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Novel Anti-tubercular 6-Dialkylaminopyrimidine Carboxamides from Phenotypic Whole-Cell High Throughput Screening of a SoftFocus Library: Structure Activity Relationship and Target Identification Studies

Colin R. Wilson^a, Richard K. Gessner^{a,†}, Atica Moosa^c, Ronnett Seldon^{a,d}, Digby F. Warner^{c,d}, Valerie Mizrahi^{c,d}, Candice Soares de Melo^a, Sandile B. Simelane^a, Aloysius Nchinda^a, Efrem Abay^b, Dale Taylor^b, Mathew Njoroge^b, Christel Brunschwig^b, Nina Lawrence^b, Helena I. M. Boshoff^e, Clifton E. Barry III^e, Frederick A. Sirgel^f, Paul van Helden^f, C John Harris^g, Richard Gordon^h, Sonja Ghidelli-Disseⁱ, Hannah Pflaumerⁱ, Markus Boescheⁱ, Gerard Drewesⁱ, Olalla Sanz^j, Gracia Santos^j, Maria José Rebollo-Lopez^j, Beatriz Urones^j, Carolyn Selenski^j, Maria Jose Lafuente-Monasterio^j, Matthew Axtman^j, Joël Lelièvre^j, Lluís Ballell^j, Rudolf Mueller^a, Leslie J. Street^a, Sandeep R. Ghorpade^{a,*} and Kelly Chibale^{a,d,k,*}

^aDrug Discovery and Development Centre (H3D), Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa; ^bDrug Discovery and Development Centre (H3D), Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Observatory, 7925, South Africa; ^cSAMRC/NHLS/UCT Molecular Mycobacteriology Research

Unit, Department of Pathology, University of Cape Town, Rondebosch 7701, South Africa;
^dInstitute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch
7701, South Africa; ^eTuberculosis Research Section, Laboratory of Clinical Infectious Diseases;
National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda,
Maryland 20892, United States; ^fDST/NRF Centre of Excellence for Biomedical TB Research,
SA MRC Centre for TB Research, Division of Molecular Biology and Human Genetics, Faculty
of Health Sciences, Stellenbosch University, Tygerberg, South Africa; ^gCJH Consultants, Ford
Cottage, South Weirs, Brockenhurst, Hampshire SO42 7UQ, UK, United Kingdom; ^hStrategic
Health Innovation Partnerships (SHIP), South African Medical Research Council, Parow Valley,
South Africa; ⁱCellzome GmbH, Molecular Discovery Research, GlaxoSmithKline,
Meyerohofstrasse 1, Heidelberg, Germany; ^jDiseases of the Developing World, GlaxoSmithKline,
Calle Severo Ochoa 2, 28760 Tres Cantos, Madrid, Spain; ^kSouth African Medical Research
Council Drug Discovery and Development Research Unit, Department of Chemistry, University
of Cape Town, Rondebosch 7701, South Africa

ABSTRACT: A BioFocus DPI SoftFocus library of ~35,000 compounds was screened against
Mycobacterium tuberculosis (Mtb) in order to identify novel hits with anti-tubercular activity.
The hits were evaluated in biology triage assays to exclude compounds suggested to function via
frequently encountered promiscuous mechanisms of action including inhibition of the QcrB
subunit of the cytochrome *bc*₁ complex, disruption of cell-wall homeostasis, and DNA damage.
Among the hits which passed this screening cascade, a 6-dialkylaminopyrimidine carboxamide
series was prioritized for hit to lead optimization. Compounds from this series were active
against clinical Mtb strains while no cross-resistance to conventional anti-tuberculosis drugs was
observed. This suggested a novel mechanism of action, which was confirmed by

chemoproteomic analysis leading to the identification of BCG_3193 and BCG_3827 as putative targets of the series with unknown function. Initial structure-activity relationship studies have resulted in compounds with moderate to potent anti-tubercular activity and improved physicochemical properties.

INTRODUCTION

Tuberculosis (TB) is one of the world's most deadly infectious diseases, leading to 1.4 million deaths in 2015.¹ The disease is caused by *Mycobacterium tuberculosis* (Mtb) and primarily affects lower to middle income countries such as those in sub-Saharan Africa. A standard first-line 4-drug regimen of rifampin, isoniazid, pyrazinamide, and ethambutol is used to treat drug-susceptible TB.² Although these drugs form the core of TB treatment regimens, they have become ineffective against resistant Mtb strains. TB that does not respond to at least isoniazid and rifampin, the two frontline drugs, is defined as multi-drug resistant (MDR) TB. MDR TB that develops additional resistance to any fluoroquinolone and at least one of the three injectable second-line drugs (amikacin, kanamycin, or capreomycin) is referred to as extensively drug resistant (XDR) TB and is virtually untreatable.³ One of the main drivers behind the rise of resistance is the 6-month long treatment required for drug-sensitive disease, resulting in poor patient adherence. Thus, the main goal of many TB drug discovery programs is the identification of compounds that will potentially contribute to treatment shortening; albeit there is no clear evidence as to how this can be accomplished.⁴ One possible approach is to identify compounds that inhibit novel targets, and do not show cross-resistance to currently used anti-TB drugs. Various novel drug combinations can then be evaluated for their potential towards treatment shortening while establishing clinical effectiveness. Identifying compounds which exert their

anti-TB effect through novel targets is not a trivial process. This is exemplified by the approval in 2012 of bedaquiline, the first new drug approved for the treatment of TB in more than 40 years with a novel mechanism of action (MOA) that involves inhibition of the mycobacterial ATP synthase.^{5–7}

Two hit-generation approaches that have been traditionally used are target-based and whole-cell screening.^{8,9} Historically, there has been extremely limited success with the target-based approach owing to the lack of translation from target activity to whole-cell activity. Nevertheless, efforts to discover and validate novel drug targets using various approaches must continue. This is exemplified by the recently reported validation of CoaBC as a bactericidal target using a chemical biology approach.¹⁰ On the other hand, a more successful approach has been phenotypic whole-cell high-throughput screening (HTS) of chemical libraries to identify novel chemotypes whose target is then identified retrospectively. There are several compounds in the TB drug pipeline that have been identified through whole-cell HTS. Examples include Q203 (Figure 1), an inhibitor of the QcrB subunit of the cytochrome *bc*₁ complex which is involved in electron transport.¹¹ Another example is SQ109 (Figure 1) whose precise MOA remains to be elucidated but is believed to involve the Mycobacterial Membrane Protein Large 3, MmpL3, a mycolic acid transporter involved in cell wall synthesis.^{12,13} Similarly, some inhibitors of decaprenylphosphoryl- β -D-ribose 2'-epimerase (DprE1) e.g. pBTZ,¹⁴ and azaindoles TBA7371^{15,16} are poised to enter clinical trials (Figure 1). While these compounds show promise as new anti-TB agents, their targets are yet to be clinically validated. This necessitates the need to continuously identify new chemical matter with alternate novel MOAs towards populating the TB drug pipeline.

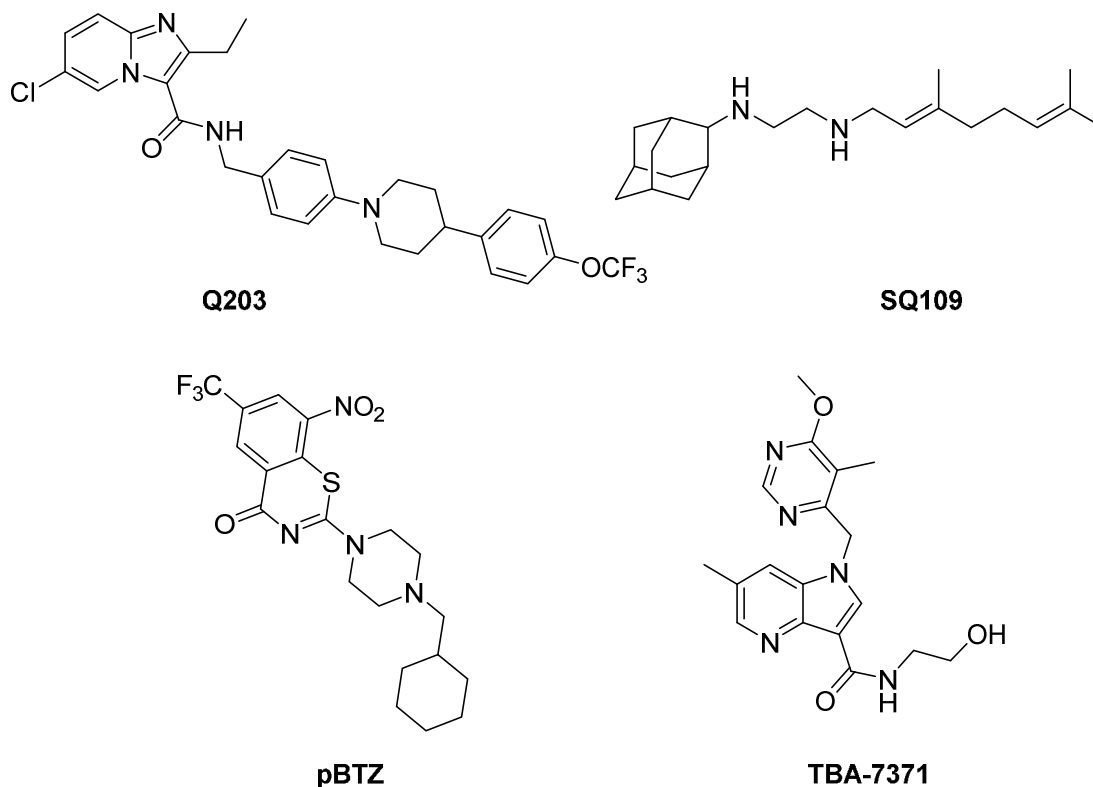
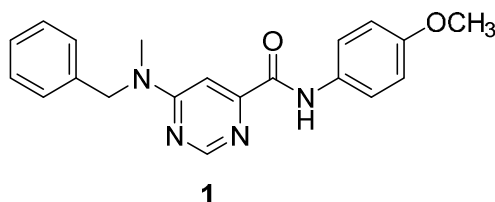


