starting point, and with substituents at the 3-position fixed as a phenyl, the 7-position was explored, to evaluate the importance of the pyridin-2-ylmethylamine substituent. The synthesis of the desired target compound **9** was carried out via advanced intermediate **8** as shown in Scheme 1, following a modified literature procedure.²⁷ 7-Hydroxypyrazolo[1,5-a] pyrimidine **7** was prepared in good yield from the acid-catalysed cyclisation of 5-amino-3-methyl-4-phenyl-1*H*-pyrazole and ethyl 3-oxobutanoate.

Scheme 1. Synthesis of 7-substituted aminopyrazolo[1,5-a]pyrimidines^a

^aReagents and conditions: (a) AcOH, reflux, 16h; (b) POCl₃ (3 eq), *N,N*-Dimethylaniline (1.2 eq), toluene, 120 °C, 16 h; (c) RNH₂ (1.2 eq), DiPEA (1.5 eq), *i*PrOH, 80°C, 16 h.

Chlorination of **7** under standard conditions using phosphorous oxychloride afforded 7-chloro-2,5-dimethyl-3-phenylpyrazolo[1,5-a]pyrimidine **8**. Subsequent displacement of the chloro group with a range of amines under standard thermal conditions gave the desired compounds. Pyridin-2-ylmethylpyrazolo[1,5-a]pyrimidine derivatives **13**, where the substituent (R¹) at the 2-position was either H or Me, were prepared using a similar synthetic route via known 7-chloropyrazolo[1,5-a]pyrimidine²⁸ (**10**) for investigation of the SAR at the 3-position (Scheme 2). Synthesis of intermediate **11** was completed by C-3 bromination using

N-bromosuccinimide. Coupling of 2-picolylamine, followed by protection of the free amine with a *tert*-butoxycarbonyl group afforded advanced intermediate **12**. Subsequent Suzuki coupling with an array of boronic acids gave the desired products **13** after deprotection.

Scheme 2. Synthesis of 3-substituted aminopyrazolo[1,5-*a*]pyrimidines^b

3. Results and discussion

A representative hit **1** identified from whole-cell screening displayed promising antimycobacterial activity (MIC₉₉ = 5 μM at day 14) but showed low kinetic solubility (<5 μM at pH 7.4) and poor metabolic stability in human liver microsomes (HLM *t*_{1/2} = 31.7 min). Initial medicinal chemistry efforts focused on exploration of the R substituent at position 7 and the R¹ substituent at position 3 to establish SAR (Tables 1 and 2). Profiling of compounds was carried out to determine aqueous solubility, hepatic microsomal stability, and mammalian cytotoxicity (Tables 3 and 4). The *in vitro* antimycobacterial activity for synthesised compounds was measured against *M. tuberculosis* H37RvMA (ATCC 27294 strain)²⁹ using the broth microdilution method³⁰ in both Middlebrook 7H9/ADC and GAST-Fe media.

3.1 Modifications to the 7-position

In order to determine the importance of the 7-pyridin-2-ylmethylamine substituent which featured in the hit compounds **1–6**, analogues were synthesised using an array of commercially available amines and alcohols. The results for the antimycobacterial activity and kinetic solubility of a selection of synthesized compounds are presented in Table 1. The 2-pyridyl ring was shown to be essential for antimycobacterial activity. The 3-pyridyl (**14**)

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and 4-pyridine (**15**) derivatives resulted in complete loss of *Mtb* activity ($\text{MIC}_{99} > 160 \mu\text{M}$). However, the pyrimidine (**16**) and pyrazine (**17**) analogues retained some *Mtb* activity albeit 16-fold lower than **1**. The poor solubility of **1** ($< 5 \mu\text{M}$ at pH 7.4) may be explained by implicating intramolecular H-bonding between the 2-pyridyl N and the NH of the methamine. By locking the 2-pyridylmethanamine in the same plane as the bicyclic ring system, this would result in the formation of a planar system capable of π -stacking to form an insoluble crystal lattice. Disruption of this molecular planarity by moving the N around the ring resulted in a significant increase in aqueous solubility ($110.2 \mu\text{M}$ for **14** and $133.6 \mu\text{M}$ for **15**). Extension of the methamine tether by a two-carbon methylene spacer (**18**) or 3-carbon methylene space (**19**) led to a decrease in *Mtb* activity ($\text{MIC}_{99} > 160 \mu\text{M}$ and $80 \mu\text{M}$ respectively). The calculated physicochemical properties of **1** confirm the trend in solubility with **1** being more lipophilic (clogP 4.09) than **14** and **15** (clogP 3.67). Removal of the methylene spacer to form compound **20** was detrimental to activity at the highest concentration tested. Also, methylation of the NH to NMe (**21**) resulted in a complete loss of activity. Similarly, replacing the arylmethyl amines with the corresponding ether analogue **22** was not tolerated. Replacement of the 2-pyridyl moiety with other heterocyclic systems such as imidazoles (**24** and **25**), methylpyridinyl (**26**), furan (**27**), thiophene (**28**), thiazole (**29**) and quinoline (**30**) generally resulted in a lack of antimycobacterial activity at the highest concentration tested ($\text{MIC}_{99} > 160 \mu\text{M}$). The exception was the methylimidazole analogue **23**, which displayed good ($\text{MIC}_{99} = 5 \mu\text{M}$) activity. The imidazoles (**23** and **25**) did offer an improvement in kinetic solubility. Changes to the 7-position of the aminopyrazolo[1,5-a]pyrimidine ring, which resulted in improved aqueous solubility, were not compatible with anti-*Mtb* activity and no compounds were identified with improved activity over the initial hit compound **1**. This suggests that the pyridin-2-ylmethanamine substituent is important for the observed antimycobacterial activity.

Table 1. Antimycobacterial activity, aqueous solubility and clogP for 7-substituted aminopyrazolo[1,5-a]pyrimidines

Compd	R	$\text{MIC}_{99}(\mu\text{M})^a$	Kinetic Solubility (pH 7.4) ^b	clogP ^c
Rifampicin		0.012		
Kanamycin		6.45		
1		5	<5	4.09
14		>160	110.2	3.67

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15		>160	133.6	3.67
16		80	37.9	2.34
17		80	7.1	2.34
18		>160	<5	3.94
19		80	5.9	4.32
20		>160	<5	4.06
21		>160	17.5	3.78
22		>160	25.6	3.43
23		5	26.9	2.64
24		37	<5	2.62
25		>160	14.6	2.62
26		>160	<5	3.79
27		>160	<5	3.96
28		>160	<5	4.67
29		>160	107.5	3.36
30		>160	<5	4.47

^a 14 day Alamar Blue readout against H37Rv in 7H9/ADC/Tween

^b Kinetic solubility assay at pH 7.4 using HPLC method

^c clogP calculated using Chemdraw

3.2 Exploration of 3-position changes

The first round of compounds to explore the 3-position of the pyrazolo[1,5-a]pyrimidine included examples with a methyl group in the 2-position of the scaffold or with no substituent at this position. This was envisaged to explore the effect of the methyl substituent on antimycobacterial activity. Table 2 summarizes the *in vitro* antimycobacterial activity and solubility data. The *in vitro* antimycobacterial activity for analogues where R₁ = Me or H (**1**, **2**, **3**, **4**, **31**, **32**) are comparable and, for this reason, we decided to focus on the series lacking the R¹ substituent in order to remove the potential metabolic liability of the methyl group.

Table 2. Antimycobacterial activity and aqueous solubility for 3-substituted aminopyrazolo [1,5-a]pyrimidines

Compd	R ¹	R ²	MIC ₉₉ (μM) ^a	Kinetic Solubility (pH 7.4) ^b
2	H		10	<5
3	CH ₃		5	<5
4	H		5	<5
31	CH ₃		20	<5
32	H		20	<5
5	H		10	<5
6	H		5	<5
33	H		20	<5
34	H		>160	<5
35	H		>160	<5
36	H		2.5	<5
37	H		2.5	5
38	H		2.5	5
39	H		1.25	<5
40	H		1.25	<5
41	H		>160	<5
42	H		>160	<5

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43	H		2.5	<5
44	H		40	48.8
45	H		20	9
46	H		>160	161.1
47	H		>160	188
48	H		>160	5
49	H		40	7.8
50	H		5	5
51	H		80	170.9
12	H	Br	>160	89.3
52	CH ₃	H	5	>200

^a 14 day Alamar Blue readout against H37Rv in 7H9/ADC/Tween

^b Kinetic solubility assay at pH 7.4 using HPLC method

A range of electron-withdrawing and electron-donating aromatic groups were prepared and tested for antimycobacterial activity. The data from alkyl, *O*- and *N*-substituted phenyls **31**, **32**, **6** and **33** (see Table 2) suggests that an electron-rich phenyl substituent is beneficial for activity. On the other hand, electron-withdrawing halogenated phenyl analogues (**4**, **5**, **36**, **37** and **38**) displayed good activity against *Mtb* (MIC₉₉ = 2.5–5 µM, when R₁ = H). It was observed that *ortho* and *meta* halogenated compounds (**36**, **37**, **38**; MIC₉₉ = 2.5 µM) were 2- to 4-fold more potent than the corresponding *para* analogue (**3**, **5**; MIC₉₉ = 5–10 µM). The most potent compounds were the di-substituted **39** and *o*-tolyl **40** analogues which were equipotent (MIC₉₉ = 1.25 µM). Di-substituted analogues **41** and **42**, however, did not show comparable activity to **39**. However, tri-fluoro analogue **43** displayed comparable activity to its *meta*- fluoro counterpart **37**. Not surprisingly, there was no improvement in aqueous solubility with these compounds (< 5 µM). However, 5-ring heterocyclic variants **44**, **45** and **47** showed improved aqueous solubility albeit this was accompanied by a decrease in antimycobacterial activity. Furan analogue **44** (MIC₉₉ = 20 µM) and thiophene analogue **45** (MIC₉₉ = 20 µM) retained some activity. Replacement of the phenyl group with a pyridyl as in compound **46** or methylpyrazole **47** led to a complete loss of activity. Phenyl amide analogue (**48**) showed no activity at the highest concentration tested. Insertion of a NH group between the pyrazolopyridine core and the phenyl ring (**49**) resulted in a 32-fold reduction in activity compared to **1**. The non-aromatic difluorocyclohexene compound **50** exhibited comparable activity to the fluorophenyl analogue (**4**) but did not offer an improvement in aqueous solubility despite the reduction in lipophilicity (**4**; clogP = 4.25 and **50**; clogP =