Figure 1. New TB drug Development candidates identified from whole cell screening

In line with this, we initiated a HTS campaign using a diverse SoftFocus™ compound library¹⁷ acquired from BioFocus and complemented this with a biology triage process that aimed specifically to exclude compounds acting on targets such as QcrB and the cell wall (e.g. MmpL3 and DprE1), as well as DNA damaging agents. The rationale was to exclude targets known for their promiscuity, with a large proportion of HTS hits having these as part of their MOA.^{18,19} There is also the question around whether or not compounds targeting cell wall metabolism can contribute to the much sought after treatment shortening.¹⁹ Compounds that induce DNA damage were also excluded in order to avoid general cytotoxicity, as well as targets such as DNA gyrase whose clinically used inhibitors are exemplified by the fluoroquinolones. Along with an aminopyrazolo[1,5-a]pyrimidine chemical series²⁰, a 6-dialkylaminopyrimidine carboxamide

scaffold was identified as a hit series with a potentially novel MOA, based on the initial biology triage process (Figure 2). Herein, we describe the synthesis, structure activity relationship (SAR), *in vitro* absorption, distribution, metabolism and excretion (ADME), *in vivo* pharmacokinetic (PK) and biological profiles of this chemical series.



7H9/ADC MIC ₉₉	20 μM
GAST/Fe MIC ₉₉	10 μM
Δcyd GAST/Fe MIC ₉₉	20 μM
Δcyd 7H9 GLU ADC MIC ₉₉	>20 μM
qcrBA317T GAST/ Fe MIC ₉₉	20 μM
Vero IC ₅₀	287 μM
Kinetic Solubility (PBS pH 7.4)	<5 μM
cLogP	4.07

Figure 2. Compound **1** and biology triage data.

RESULTS AND DISCUSSION.

Phenotypic whole-cell HTS and biology triage.

A screen of a BioFocus DPI SoftFocus library of ~35,000 compounds against virulent Mtb H37Rv conducted at the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIAID/NIH, USA) led to a number of confirmed hits, which included compound **1** with a moderate Minimum Inhibitory Concentration (MIC) value of 20 μM in albumin-dextrose complex (ADC) medium containing ~0.4% Bovine serum albumin (BSA). The compound was slightly more potent in GAST-Fe (Glycerol-alanine-salts)²⁰ minimal medium, likely driven by the absence of BSA which allowed for higher free concentration of the

compound under the assay conditions. Hence, GAST-Fe was used alongside the 7H9/ADC medium during further SAR exploration of the series. Compound **1** was tested in the three biology triage assays in order to assess activity against promiscuous targets. The compound did not show hypersensitivity against a cytochrome *bd* oxidase knockout mutant strain (*cydKO*)^{21,22} and also retained activity against a QcrB mutant (*qcrB*^{A317T}), thereby eliminating this as potential target. Compound **1** also did not elicit a positive response in two standard bioluminescence reporter assays: *PiniB*-LUX²³, which is designed to detect compounds targeting Mtb cell wall biosynthesis, and *PrecA*-LUX²³ which allows detection of genotoxic compounds (data in supporting information). Similar results were obtained with other analogues in the series obtained during SAR studies (data in supporting information). These results, along with chemoproteomics and cross-resistance studies (see biology results below), indicate that the 6-dialkylaminopyrimidine carboxamide series of compounds exemplified by compound **1** possesses a novel MOA that is not shared with known TB drugs.

SAR exploration strategy. Compound **1** was identified as a suitable hit with a moderate minimum inhibitory concentration (MIC₉₉) of 20 μ M in 7H9/ADC medium, low toxicity against the mammalian Vero cell line (IC₅₀ of 287 μ M) yet poor kinetic solubility (KS, < 5 μ M). The poor solubility was assumed to be related to the high lipophilicity and flat aromatic character of the molecule. Since there were very few active near neighbors identified from the screen, very limited information was available on further optimization scope with the scaffold to improve anti-TB activity while addressing the poor physicochemical properties and, potential safety issues, which may be associated with the scaffold. A detailed SAR plan as summarized in figure 3 was embarked upon. The molecule was divided into three parts for convenience, the central pyrimidine core, right-hand side (RHS) amide and left-hand side (LHS) hydrophobic *N*-benzyl

moiety (Figure 3). The major SAR exploration focus was on understanding key hydrogen bond (HB) donor-acceptor interactions critical for activity, shape and size of the central core, and scope for substitution/modification on either the RHS or LHS parts of the molecule to improve potency. The scope for addition of heteroatoms or polar groups into the molecule was also explored with the aim of reducing lipophilicity along with addressing related issues such as poor physicochemical properties and structure alerts such as the presence of potentially AMES positive anilines upon cleavage of the amide group.

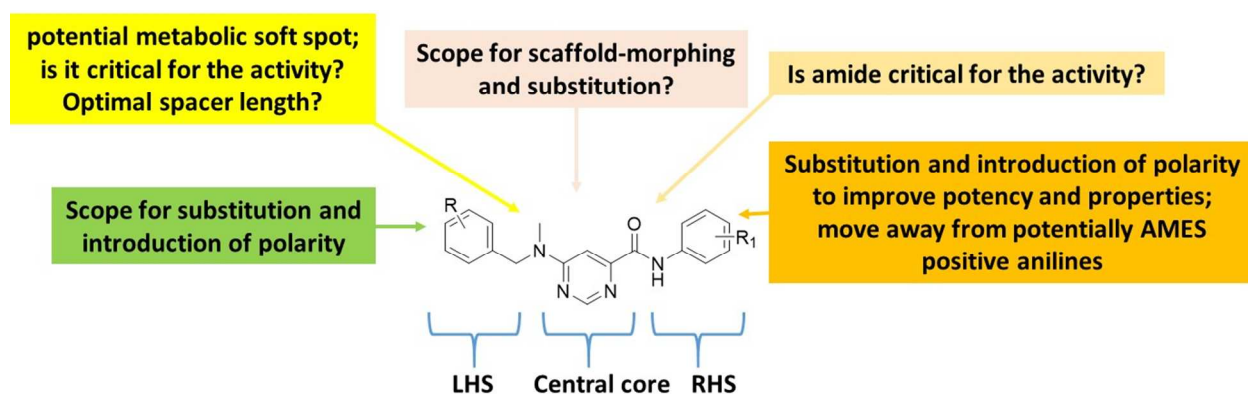
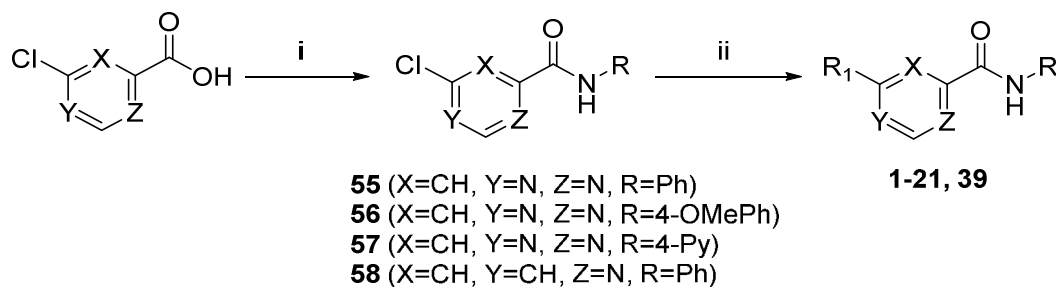


Figure 3: SAR exploration strategy

Synthesis. Compounds **1-20** were synthesized to explore the SAR around the *N*-methyl-1-phenylmethanamine motif (SAR on LHS, Table 1), compounds **21-30** were synthesized to explore the central pyrimidine core of the scaffold (SAR on central core, Table 2), and compounds **31-54** were synthesized to explore the SAR around the amide portion of the scaffold (SAR on RHS, Table 3). In general, target compounds were synthesized using either one of two routes. Scheme 1 was used to synthesize an advanced intermediate with a specific amide in place allowing for the exploration of the LHS of the molecule. The same route was also used to synthesize several of the core modifications. The route involved a straightforward 2-step