3.5). A dramatic increase in solubility was observed for dihydropyran analogue **51** but this compound displayed poor *in vitro* antimycobacterial activity. However, the bromo intermediate **12**, without the Boc protecting group, is devoid of antimycobacterial activity. Interestingly when the R² substituent was removed entirely (R² = H, **52**) the antimycobacterial activity was retained (MIC₉₉ = 5 µM), and is comparable to the original hit compound **1** but with good solubility. This suggests that the R₂ substituent is not essential for antimycobacterial activity in this series.

3.3 *In vitro* ADME profiling

In an attempt to address liabilities of poor solubility and metabolic stability, whilst maintaining potency, a further set of pyrazolo[1,5-a]pyrimidines were synthesised (Table 3). Strategies to improve aqueous solubility included introduction of hydrophilicity and removal of aromaticity and planarity³¹. A single time-point assay (% compound remaining after 0.5 h) was used to establish a ranking of compounds to predict half-life and this was measured in human, rat and mouse liver microsomes. Hit compound **1** exhibited poor metabolic stability in human liver microsomes (HLM *t*_{1/2} = 31.7 min). It was hypothesized that the electron rich 2-pyridyl, methyl and phenyl groups of **1** are metabolic hot spots. The *in vitro* metabolic profiling of **1** resulted in formation of 3 hydroxylation metabolites in HLMs on the phenyl and 2-pyridyl ring systems. Therefore, we reasoned that blocking or modifying these groups should help to improve metabolic stability. Removal of aromaticity as for example with the piperidine (**53**) and pyrrolidine (**54**) analogues resulted in an improvement in kinetic solubility but with loss of antimycobacterial activity and no improvement in microsomal stability compared to **1**. In order to potentially block metabolism, the metabolically labile 2-pyridyl ring was replaced with a fluoro-substituted 2-pyridine ring to deliver compound **55**, which retained activity against *Mtb* (MIC₉₉ = 5 µM). However, this compound exhibited similar metabolic stability issues in rat and mouse liver microsomes and reduced stability in human liver microsomes. The *N*-oxide analogue (**56**) was synthesized to ascertain if the predicted metabolite would be active against *Mtb*. No activity was seen with this derivative, suggesting that the basic pyridine N is important for activity. The promising antimycobacterial activity and improved solubility of **52** prompted the synthesis of compound **57** which resulted in a significant increase in aqueous solubility and metabolic stability but at the expense of antimycobacterial activity. Introduction of hydrophilicity by replacement of the R substituent with a triazole moiety **58** and R² with polar phenyl substituents (**59–62**) all led to a marked improvement in solubility and metabolic stability, with the exception of the metabolically unstable NMe₂ analogue **61**. Unfortunately loss of potency was once again observed. A basic amine side chain (**63**) was also not tolerated. *Ortho*-substituted analogues **64** and **65** were synthesized to improve solubility by disruption of molecular planarity, which greatly improved the solubility of **65**. However, *o*-fluoropyridyl **64** did not offer improved solubility but it was nevertheless metabolically stable and showed good antimycobacterial activity (MIC₉₉ = 10 µM). It was unfortunate that any improvement in aqueous solubility led to simultaneous loss of *in vitro* antimycobacterial activity. This correlates with other series of TB compounds where increased hydrophobicity generally leads to higher *Mtb* activity.³²

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Table 3. Inhibition of *Mtb* Growth, kinetic solubility and *in vitro* Microsomal Metabolic Stability

Compd	R	R ¹	R ²	MIC ₉₉ (μM) ^a	Kinetic Solubility (pH 7.4) ^b	Projected half life (min;h/r/m) ^c	% Remaining at 30 min (h/r/m) ^d
1		CH ₃		5	<5	31.7/22.1/7.5	51.9/39.0/6.2
53		CH ₃		>160	188.6	33.4/7.0/10.7	51.8/5.1/14.0
54		CH ₃		>160	172	33.7/-/-	54.0/-/-
55		CH ₃		5	<5	18/26.4/7.1	31.4/45.5/5.3
56		H		>160	159	>100/42.3/9.9	99.7/61.2/12.3
57		CH ₃	H	>160	182.3	>100/96.9/>100	100/80.6/85.1
58		CH ₃		>160	184.7	>100/55.7/12.5	99.5/68.8/18.9
59		H		>160	10.9	>100/41.0/23.6	99.3/60.1/41.4
60		H		>160	159.9	>100/36.7/35.1	89.5/56.8/55.1
61		H		80	94.6	19.1/6.7/8.2	33.7/4.5/7.9
62		H		160	111	-	-
63		H		>160	189.3	-	-
64		CH ₃		10	<5	>100/>100/>100	100/96.5/100
65		H		>160	42.3	-	-

^a 14 day Alamar Blue readout against H37Rv in 7H9/ADC/Tween

^b Kinetic solubility assay at pH 7.4 using HPLC

^c Projected half-life (min) at 30 minutes assessed in human, rat and mouse liver microsomes.

^d Metabolic Stability or Percentage remaining at 30 minutes assessed in human, rat and mouse liver microsomes.

3.4 Further Screening of Hit Compounds

The most active compounds in the series were screened in GAST-Fe³³ minimal media to confirm anti-*Mtb* activity in an additional growth media. We reasoned that this would allow insight into protein binding by comparison of the BSA-rich media (7H9) vs. non-BSA media

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(GAST-Fe), Fe-dependence (GAST-Fe vs. Low Fe GAST data) and provide supportive evidence that *Mtb* growth inhibition may be due to inhibition of a particular target or pathway. As shown in Table 4, all compounds were significantly less active in the GAST-Fe medium and activity of this series seems to be condition dependent. The compounds tested did not show iron dependence with comparison of the MIC in GAST-Fe and GAST without Fe displaying similar values with no impact on the already weak activity observed in this medium. In order to potentially identify the drug target or mechanism of action, whole-cell screening is coupled with whole-genome sequencing of resistant mutants.³⁴ The leading compounds were evaluated for their activity on promiscuous targets³⁵ including QcrB, a component of the respiratory *bc₁* complex. The compounds showed no differential activity against the *cyd* mutant; moreover, given that all known QcrB inhibitors tested to date are more active in GAST/Fe medium,^{35b} these data indicated that this compound series does not appear to be inhibiting this cell wall target. Additionally, a reporter assay for inhibition of targets involved in cell wall mycolyl-arabinogalactan biosynthesis³⁶ indicated that these inhibitors were unlikely to affect promiscuous targets in cell wall biosynthesis^{35a,36} (results not shown). Efforts to determine the mechanism(s) of action are underway. In this regard, we will attempt to “de-construct” 7H9 to identify the components that are essential for the observed effect; in addition, we will continue to pursue the mutant generation work in order to identify a mechanism of resistance. The leading compounds and selected inactive compounds were tested for cytotoxicity against CHO cells. The SAR for cytotoxicity tracked that of the antibacterial activity with the active compounds displaying a narrow selectivity index, thus none of the leading compounds was progressed to *in vivo* studies.

Table 4. Variation of MIC values with growth media and cytotoxicity of leading compounds

Compd	R	R ¹	R ²	MIC ₉₉ 7H9/ADC (μM) ^a	MIC ₉₉ GAST-Fe (μM) ^b	MIC ₉₉ Low Fe GAST (μM) ^c	Cytotoxicity IC ₅₀ (μM) ^d
1		CH ₃		5	>160	>160	34
23		CH ₃		5	37	20	<9.0
36		H		2.5	>160	>160	3.78
38		H		2.5	>160	>160	5.45
39		H		1.25	>160	>160	19.98
55		CH ₃		5	20	>160	22

^a 14 day Alamar Blue readout against H37Rv in 7H9/ADC/Tween

^b 14 day Alamar Blue readout against H37Rv in GAST-Fe

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^b 14 day Alamar Blue readout against H37Rv in low Fe GAST

^d Cytotoxicity against Chinese Hamster Ovarian cells (CHO)

4. Conclusion

In conclusion, a novel series of pyrazolo[1,5-a]pyrimidines with promising *in vitro* antimycobacterial activity has been identified from a whole-cell high-throughput screening campaign. Medicinal chemistry efforts focused on exploration of the R substituent at position 7 and the R² substituent at position 3 to identify a number of promising hit compounds **1**, **23**, **36**, **38** and **39** displaying a MIC₉₉ values of 1.25-5 µM in 7H9 media. The series displays SAR trends, and key substituents important for antimycobacterial activity have been identified to inform a future optimisation campaign. However, this series suffers from the liabilities of poor aqueous solubility and *in vitro* cytotoxicity, both of which will need to be addressed without compromising *in vitro* antimycobacterial activity in any future optimisation campaign.

5. Experimental section

5.1 Chemistry

5.1.1 General

All reagents and solvents were obtained from commercial sources and used as received. All dry solvents were obtained from an SP-1 standalone solvent purification system from LC Technology Solutions Inc. Reactions were monitored by thin layer chromatography (TLC) using Merck TLC silica gel 60 F₂₅₄ aluminium-backed precoated plates and were visualized by ultraviolet light at 254 nm. Purification of compounds was carried out by either column chromatography on silica gel 60 (Fluka), particle size 0.063–0.2 mm (70–230 mesh ASTM), as the stationary phase, or by Waters' HPLC using X-bridge C18 5µm column (4.6 x 150 mm); organic phase: 10 mM Ammonium acetate (pH 3.7) in HPLC grade methanol, aqueous phase: 10 mM Ammonium acetate (pH 3.7) in HPLC grade water; flow rate = 15.00 mL/min; detector: photodiode array (PDA). All target compounds and intermediates were characterised by ¹H NMR, ¹³C NMR and MS. NMR spectra were recorded on either a Varian Mercury-300 (¹H 300.1 MHz, ¹³C 75.5 MHz) or Bruker-400 (¹H 400.2 MHz, ¹³C 100.6 MHz) instrument using CDCl₃, MeOH-*d*₄ and DMSO-*d*₆ as solvents. The ¹H NMR data are reported as follows: chemical shift in parts per million (δ) downfield of tetramethylsilane (TMS), multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, dd = doublets of doublets, dt = triplets of doublets and m = multiplet), coupling constant (Hz), and integrated value. The ¹³C NMR spectra were measured with complete proton decoupling. LC–MS/MS analysis was performed using an Agilent® 1260 Infinity Binary Pump, Agilent® 1260 Infinity Diode Array Detector (DAD), Agilent® 1290 Infinity Column Compartment, Agilent® 1260 Infinity Standard Autosampler, and a Agilent® 6120 Quadrupole (Single) MS, with APCI/ESI multimode ionisation source. The purities were determined by Agilent® LCMS/MS or Waters' HPLC using X-bridge C18 5µm column (4.6 x 150 mm); organic phase: 10 mM Ammonium acetate (pH 3.7) in HPLC grade methanol, aqueous phase: 10 mM