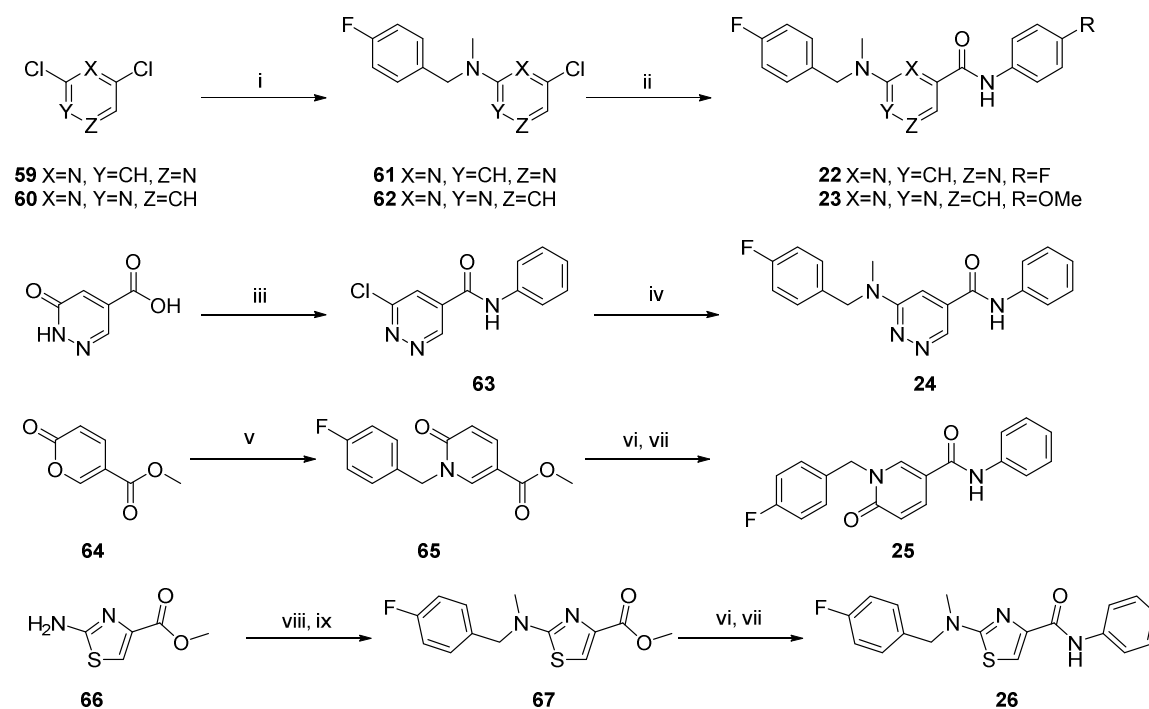
procedure starting from a chloro-heterocyclic-carboxylic acid, where coupling with corresponding amine using 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluoro-phosphate (HATU) was first performed, followed by nucleophilic displacement of the relevant chloro group by an appropriate amine. Thus, synthesis of compounds **1-20** and **39** (Table 1) was achieved by coupling of 6-chloropyrimidine-4-carboxylic acid with aniline, 4-methoxyaniline or 4-aminopyridine to give intermediates **55**, **56** and **57** respectively, followed by displacement of 6-chloro group with various amines. Compound **21** was synthesized by coupling of 4-chloropicolinic acid with aniline to form intermediate **58** followed reaction with 1-(4-fluorophenyl)-*N*-methylethanamine.

Scheme 1. General route for the synthesis of 6-Amino-pyrimidine-4-carboxamides as well as alternative cores.



Reagents and conditions: (i) Amine, DIPEA, HATU, DMF, 25°C (ii) Amine, *i*PrOH, DIPEA, 100 °C

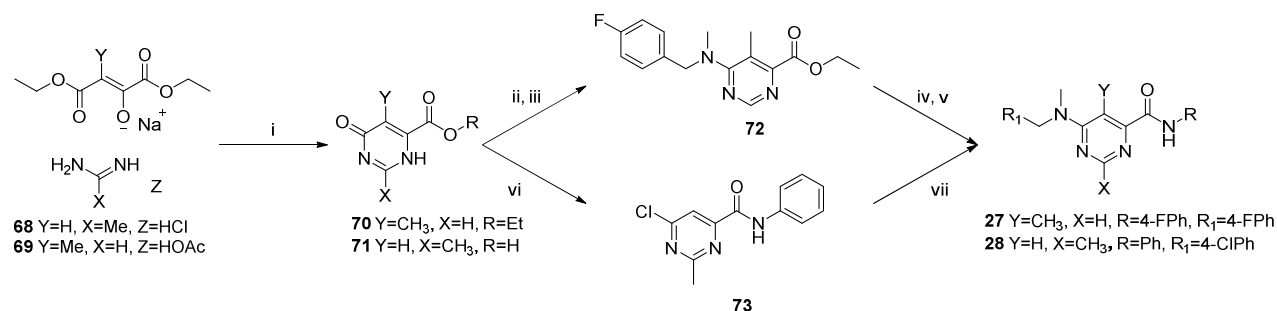
Scheme 2. Synthetic routes used to access core modifications



Reagents and conditions: (i) 1-(4-fluorophenyl)-*N*-methylmethanamine, Et₃N, Dioxane, 60 °C (ii) Aniline, Mo(CO)₆, Et₃N, TBAC, Toluene, Tetraglyme, 150 °C (iii) a) POCl₃, 110 °C b) Aniline, Et₃N (iv) 4-Fluorobenzylamine, Et₃N, Dioxane, microwave, 125 °C (v) 4-Fluorobenzylamine, H₂O:EtOH (1.5:1), 25°C (vi) LiOH, MeOH (vii) Aniline, HATU, DMF, Et₃N (viii) 4-Fluorobenzaldehyde, NaBH₄, EtOH (ix) MeI, K₂CO₃, DMF.

Synthesis of compounds **22-26** (Table 2) with changes in the central core ring are summarized in scheme 2. Compound **22** was synthesized by nucleophilic displacement of **59** with 1-(4-fluorophenyl)-*N*-methylmethanamine followed by carbamoylation according to the procedure described by Ren et al.²⁴ Compound **23** was synthesized in a similar manner except the initial nucleophilic displacement step of **60** resulted in a mixture of the intended 4-chloro-*N*-(4-fluorobenzyl)-*N*-methylpyrimidin-2-amine (**61**) as well as the 2-chloro regioisomer in a 1:2 ratio.

The pyridazine core replacement analogue was synthesized by heating 6-oxo-1,6-dihydropyridazine-4-carboxylic acid with POCl_3 followed by quenching of the 6-chloropyridazine-4-carbonyl chloride intermediate with aniline to afford **63**. Intermediate **63** was subsequently subjected to nucleophilic displacement with 1-(4-fluorophenyl)-*N*-methylmethanamine under microwave heating to deliver the final compound **24**. The pyridone analogue (**25**) was synthesized from the commercially available precursor, methyl coumalate (**64**), which was reacted with 4-fluorobenzylamine at room temperature (25 °C) to give the *N*-substituted pyridinone-ester (**65**). The ester was then hydrolyzed using LiOH followed by an amide coupling to afford **25**. The thiazole analogue **26** was synthesized by reductive amination of methyl 2-aminothiazole-4-carboxylate **66** with 4-fluorobenzaldehyde and NaBH_4 , followed by methylation under basic conditions to afford **67**. Intermediate **67** was then subjected to the same hydrolysis and amide coupling conditions as **65**, to afford **26**.