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Ammonium acetate (pH 3.7) in HPLC grade water; flow rate = 1.20 mL/min; detector: photodiode array (PDA). The purities of all compounds were found to be >95%.

5.1.2 Scaffold Synthesis

Synthesis of 2,5-dimethyl-3-phenylpyrazolo[1,5-a]pyrimidin-7(4*H*)-one (7)

Ethyl acetoacetate (7.6 ml, 60.3 mmol) was added dropwise to a stirred solution of 3-methyl-4-phenyl-1*H*-pyrazol-5-amine (10.0 g, 54.8 mmol) in acetic acid (50 ml) and refluxed at 120°C for 16hrs. The reaction mixture was cooled and poured into cooled TBME (300 ml) and stirred at 0°C for 1h. The resulting precipitate was filtered, washed with TBME (100 ml) and dried at 70°C to obtain a cream solid (quantitative, 14.45 g, 60.3 mmol). δ_{H} (400 MHz; CDCl₃) 11.72 (br s, 1H), 7.48 – 7.35 (m, 5H), 5.55 (s, 1H), 2.26 (s, 3H), 2.25 (s, 3H); δ_{C} (101 MHz; CDCl₃) 156.3, 151.1, 150.2, 139.2, 131.3, 129.9 (x2), 129.2 (x2), 127.3, 103.3, 96.1, 19.1, 13.5. LCMS (ESI): m/z 240 [M + H]⁺; HPLC purity 97%.

5.1.3 Intermediate Synthesis

Synthesis of 7-chloro-2,5-dimethyl-3-phenylpyrazolo[1,5-a]pyrimidine (8)

Phosphoryl trichloride (5.7 ml, 62.7 mmol) and *N,N*-dimethylaniline (3.3 ml, 26.1 mmol) were added dropwise to a stirred solution of [2,5-dimethyl-3-phenylpyrazolo[1,5-a]pyrimidin-7(4*H*)-one (7) (5.00 g, 20.9 mmol) in toluene (50 ml), and refluxed at 120°C. After 2.5 h, the reaction mixture was allowed to cool and was concentrated. The black residue was poured into a beaker containing crushed ice (100 mL) and the resulting solution was neutralised with NaHCO₃. The aqueous solution was extracted with EtOAc (3 × 150 mL) and the combined organic phase was washed with brine (100 mL). The organic phase was dried over Na₂SO₄ and concentrated to yield the desired crude product which was purified by column chromatography (10% EtOAc/hexane) to obtain a light green powder (Yield = 96%, 5.16 g, 20.02 mmol). δ_{H} (400 MHz; CDCl₃) 7.31 – 7.17 (m, 5H), 6.80 (s, 1H), 2.71 (s, 3H), 2.61 (s, 3H); LCMS (ESI): m/z 260 [M + H]⁺; HPLC purity 95 %.

5.1.4 Library Production

General procedure A for synthesis of 7-substituted aminopyrazolo[1,5-a]pyrimidines (9)

To a solution of 7-chloro-2,5-dimethyl-3-phenylpyrazolo[1,5-a]pyrimidine (1 equiv) and diisopropylethylamine (1.5 equiv) in 2-propanol was added amine (1.2 equiv). After refluxing for 16 h, the reaction mixture was concentrated *in vacuo* and the appropriate solvent added to precipitate the crude product, which was collected by filtration and then purified by recrystallization, prep HPLC or column chromatography.

5.1.5 Synthesis of 2,5-dimethyl-3-phenyl-*N*-(pyridin-3-ylmethyl)pyrazolo[1,5-a]pyrimidin-7-amine (14)

A representative experimental for General Procedure A. Characterization data of **15-30** is provided in the Supplementary data. The title compound was synthesized from **8** (100 mg,

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0.388 mmol) using general procedure A to afford (127 mg, 98 %) of a yellow coloured powder recrystallized from DCM/hexane. δ_{H} (400 MHz; DMSO- d_6) 8.68 (d, $J = 4.0$ Hz, 1H), 8.49 (dd, $J = 8.0$ and 4.0 Hz, 1H), 8.27 (t, $J = 8.0$ Hz, 1H), 7.82 (dt, $J = 8.0$, 4.0 and 1.6 Hz, 1H), 7.74 (dd, $J = 8.0$ and 4.0 Hz, 2H), 7.42 (m, 2H), 7.36 (ddd, $J = 8.0$, 4.0 and 1.6 Hz, 1H), 7.23 (m, 1H), 6.09 (s, 1H), 4.68 (d, $J = 8.0$ Hz, 1H), 2.56 (s, 3H), 2.37 (s, 3H). δ_{C} (101 MHz; DMSO- d_6) 206.3, 159.4, 150.6, 149.2, 148.9, 145.6, 145.3, 135.4, 134.3, 133.8, 128.7, 128.5, 125.6, 123.9, 106.2, 85.2, 42.8, 31.0, 25.2, 14.8. LCMS (ESI): m/z 330.1 $[\text{M} + \text{H}]^+$; HPLC purity 96 %.

5.1.6 Scaffold Synthesis

General procedure for the synthesis of 7-chloro-5-methylpyrazolo[1,5-a]pyrimidine (10a) and 7-chloro-5-methylpyrazolo[1,5-a]pyridimidine (10b)

Methyl acetoacetate (1.1 equiv) or ethyl acetoacetate (1.1 equiv) was added dropwise to a stirred solution of 3-amino-1*H*-pyrazole (1 equiv) or 3-amino-5-methyl-1*H*-pyrazole (1 equiv) in acetic acid and refluxed at 120°C for 16hrs. The reaction mixture was cooled and poured into cooled TBME and stirred at 0°C for 1h. The resulting precipitate was filtered, washed with TBME and dried at 70°C to obtain a cream solid. **a** R = H, (Yield = 87 %, 18.35 g, 123.13 mmol). δ_{H} (400 MHz; CDCl₃) 12.22 (br s, 1H), 7.79 (dd, $J = 2.0$ and 0.8 Hz, 1H), 6.06 (dd, $J = 2.0$ and 0.8 Hz, 1H), 5.54 (s, 1H), 2.27 (m, 3H). **b** R = Me, (Yield = 82 %, 16.68 g, 101.87 mmol). δ_{H} (400 MHz; DMSO- d_6) 12.01 (bs, 1H), 5.90 (d, $J = 0.4$ Hz, 1H), 5.49 (d, $J = 0.8$ Hz, 1H), 2.27 (d, $J = 0.4$ Hz, 3H), 2.26 (d, $J = 0.4$ Hz, 3H).

N,N-Dimethylaniline (1.25 equiv) was added to a stirred solution of 5-methylpyrazolo[1,5-a]pyrimidin-7(4*H*)-one (**a**) or 2,5-dimethylpyrazolo[1,5-a]pyrimidin-7(4*H*)-one (**b**) (1 equiv) in toluene, followed by the addition of phosphorus oxychloride (3 equiv). The reaction mixture was heated at reflux for 4 h. The reaction mixture was allowed to cool and was neutralised with NaOH (2M). The aqueous solution was extracted with DCM (3 ×) and the combined organic phase was washed with brine. The organic phase was dried, concentrated and the crude product was purified by column chromatography (10% EtOAc/hexane) to yield the desired light yellow solid **10a** Yield = 41 %. δ_{H} (400 MHz; CDCl₃) 8.15 (d, $J = 2.4$ Hz, 1H), 6.83 (s, 1H), 6.65 (d, $J = 2.4$ Hz, 1H), 2.59 (s, 3H). MS: m/z 168 $[\text{M}]^+$ **10b** Yield = 46 %. δ_{H} (400 MHz; CDCl₃) 6.74 (s, 1H), 6.43 (s, 1H), 2.56 (s, 3H), 2.54 (s, 3H). LCMS (ESI): m/z 182 $[\text{M} + \text{H}]^+$

5.1.7 General procedure for the synthesis of 3-bromo-7-chloro-5-methylpyrazolo (11a) and 3-bromo-7-chloro-2,5-dimethylpyrazolo[1,5-a]pyrimidine (11b)

A solution of **10a** or **10b** (1 equiv) in dichloromethane was cooled to 0 °C using an ice bath. *N*-bromosuccinimide (1.1 equiv) was added slowly to the solution that was stirred for 16 h at room temperature. The reaction mixture was washed diluted with water and the reaction mixture extracted with dichloromethane (3 x). The organic phase was dried over Na₂SO₄ and concentrated to yield the desired product as a yellow solid. **11a** Yield = quantitative. δ_{H} (400 MHz; CDCl₃) 8.13 (s, 1H), 6.88 (s, 1H), 2.63 (s, 3H). LCMS (ESI): m/z 247 $[\text{M} + \text{H}]^+$ **11b**

Yield = quantitative. δ_{H} (400 MHz; CDCl_3) 8.13 (s, 1H), 2.85 (s, 3H), 2.63 (s, 3H). LCMS (ESI): m/z 261 $[\text{M} + \text{H}]^+$

5.1.8 Intermediate synthesis

General procedure for the synthesis of *tert*-butyl (3-bromo-5-methylpyrazolo[1,5-a]pyrimidin-7-yl)(pyridin-2-ylmethyl)carbamate (12a) and *tert*-butyl (3-bromo-2,5-dimethylpyrazolo[1,5-a]pyrimidin-7-yl)(pyridin-2-ylmethyl)carbamate (12b)