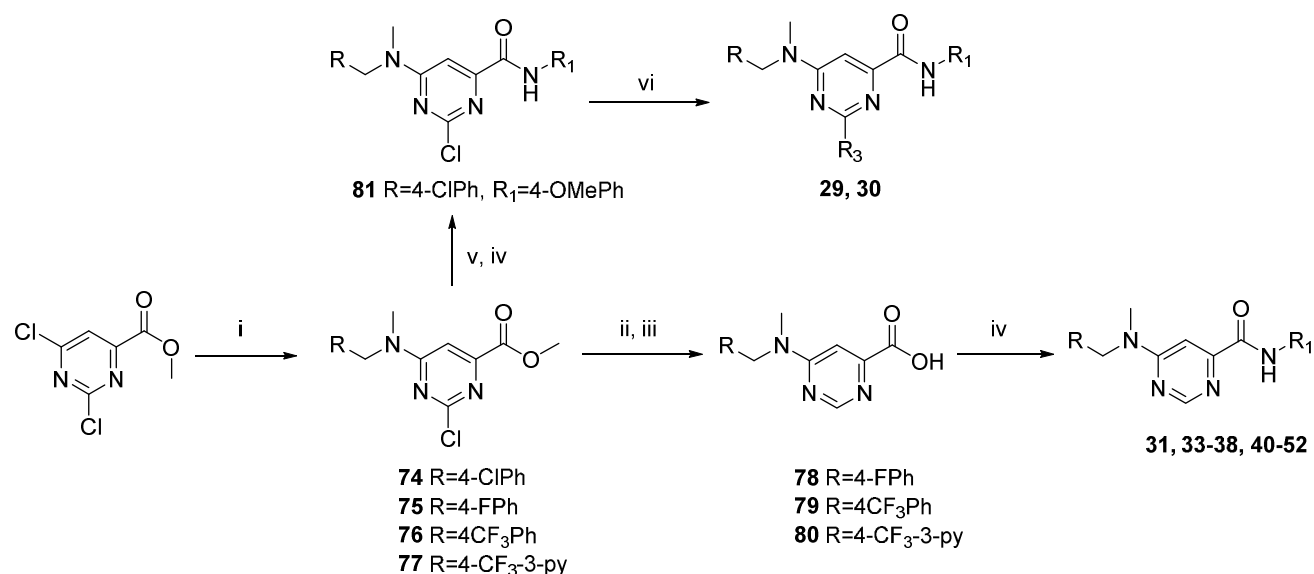


(x) a) KOH, EtOH, b) HCl, (ii) SOCl_2 , DMF (iii) 1-(4-fluorophenyl)-*N*-methylmethanamine, K_2CO_3 , DMF (iv) LiOH, $\text{H}_2\text{O}:\text{THF}$ (2:1), 25 °C (v) HATU, DIPEA, DMF, 25 °C (vi) $(\text{COCl})_2$, EtOAc, Aniline (vii) 1-(4-Chlorophenyl)-*N*-methylmethanamine, *i*PrOH, DIPEA, 100 °C.

The 2- and 5-methylpyrimidine analogues **27** and **28** (Table 2) were synthesized in a similar fashion by condensation of the appropriate 1,4-diethyl ester and imidamide **68** and **69** to form the corresponding dihydropyrimidine carboxylic ester and acid intermediates, **70** and **71**,

respectively. Intermediate **70** was chlorinated using SOCl_2 followed by nucleophilic displacement of the 6-chloro with 1-(4-fluorophenyl)-*N*-methylethanamine delivered **72**, which was then hydrolyzed under standard basic conditions followed by an amide coupling with HATU to produce **27**. Intermediate **71** was subjected to chlorination of both the hydroxyl and carboxylic acid groups using oxalyl chloride. The reaction was then quenched with aniline to afford **73** which was subsequently aminated with 1-(4-chlorophenyl)-*N*-methylethanamine to afford **28**.

Scheme 3. General route for the synthesis of 6-Amino-pyrimidine-4-carboxamides as well as selected core modifications.

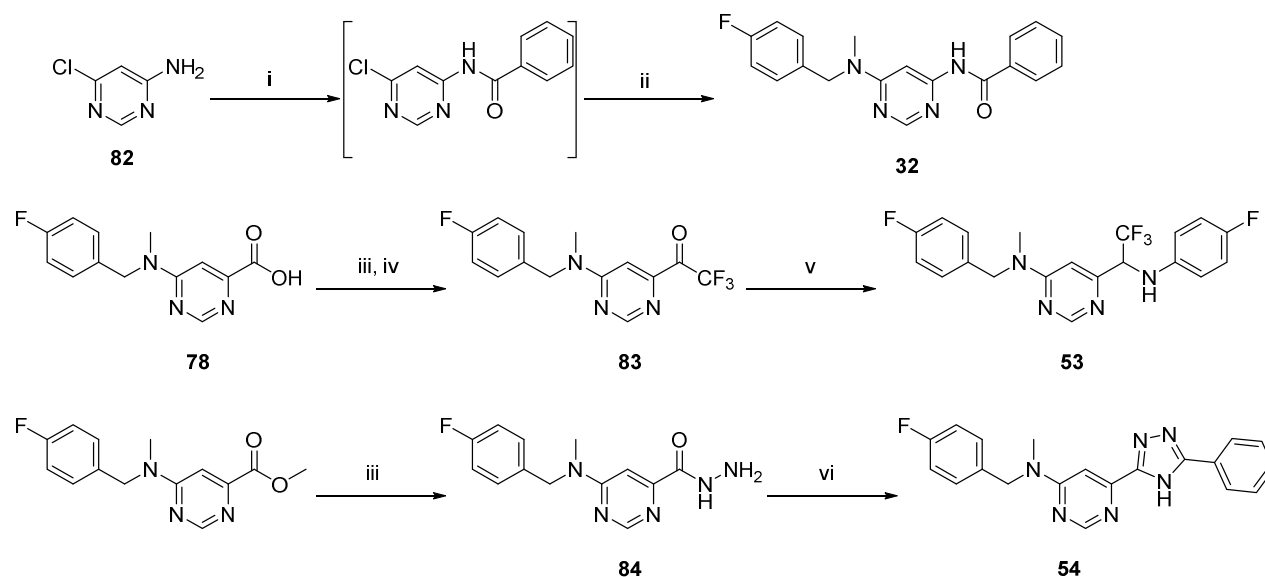


Reagents and conditions: (i) $\text{R-CH}_2\text{NHCH}_3$, THF, 0 °C (ii) 10% Pd/C, H_2 , MeOH (iii) LiOH, MeOH (iv) Amine, DIPEA, HATU, DMF (v) LiOH, THF:H₂O (1:1) (vi) NH_3 or cyclopropylamine, *i*-PrOH, Et_3N , 100 °C

In order to access an advanced intermediate that would allow for exploration of the RHS of the molecule (**31**, **33-38**, **40-52**), another relatively straightforward approach (Scheme 3), involving 4 steps from the commercially available methyl 2,4-dichloropyrimidine-6-carboxylate was used.

Briefly, quantitative amination by selective displacement of the 6-chloro group using an appropriate *N*-methyl-1-phenylmethanamine at 0 °C in THF, followed by hydrogenation with 10% Pd/C and subsequent hydrolysis using LiOH in methanol afforded the carboxylic acid (**78-80**). Lastly, amide coupling with appropriate amines using HATU gave the desired carboxamide final compound. The intermediate as represented by **74** could be utilized to access 2-amino substituted analogues such as **29** and **30**. Intermediate **74** was hydrolyzed using LiOH in THF/MeOH (1:1) followed by amide coupling with HATU, and substitution of the 2-chloro group with an appropriate amine through heating in 2-propanol in the presence of triethylamine in a sealed tube, affording compounds **29** and **30**.

Scheme 4. Synthetic schemes used to access reverse amide and amide isosteres.



Reagents and conditions: (i) BzCl, DIPEA, THF, RT-50 °C (ii) 1-(4-Fluorophenyl)-*N*-methylmethanamine, *i*-PrOH, Et₃N, 100 °C (iii) HATU, Et₃N, *N,O*-dimethylhydroxylamine, DMF, RT (iv) a) TMS-CF₃, Toluene, 20% CsF b) TBAF, H₂O, 50 °C (v) 4-Fluoroaniline, NaCNBH₃, Et₃N, TiCl₄, MeOH, DCM (50:50), N₂ (vi) Benzonitrile, K₂CO₃, BuOH, 120 °C.

The reverse amide **32** was synthesized by reacting 6-chloropyrimidin-4-amine, **82** with benzoyl chloride in the presence of a base followed by nucleophilic substitution of the 6-chloro with 1-(4-

fluorophenyl)-*N*-methylethanamine (Scheme 4). The trifluoromethylketone, **83**, was synthesized from the free acid, **78**, via formation of a Weinreb amide followed by trifluoromethylation using TMS-CF₃.²⁵ Reductive amination using NaCNBH₃ in the presence of TiCl₄ then afforded compound **53**. Finally, triazolyl compound **54** was synthesized by heating the hydrazide **84** with benzonitrile in refluxing butanol in the presence of potassium carbonate.

SAR at LHS. After ascertaining that the 4-methoxy substituent of the phenyl carboxamide was not crucial for activity (Table 1; compound **2**; R₁ = H) the LHS (Table 1) of the scaffold was varied to identify the key pharmacophore needed for activity whilst keeping the central pyrimidine core unchanged and the RHS constant as 4-methoxyphenyl or phenyl.