To a solution of **11a** or **11b** (1 equiv) and diisopropylethylamine (1.5 equiv) in 2-propanol was added pyridin-2-ylmethanamine (1.2 equiv). After heating for 16 h at 80 °C, the precipitate that formed was collected by filtration. The product was washed with water and 2-propanol and dried to yield the product as a white solid, which was sufficiently pure to be used in the next step. Di-*tert*-butyl dicarbonate (1.5 equiv) was added to a stirred solution of the free amine (1 equiv) in dichloromethane, followed by the addition of dimethylaminopyridine (0.1 equiv), and diisopropylamine (1.5 equiv). After stirring for 24 h, the reaction mixture was concentrated and partitioned between EtOAc and water. After separation, the organic phase was washed with 10% citric acid and brine, dried and concentrated. The crude product was purified by column chromatography. **12a** Yield = 78 % over two steps. δ_{H} (400 MHz; $\text{DMSO}-d_6$) 8.58 (d, J = 4.0 Hz, 1H), 8.0 (s, 1H), 7.73 (td, J = 8.0 and 4.0 Hz, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.28 (t, J = 8.0, 1H), 5.91 (s, 1H), 4.7 (s, 2H), 2.57 (s, 3H), 1.30 (s, 9H). **12b** Yield = 75 % δ_{H} (400 MHz; $\text{DMSO}-d_6$) 8.58 (d, J = 8.0 Hz, 1H), 7.73 (td, J = 8.0 and 2.0 Hz, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.28 (t, J = 8.0, 1H), 5.91 (s, 1H), 4.7 (s, 2H), 2.87 (s, 3H), 2.57 (s, 3H), 1.30 (s, 9H).

5.1.9 Library production

General procedure B for synthesis of 3-substituted aminopyrazolo[1,5-a]pyrimidines (13)

To a solution of **12** (1 equiv) in dioxane and water (5:1) was added boronic acid (1.2 equiv) followed by addition of cesium carbonate (1 equiv) and $\text{PdCl}_2(\text{dppf})$ (0.1 equiv). The reaction mixture was degassed and heated at 80 °C overnight. The reaction was diluted with EtOAc and washed with water (2 x). After drying over Na_2SO_4 , filtration and concentration, the crude product was dissolved in DCM (4 ml) and TFA (8 ml) was added. The reaction was stirred for 4 hrs at rt followed by concentration. The residue was dissolved in DCM and washed with 10% Na_2CO_3 (2 x), the organic layer was dried (MgSO_4), filtered and concentrated. The crude product was purified by flash column chromatography (80% EtOAc/Hex) or recrystallization from DCM/Hexane or by prep TLC (EtOAc) to give the desired product.

5.1.10 Synthesis of 2,5-dimethyl-3-phenyl-*N*-(pyridin-2-ylmethyl)pyrazolo[1,5-a]pyrimidin-7-amine (1)

A representative experimental for General Procedure B. Characterization data of **2-6** and **31-52** is provided in the Supplementary data. The title compound was synthesized from **12b** (400

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mg, 0.925 mmol) using general procedure B to afford (84 mg, 27%) as an off white solid after purification by flash column chromatography (80% EtOAc/Hex) followed by recrystallization from DCM/Hexane and finally by prep TLC (EtOAc). δ_{H} (400 MHz; DMSO- d_6) 8.68 (ddd, $J = 4.0, 1.6$ and 0.8 Hz, 1H), 7.78-7.71 (m, 3H), 7.45 (t, $J = 8.0$ Hz, 2H), 7.38 (d, $J = 8.0$ Hz, 1H), 7.30-7.26 (m, 2H), 5.86 (s, 1H), 4.75 (d, $J = 8.0$ Hz, 2H), 2.63 (s, 3H), 2.53 (s, 3H). δ_{C} (101 MHz; CDCl_3) 159.5, 155.5, 151.5, 149.6, 145.9, 145.8, 137.0, 133.0, 128.8 (x2), 128.4 (x2), 125.8, 122.8, 121.2, 107.5, 85.9, 46.9, 25.2, 14.4. LCMS (ESI): m/z 330.1 $[\text{M} + \text{H}]^+$; HPLC purity 96.3 %.

5.2 *M. tuberculosis* H37Rv Minimum Inhibitory Concentration (MIC) assays

The broth microdilution method²⁷ allows a range of antibiotic concentrations to be tested on a single 96-well microtitre plate in order to determine the minimum inhibitory concentration (MIC). MICs were determined according to the broth microdilution method in liquid 7H9 medium supplemented with ADC (bovine albumin fraction V [5mg/ml], D-Glucose [2mg/ml], sodium chloride [0.81mg/ml]) and/or in the glycerol–alanine–salts (GAST-Fe) medium pH 6.6, supplemented with [per liter: 0.3 g of Bacto Casitone (Difco), 0.05 g of ferric ammonium citrate, 4.0 g of dibasic potassium phosphate, 2.0 g of citric acid, 1.0 g of L-alanine, 1.2 g of magnesium chloride hexahydrate, 0.6 g of potassium sulfate, 2.0 g of ammonium chloride, 1.80 ml of 10 g sodium hydroxide, and 10.0 ml of glycerol, Tween80 was added to 0.05%].³³ Low iron GAST medium is normal GAST medium but without the ferric ammonium citrate.