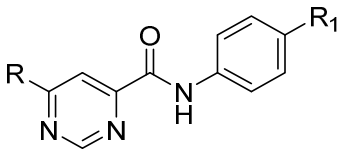
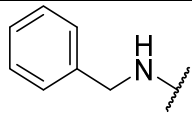
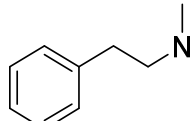
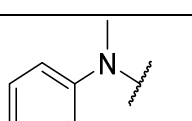
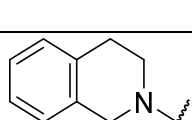
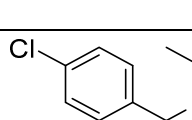
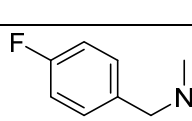
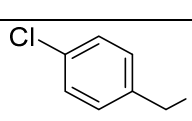
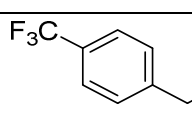
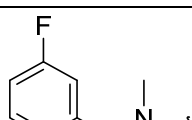
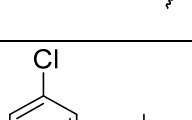
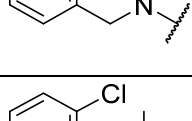
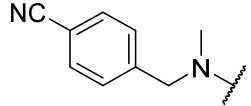
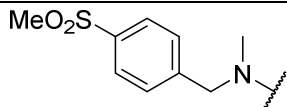
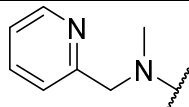
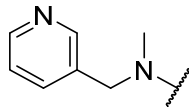
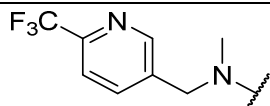


Table 1: SAR on the LHS

Compound	R	R ₁	MIC ₉₉ 7H9/A DC (μM)	MIC ₉₉ GAST/Fe (μM)	Solubility (μM) ^a	Cytotoxicity (μM) ^b
1		OMe	20	10	<5	287
2		H	20	10	<5	314
3		H	>50	40	ND	178
4		H	>160	ND	ND	ND

5		H	>160	ND	<5	ND
6		H	80	ND	<5	ND
7		H	>160	ND	<5	ND
8		H	>160	>160	<5	ND
9		H	>50	40	5	272
10		H	6.25	1.56	<5	297
11		H	5	2.3	<5	207
12		H	5	5	<5	258
13		OMe	37	5	5	272
14		H	20	10	<5	ND
15		H	>160	ND	<5	ND

16		H	>50	20	<5	ND
17		H	>125 ^c	>125 ^c	10	ND
18		H	>160	ND	ND	ND
19		H	48 ^c	37 ^c	150	ND
20		H	37	10	<5	258

^aKinetic solubility at pH 7.4; ^bIC₅₀ on Vero cell lines; ^cMIC₉₉ from GFP strain based assay; ND – Not determined

Replacement of the aryl ring with a saturated cyclohexyl ring (compound **3**) led to considerable loss of activity, suggesting essentiality of an aromatic ring on the LHS. Similarly, replacement of the *N*-CH₃ linker with “O” or “NH” led to inactive compounds **4** and **5** respectively, suggesting the critical importance of a tertiary nitrogen at the linker position or intolerance toward more polar “O” or “NH” groups. Furthermore, linker modifications such as addition of a methylene group (*N*-methylphenethyl, **6**) or removal of CH₂ (*N*-methylaniline, **7**), cyclization of the *N* onto the phenyl ring as tetrahydroisoquinoline moiety (**8**) or extension of *N*-methyl to *N*-ethyl (**9**), all led to loss or deterioration of MICs. This all indicated stringent shape requirements at this presumably hydrophobic part of the molecule.

Realizing the essential nature of the aryl ring and the linker, we focused efforts on exploring substitution on the phenyl ring to improve potency and introduce polarity. *Para*-halogenated compounds, **10** (4-F), **11** (4-Cl) and **12** (4-CF₃) were at least ≥ 4 -fold and ≥ 2 -fold more potent in 7H9/ADC and GAST/Fe, respectively, compared to **1**. This was encouraging and the first sign of a SAR trend emerging, and leading to the conclusion that a *para*-halogenated phenyl ring gave the best activity with 4-F phenyl compound **10** showing the most promise. Compounds with 3-F (**13**) and 3-Cl (**14**) were less potent compared to corresponding 4-halogenated analogues whereas the 2-Cl analogue (**15**) was inactive up to the highest concentration tested, once again suggesting stringent shape requirements on the LHS. Addition of more polar groups such as 4-CN (**16**) and 4-SO₂Me (**17**) led to deterioration or loss of MICs.

Up to this point, all compounds tested had poor aqueous solubility of ≤ 5 μ M. In an attempt to improve solubility by introducing polarity, the phenyl ring was replaced with a pyridyl ring. Compound **18** with a 2-pyridyl ring was found to be inactive whereas compound **19** with a 3-pyridyl ring was equipotent to compound **1** albeit with a much improved aqueous solubility of 150 μ M at pH 7.4. Further substitution of the 3-pyridyl ring as in compound **20** with a 3-pyridyl-4-trifluoromethyl group retained similar activity, but now with loss of solubility albeit this substitution improved the *in vivo* pharmacokinetic properties as will be discussed in the DMPK section.

SAR at the central core: Exploration of the SAR around the central core of the scaffold was aimed at understanding whether or not the 4,6-disubstituted pyrimidine was essential for activity whilst retaining a 4-halo group on the LHS phenyl ring and a 4-methoxy, halogen or hydrogen on the RHS phenyl ring. Initial efforts looked at shuffling the nitrogen atoms around the core ring, including the pyridine (**21**), pyrazine (**22**), and pyrimidine (**23**), with one of the nitrogen

atoms shifted between the aminobenzyl and carboxamide groups, and the pyridazine (**24**), analogues. Only the pyridine retained partial activity at 10 μ M in the GAST/Fe medium, which was not sufficient to warrant a scaffold hop. Compounds based on the pyrimidinone isostere (**25**), and thiazole (**26**) all lost activity. Lastly 5-, and 2-substitutions (**27**, **28**, **29** and **30**) on the pyrimidine core were evaluated and found to be similarly inactive. It was thus concluded that the original pyrimidine core was optimal for activity and could not be replaced with other diazine and heteroaromatic rings.

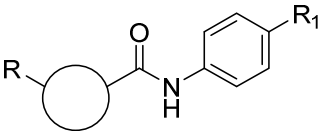
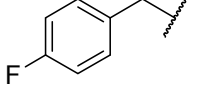
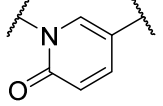
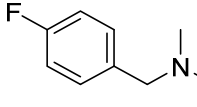
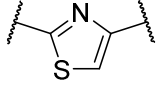
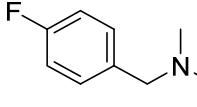
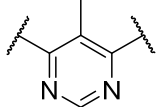
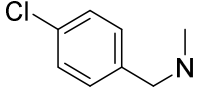
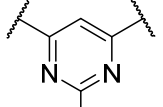
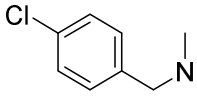
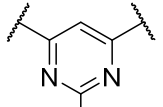
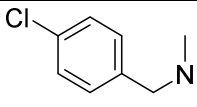
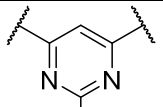


Table 2: SAR on the central core

Compound	R	R ₁	Core change	MIC ₉₉ 7H9/ADC (μ M)	MIC ₉₉ GAST/Fe (μ M)	Solubility (μ M) ^a	Cytotoxicity (μ M) ^b
21		H		ND	10	<5	298
22		F		>50	>50	<5	282
23		OMe		>50	>50	<5	273
24		H		>50	>50	40	279

25		H		>50	>50	170	310
26		H		>50	>50	<5	279
27		F		>50	37	<5	271
28		H		>160	>160	ND	ND
29		OMe		>50	>160	<5	ND
30		OMe		>50	>160	5	ND

^aKinetic solubility at pH 7.4; ^bIC₅₀ on Vero cell lines; ND – Not determined

SAR on the RHS: An extensive SAR exploration on the RHS was undertaken in order to evaluate the scope for optimization of potency and physicochemical properties (Table 3). The free amide NH was deemed critical for potency as *N*-methylation (**31**) led to a complete loss in activity. Similarly, reversal of the amide functionality led to inactive compound **32**. For further exploration of substitution on the aryl ring of the amide, a 4-halophenyl or 4-CF₃-3-pyridyl was fixed on the LHS of the scaffold. Replacement of the phenyl ring with an aliphatic chain such as isopropyl (**33**) was detrimental to activity, while replacement with a saturated cyclohexyl ring

(**34**) resulted in retention of activity in the GAST/Fe medium albeit it was completely inactive in 7H9/ADC. The overall trend suggested a preference for an aryl ring on the RHS. Since the original hit contained a methoxy group, additional ethers were explored in the *para* position, including isopropoxy (**36**), 2-methoxyethoxy (**37**), and a *N,N*-dimethyl-2-ethoxy ether (**38**). Encouragingly, compounds **36** and **37**, were some of the most active compounds synthesized up to this point, but were poorly soluble (solubility pH 7.4 ≤ 5 μ M). Compound **38** with the basic dimethylaminoethoxy side-chain (predicted pKa 8.77) was less active (7H9/ADC MIC 25 μ M) even though it showed considerably improved aqueous solubility of 155 μ M. Nonetheless, these results suggested scope for substitutions on the aryl ring to modulate physicochemical properties whilst retaining potency. Replacement of the phenyl ring with a 4-pyridyl ring, as in compound **39**, was tolerated indicating a wider scope for SAR exploration at this position with heterocycles and weakly basic substituents. Addition of a less basic *N*-methylpiperazine ring at the 4-position of the phenyl ring (predicted pKa 7.85) led to potent compound **40** which was poorly soluble at pH 7.4 but sufficiently soluble at a slightly acidic pH of 6.5 (refer DMPK section). Solubility could be improved further by combining *N*-methylpiperazinylphenylamide on the RHS with a 4-CF₃-3-pyridyl group on the LHS as in compound **41** with some loss in potency (MIC 12.5 μ M, solubility pH 7.4 165 μ M). Moving the *N*-methylpiperazine ring to the 3-position of the phenyl ring (**42**) was detrimental to activity with MIC values dropping by 10-30 fold. Similarly, 3-pyridyl-4-(*N*-methylpiperazinyl) analogue **43** was found to be much less potent (7H9/ADC MIC >50 μ M; GAST/Fe MIC 19 μ M). Compounds with neutral substituents at the 4-position of the phenyl ring like *N*-acetylpiperazinyl (**44**) or, morpholino (compound **45**), retained good MICs (<5 μ M) but were much less soluble. Interestingly, the 4-morpholino-2-pyridyl analogue (**46**) retained good MIC activity (2-3 μ M) indicating scope for the introduction of a nitrogen into the

phenyl ring to address potential reactivity issues of electron-rich anilines during further optimization.

A small set of amides with 5-membered heterocycles was synthesized to evaluate the scope for further SAR exploration at this position. Compounds with *N*-methylpyrazole (**47**), *N*-methyltriazolyl (**48**) and isoxazolyl (**49**) substituents were either inactive or weakly active. Compound **50** containing a 4-methylthiazolyl group showed a GAST MIC equivalent to its phenyl counterpart (5 μ M). However, its MIC in 7H9/ADC medium (37 μ M) was weaker and had a poor solubility (<5 μ M). Similarly, compounds containing 1,3,4-thiadiazole moieties (**51** and **52**) showed reasonable MIC in GAST/Fe medium (~5 μ M), but had weaker potency in 7H9/ADC medium (12.5 and 50 μ M respectively). Unfortunately compounds with these moieties were cytotoxic *in vitro* and were also found to be toxic at higher doses in animal studies. Hence, these modifications were abandoned even though more lead-like DMPK properties were achieved with these compounds.

Lastly, two amide isosteres CF₃-amine (**53**)²⁶ and triazole (**54**)²⁷ were synthesized in an attempt to explore the scope for variation and in line with metabolite identification studies (supplementary information Table S1). Metabolite identification, performed on **12** and **51**, indicated cleavage of the amide bond on the RHS as a primary route of metabolism compared to *N*-demethylation on the LHS. Even though both these modifications resulted in compounds showing some activity, there was no improvement in other properties like solubility and hence, were not explored further. Overall, as summarized in Figure 4, the SAR around the LHS and the central core was narrow with fewer modifications tolerated; SAR around the RHS was more

dynamic allowing scope for further optimization. From this, several compounds were identified for further profiling both *in vitro* and *in vivo*.

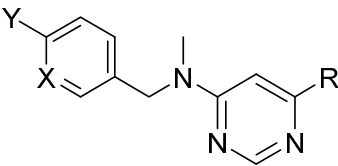
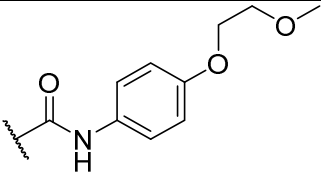
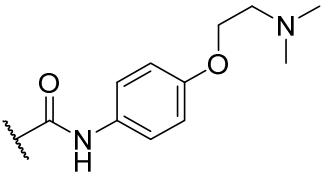
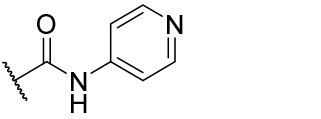
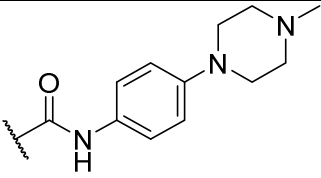
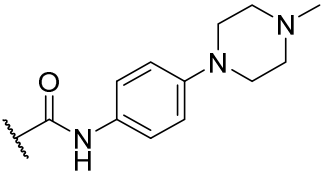
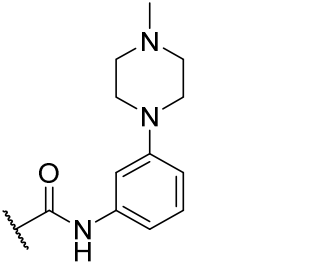
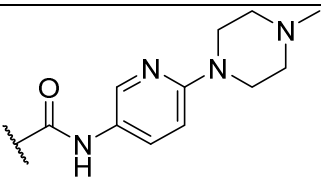
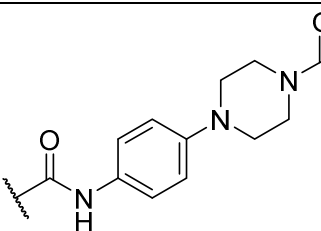
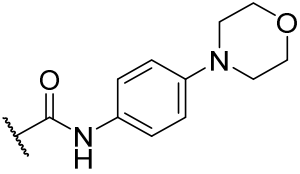
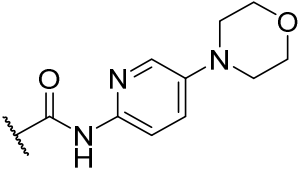
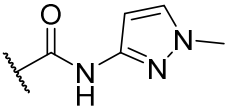
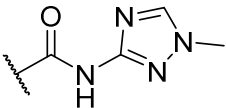
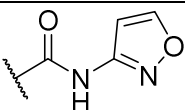
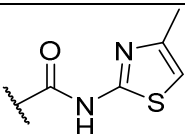
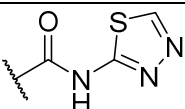
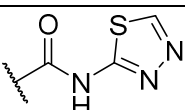
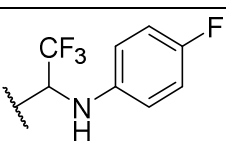
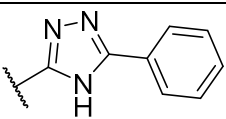


Table 3: SAR on the RHS (R)

Compound	Y	X	R	MIC ₉₉ 7H9/ ADC (μM)	MIC ₉₉ GAST/Fe (μM)	Solubility (μM) ^a	Cytotoxicity (μM) ^b
31	H	C		>160	ND	ND	ND
32	F	C		N/A	>160	5	ND
33	CF ₃	C		>50	>160	13	217
34	CF ₃	C		>125 ^c	12 ^c	5	ND
35	F	C		5	2.5	62	60.48
36	F	C		5	1.2	5	254*

37	F	C		2.3	1.56	<5	244
38	F	C		25	12.5	155	ND
39	Cl	C		ND	10	<5	282
40	CF ₃	C		3.13	1.25	<5	161
41	CF ₃	N		12.5	12.5	165	45.2
42	F	C		37	37	74	118
43	F	C		>50	19	150	126
44	CF ₃	N		4.7	2.3	<5	>50

45	F	C		0.78	0.60	<5	237
46	CF ₃	N		3.13	2.3	<5	211
47	F	C		>50	40	90	ND
48	CF ₃	C		50	10	173	28
49	F	C		>50	160	ND	ND
50	F	C		37	5	<5	ND
51	F	C		12.5	4.7	128	21.9
52	CF ₃	N		50	5	191	28
53	F	C		37	19	<5	41.7
54	F	C		>50	25	<5	277

^aKinetic solubility at pH 7.4; ^bIC₅₀ on Vero cell lines; ^cMIC₉₉ from Mtb-GFP strain based assay
ND – Not determined

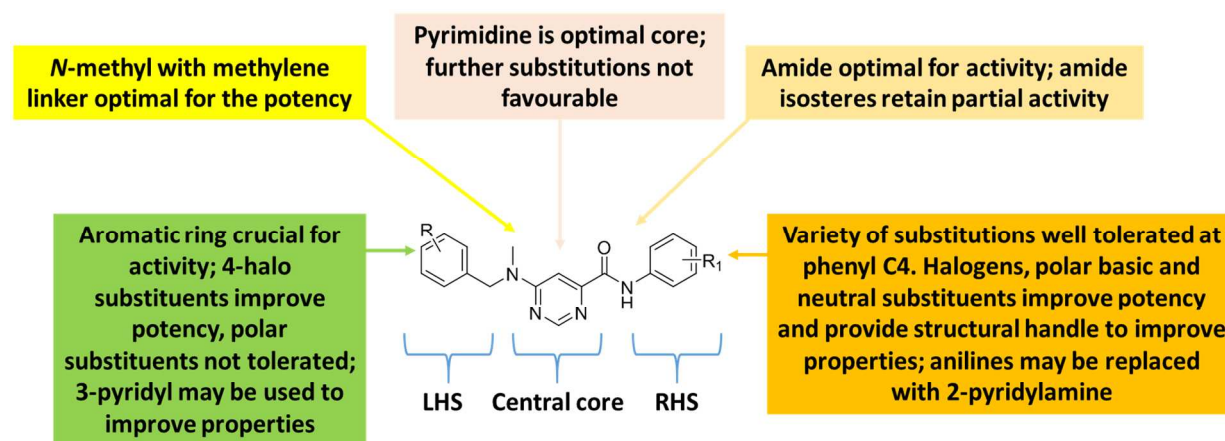


Figure 4: Summary of SAR

Biology profiling. A spontaneous resistant mutant of *Mtb* H37Rv was successfully raised against compound **13**. Most of the analogues from the series were cross-resistant against this mutant except the thiadiazole analogues **50** and **51**, indicating additional targets and/or a different MOA (Table 4). Additionally, these mutants were not cross-resistant to any known TB drugs suggesting a potential novel MOA for the series. Unfortunately, whole genome sequencing of the mutant did not reveal any genetic polymorphism(s) suggestive of the target or MOA. Hence, further studies using different target identification approaches were explored in order to identify the molecular target of these compounds. Representative compounds were screened against drug-sensitive clinical isolates and were found to retain MICs similar to those in H37Rv (Table 5).