Briefly, a 10 ml culture of *Mycobacterium tuberculosis* (Ma)³⁸ is grown to an OD_{600} of 0.6 - 0.7. The culture is then diluted with 1:500 in liquid 7H9 or diluted 1:100 in BSA-free GAST/Fe medium. In a 96-well microtitre plate, 50 μl of medium is added to all wells from Rows 2-12. The compounds to be tested are added to Row 1 in duplicate, at a final concentration of 640 μM (stocks are made up to a concentration of 12.8 mM in DMSO, and diluted to 640 μM in medium). A two-fold serial dilution is prepared, using a multichannel pipette, by transferring 50 μl of the liquid in Row 1 to Row 2 and aspirating to mix. 50 μl of the liquid in Row 2 is then transferred to Row 3 and aspirated, and so on. This procedure is repeated until Row 12 is reached, from which 50 μl of the liquid is discarded so as to bring the final volume in all wells to 50 μl . Controls include media only, 5% DMSO, Rifampicin and Kanamycin. Finally, 50 μl of the diluted *M. tuberculosis* culture (1:500 for 7H9 or 1:100 for GAST-Fe) is added to all wells in Rows 2-12. Cells are not added to Row 1, as this serves as a contamination control. The microtitre plate is sealed in a ziplock bag and incubated at 37°C with humidifier to prevent evaporation of liquid. The lowest concentration of drug that inhibits growth of more than 99% of the bacterial population is considered to be the MIC_{99} . MIC_{99} values are scored visually at 7-days and 14-days post inoculation, and digital images captured and stored.

5.3 Kinetic solubility assay

The kinetic solubility assay was performed using a miniaturised shake flask method.³⁹ 10mM stock solutions of each of the test compounds were used to prepare calibration standards (10-

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220 μ M) in DMSO, and to spike (1:50) duplicate aqueous samples of phosphate buffered saline (pH 6.5), 0.01M HCL (pH 2) and FaSSIF (simulating fasting state biorelevant media, pH 6.5), with a final DMSO concentration of 2%. After shaking for 2 hours at 25°C, the solutions were filtered and analysed by means of HPLC-DAD (Agilent 1200 Rapid Resolution HPLC with a diode array detector). Best fit calibration curves were constructed using the calibration standards, which were used to determine the aqueous samples' solubility.

5.4 Metabolic stability assay

The metabolic stability assay was performed in duplicate in a 96-well micro titre plate. The test compounds (0.1 μ M) were incubated individually in mouse, rat and pooled human liver microsomes (0.4 mg/ml) at 37 °C for predetermined times, in the presence and absence of the cofactor NADPH (1mM). Reactions were quenched by adding 300 μ l of ice cold acetonitrile containing internal standard (carbamazepine, 0.0236 μ g/mL). Test compounds in the supernatant were quantified by means of specific LC-MS/MS (Agilent Rapid Resolution HPLC, AB SCIEX 4000 QTRAP MS) methods. Metabolite searches were not conducted during the metabolic stability assay.⁴⁰

5.5 Chinese hamster ovarian (CHO) Cytotoxicity assay

Test samples were screened for *in vitro* cytotoxicity against a mammalian cell-line, Chinese Hamster Ovarian (CHO) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays.^{41,42} The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The tetrazolium ring is cleaved in active mitochondria. Thus only viable cells are able to reduce the water-soluble yellow coloured MTT to water-insoluble purple coloured formazan. Formazan crystals are dissolved in dimethyl sulphoxide (DMSO). The test samples were tested in triplicate on one occasion. The test samples were prepared to a 20 mg/ml stock solution in 100% DMSO. Stock solutions were stored at -20°C. Further dilutions were prepared in complete medium on the day of the experiment. Samples were tested as a suspension if not completely dissolved. Emetine was used as the reference drug in all experiments. The initial concentration of emetine was 100 μ g/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 μ g/ml. The same dilution technique was applied to the all test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability (data not shown). The 50% inhibitory concentration (IC₅₀) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

Acknowledgements

This work was funded, in part, by the Intramural Research Program of NIAID, by grants from the Foundation for the National Institutes of Health with support from the Bill & Melinda Gates Foundation, and by the South African Medical Research Council (MRC), the South African Tuberculosis Research and Innovation Initiative (SATRII) and the Strategic Health

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and Innovation partnership (SHIP). The University of Cape Town, South African Medical Research Council, and South African Research Chairs Initiative of the Department of Science and Technology, administered through the South African National Research Foundation are gratefully acknowledged for support (K.C). Mathew Njoroge, Efrem Abay and Christel Brunschwig (H3-D ADME laboratories) are acknowledged for the metabolite identification studies and Carmen De Kock is acknowledged for the cytotoxicity studies.

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Original hit from HTS

$MIC_{99} = 5 \mu M$

Solubility = $< 5 \mu M$

HLM $t_{1/2} = 35.9 \text{ min}$

Graphical Abstract