Table 4: Cross-screening against **13**-resistant mutant

	14 day GAST/Fe MIC ₉₉ (μM) against WT H37Rv (μM)	14 day GAST/Fe MIC ₉₉ (μM) Against 13-resistant mutant (μM)
1	20	>160
10	10	>160
13	2.5-5	>160
20	10	>160
36	1.25-5	>160
51	5	10
Rif	0.012	0.012
INH	0.18	0.36
STREP	1.62	1.62
ETB	2.3	2.3
LEVO	0.64	1.3
KAN	0.32	0.32

Rif: Rifampicin; INH: Isoniazid; STREP: Streptomycin; ETB: Ethambutol; LEVO: Levofloxacin; KAN: Kanamycin

Table 5: Activity against clinical isolates

Compound	MIC on Reference strain μM ^a	MICs against Clinical Isolates μM ^a					
		SAWC1125	SAWC3933	SAWC3385	SAWC3200	SAWC3388	SAWC3906
36	1.25	2.5	2.5	0.31	<0.08	0.31	0.31
40	1.25-2.5	2.5-5	1.25 - 2.5	1.25	1.25	1.25 - 2.5	0.31-0.63
51	2.5	<0.08	0.625	<0.08	<0.08	0.15	<0.08
52	50	2.5	10-20	10	2.5-5	10	2.5-5

^aMIC₉₀ readout from MABA assay in Middlebrook 7H9 Medium; SAWC 1125 -Typical Beijing (lng 2.2); SAWC 3933 –T1 (lng 4.2); SAWC 3385 (Cas 1/Delhi (lng 3); SAWC - 3200 - X1 (lng 4.9); SAWC 3388 - T1, Tuscany, T5/Rus (lng4.3); SAWC 3906 -Atypic Beijing (lng 2)

Chemoproteomics. In order to supplement our MOA identification efforts, we used a chemoproteomics approach to identify the potential target proteins of the 6-dialkylaminopyrimidine carboxamides. This affinity-based approach was recently successfully employed for the direct target identification of antitubercular compounds.^{28–30} This approach is based on the modification of the compound **40** with a linker for covalent immobilization on sepharose-beads by *N*-hydroxysuccinamide (NHS) coupling. The modified compound **85** (Figure 5, synthesis scheme S1) retained antimycobacterial activity (MIC₉₀ 18.6 μ M as determined against *M. bovis* BCG) suggesting that the derivatization with the linker moiety did not interfere with target binding. The **85**-beads were incubated with *Mycobacterium bovis* BCG extract and proteins captured by the beads were digested with trypsin, labeled with isobaric mass tags (TMT 10plex), and quantitatively identified by liquid-chromatography-tandem mass spectrometry (LC-MS/MS). In order to distinguish true targets from nonspecific background binding, the bacterial extracts were incubated prior to the bead-incubation step with either the active test compound **40**, the inactive test compound **25**, or with a vehicle (DMSO only) control. Only the active compound should bind to its target proteins in the lysate and thus reduce the binding of these proteins to the beads, but not the inactive compound. Indeed, two proteins, BCG_3193 (Rv3169) and BCG_3827 (Rv3768), showed reduced binding to the compound-beads in the presence of competing “free” **40** (Fig. 3A), but not with inactive **25**, which suggests that these proteins are the potential targets of **40**. In order to estimate inhibitor potency, we performed the affinity-step in the presence of different concentrations of active compound **40** in the protein extract, which allowed the determination of an apparent dissociation constant K_d^{app} (Fig. 3C). The K_d^{app} takes

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2
3 into consideration the affinity of the target for the bead-immobilized ligand. The latter effect can
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5 be deduced by measuring the depletion of the target protein by the beads.³¹ The apparent
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7 dissociation constant of compound **40** for BCG_3193 was determined in two separate
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9 experiments as 0.61 μ M and 0.69 μ M and for BCG_3827 as 2.1 μ M and 3.8 μ M (Fig. 3C). Both
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11 potential novel target proteins, BCG_3193/Rv3169 and BCG_3827/Rv3768 (contains a
12
13 Polyketide cyclase SnoaL-like domain, IPR009959), are conserved hypothetical proteins of
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15 unknown function. Both proteins are non-essential but notably found using Triton X-114
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17 detergent-phase separation for extraction of lipophilic proteins, suggesting they are associated
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19 with the cell envelope despite lacking detectable trans-membrane helices.³²
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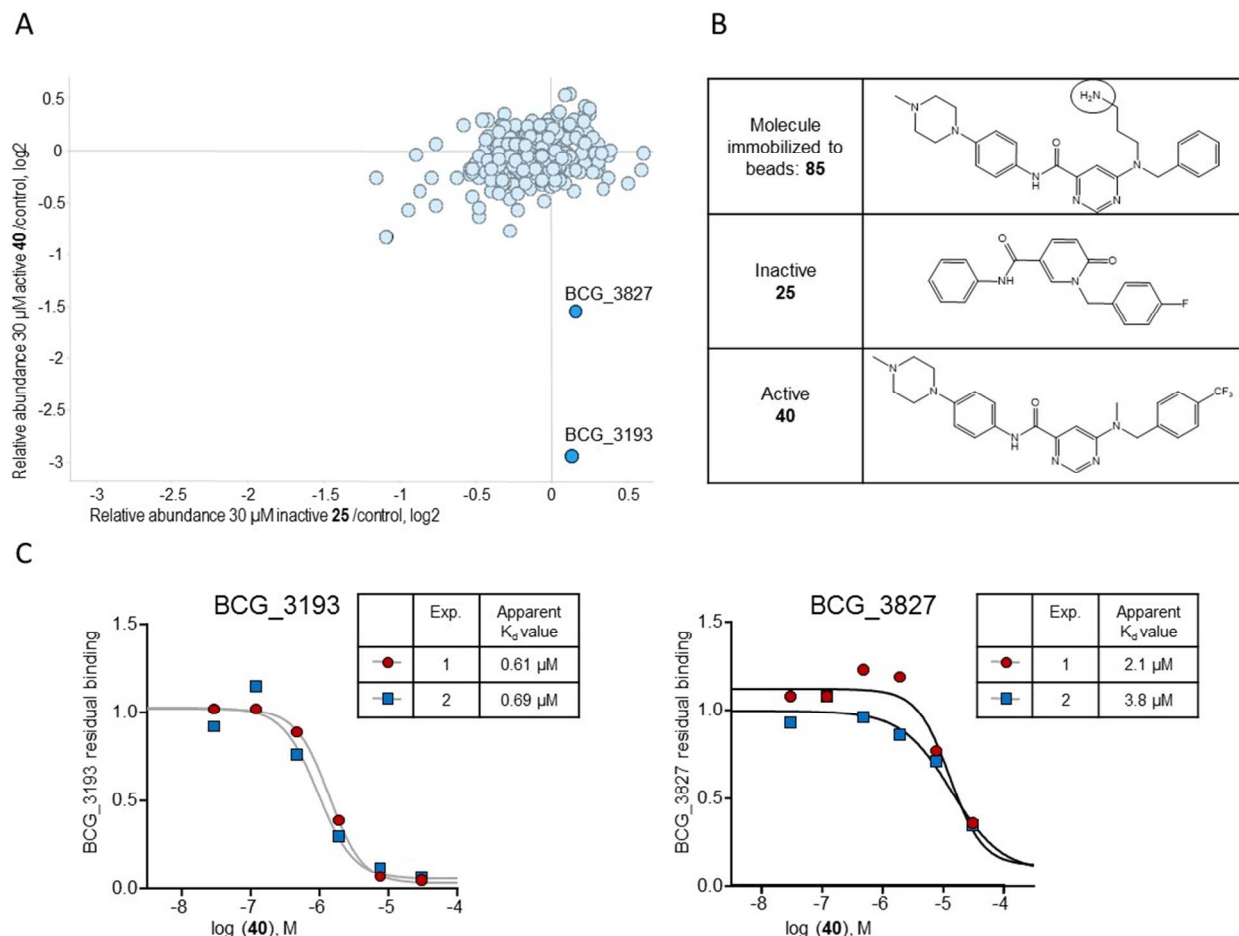


Figure 5: Affinity chemoproteomics revealed BCG_3193 and BCG_3827, novel TB targets with unknown function, as targets of the 6-dialkylaminopyrimidine carboxamides. **A)** BCG_3193 and BCG_3827 show reduced binding to 6-dialkylaminopyrimidine carboxamide analogue-beads due to compound **40** binding, but no effect on these proteins was seen by the inactive compound. Shown are relative protein amounts captured by the beads in a log₂ scale. **B)** The apparent dissociation constant of compound **40** for BCG_3193 was determined in two different experiments as 0.61 μ M and 0.69 μ M and for BCG_3827 as 2.1 μ M and 3.8 μ M. **C)** Compound structures used for the affinity chemoproteomics experiments

Physicochemical, DMPK and hERG profiles. Representative compounds were profiled for physicochemical properties and *in vitro* DMPK properties which included kinetic solubility, human plasma protein binding (PPB), microsomal metabolic stability (MS) and permeability, as shown in Table 6.

Table 6: In vitro ADME properties, hERG activity and caseum distribution

Compound	12	20	36	40	51	52
cLogP ^a	5.0	3.7	5.0	5.0	2.0	1.4
FassiF solubility pH 6.5 (μM) ^b	5	<5	<5	150	<5	195
Solubility pH 7.4 (μM) ^c	<5	<5	5	<5	128	191
PPB Human <i>fu</i> ^d	0.11	0.05	0.04	0.14	0.01	0.32
PAMPA LogP _{app} ^e	-4.9	-3.8	ND	-4.5	ND	-3.9
MS H/R/M % remaining after 30 mins ^f	55/87/60	85/72/87	64/43/37	57/37/21	67/65/74	100/88/78
hERG IC ₅₀ (μM) ^g	3.3	5.6	4.1	0.9	4.2	14
Caseum distribution Unbound (%) ^h	0.1	ND	ND	0.1	0.5	3.6

^acalculated logP (StarDrop); ^bfasted-state simulated intestinal fluid, pH 6.5; ^cKinetic solubility; ^dPlasma proteing binding *fu* - fraction unbound; ^eParallel Artificial Membrane Permeability Assay; ^fmetabolic stability H: human, R: rat M: mice; ^gTested at Essen biosciences using IonWorks patch clamp electrophysiology; ^hassay using surrogate caseum; ND: not determined

As discussed previously, most of the compounds based on phenyl amides had poor to moderate solubility at pH 7.4 except those with basic solubilizing side chains (e.g. compounds **38** and **41**). These compounds showed considerably improved solubility in slightly acidic Fassif medium (fasted-state simulated intestinal fluid, pH 6.5)³³ e.g. compound **40** (kinetic solubility pH7.4 <5 μM; K. solubility Fassif 150 μM). This may be relevant for oral absorption of these compounds. Compounds containing heterocyclic amides like thiadiazole compounds **51** and **52** had good solubility >100 μM in both media. In general, compounds in the series had 5-10% free fraction in human plasma as measured in a PPB assay while some more polar compounds like **52** had upto 30% free fraction in human plasma. The ability of compounds to cross biological membranes by passive diffusion was assessed using the PAMPA assay.³⁴ All compounds tested showed good to moderate permeability with values ranging between -3.8 and -4.9 LogP_{app}

presumably due to their lipophilic character. Caseum binding was also assessed for certain compounds in order to give some insight into the ability of the compounds to permeate the caseous necrotic regions of granulomas to access the bacteria.³⁵ Compound **52** had the highest free fraction (3.6% unbound) from the compounds assessed with phenyl compounds generally being highly bound (0.06 – 0.1 % unbound). Further safety profiling was conducted against the human ether-a-go-go-related gene (hERG) potassium channel, and compounds were found to be low μM inhibitors. While hERG activity is a well-established safety concern³⁶, compounds were not optimized to remove hERG activity due to the initial focus being on increasing potency and solubility.

Table 7: Pharmacokinetic parameters in male C57/BL6 mouse blood

	12		20		40		51		52	
Dose (mg/kg)	Oral 20	IV 5	Oral 20	IV 5	Oral 20	IV 5	Oral 20	IV 5	Oral 20	IV 5
Apparent $t_{1/2}$ (h)	8.7	4.6	7.2	0.7	15.8	10.3	4.4	5.1	3.0	3.0
CL _{total} (mL/min/kg)	—	11.3	—	11.2	—	26.3	—	11.1	—	6.6
V _d (L/kg)	—	4.3	—	0.7	—	21.8	—	4.8	—	1.7
C _{max} (μM)	2.2	—	3.4		0.42		2.58		10.8	
T _{max} (h)	1	—	4		5		1		1	
AUC _{0–∞} (min· μM)	1334	1224	1999	1179	564	540	1022	1329	5614	2106
oral bioavailability (%)	28	—	42	—	26	—	19	—	67	—

IV: intravenous; $T_{1/2}$: elimination half-life; CL: plasma clearance; V_d: volume of distribution; C_{max}, maximum (peak) plasma concentration following oral administration; AUC, area under the curve

In general, compounds showed moderate metabolism in human, mouse as well as rat liver microsomes except compound **40**, which showed rapid *in vitro* clearance. The compounds were also tested in male C57/BL6 mice for pharmacokinetic parameters at an intravenous dose of 2 mg/kg and an oral dose of 20 mg/kg (Table 7). Most of the compounds showed low *in vivo* clearance in mice in accordance with their *in vitro* microsomal metabolic stability. Compound **40** showed low clearance IV clearance despite its poor microsomal stability, probably due to its extensive tissue distribution as indicated by high volume of distribution ($V_d = 21.8 \text{ L/Kg}$). Most of the compounds had moderate to good oral bioavailability. Moderate permeability was sufficient to give good oral absorption as well as moderate bioavailability for compounds with low solubility (**12** and **20**). Compound, **52** with a thiadiazole group at LHS showed improved physicochemical and ADME parameters such as good solubility, improved microsomal stability leading to higher oral absorption, higher AUC and better bioavailability of 67%. Unfortunately, this compound was found to be toxic to mice when dosed orally above 50 mg/kg and hence was not progressed any further.

CONCLUSION

Screening of a Biofocus library led to the identification of 6-dialkylaminopyrimidine carboxamides with potent anti-tubercular properties and a novel mode of action. A biology triage cascade allowed deprioritization of compounds potentially acting through inhibition of QcrB, cell-wall targets and DNA damage. Cross-resistance studies of TB drugs against the resistant mutant raised against this series indicates a potentially novel MOA for the series, and this was reinforced by chemoproteomic studies albeit the exact target (and MOA) remains unknown. Detailed SAR studies have demonstrated limitations as well as scope for further optimization of

these series to deliver lead-like compounds with potent anti-TB properties and oral bioavailability.

EXPERIMENTAL

Biology. Biology triage assays were carried out as described in reference²³

DMPK: All protocols for *in vitro* DMPK studies and mouse PK studies are available in the supplementary document. Animal studies were conducted following guidelines and policies as stipulated in the UCT Research Ethics Code for Use of Animals in Research and Teaching, after review and approval of the experimental protocol by the UCT Senate Animal Ethics Committee (protocol FHS-AEC 013/032)

Chemoproteomics. Chemoproteomics experiments were performed as previously described³¹. Briefly, sepharose beads were derivatized with compound **85** at a concentration of 0.04 mM, and subsequently washed and equilibrated in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.4 % Igepal-CA630, 1.5 mM MgCl₂, 5 % Glycerol, 150 mM NaCl, 25 mM NaF, 1 mM Na₃VO₄, 1 mM DTT, and one Complete EDTA-free protease inhibitor tablet (Roche) *per* 25 mL). The **85**-beads were incubated at 4°C for 1 h with 0.1 mL (0.25 mg) *M. bovis* BCG extract, which was pre-incubated with compound or DMSO (vehicle control). Our experimental design employed isobaric tandem mass tags (TMT)³⁷ which allowed us to analyze 10 samples together by LC-MS/MS enabling the relative quantification of proteins across these samples. The experiments were configured to generate values for the affinity of the beads to the bound proteins (“depletion” values, 4 samples) and to allow determination of IC₅₀ values (6 samples) in a single experiment. Samples 1 and 2 were vehicle control duplicates, samples 3 and 4 were duplicates of the “rebinding” experiment and samples 5-10 served to generate IC₅₀ values by adding compound over a range of 5

concentrations (highest concentration 30 μ M, then 4 dilution steps of 1:4 each). In the “rebinding” experiment, the nonbound fraction from the first bead incubation step was incubated again with “fresh” beads, allowing the determination of target depletion by the beads. Apparent dissociation constants were determined by taking into account the protein depletion by the beads.³¹ A repeat experiment was performed with vehicle control versus 20 μ M and 5 μ M compound. The beads were transferred to filter plates (Durapore PVDF membrane, Merck Millipore), washed extensively with lysis buffer and eluted with SDS sample buffer. Proteins were alkylated, separated on 4–12 % Bis-Tris NuPAGE (Life technologies) and stained with colloidal Coomassie. Gel lanes were cut into three slices and subjected to in-gel digest using LysC for 2 h and trypsin overnight. Digestion, labeling with TMT isobaric mass tags, peptide fractionation, and mass spectrometric analyses were performed.³¹ Proteins were quantified by isobaric mass tagging and LC-MS/MS. The proteins.fasta file for *M. bovis* BCG was downloaded (May 11th 2011) from <http://genome.tdbb.org/annotation/genome/tbdb/MultiDownloads.html> and supplemented with the sequences of bovine serum albumin, porcine trypsin and mouse, rat, sheep and dog keratins. Decoy versions of all proteins were created and added. The search database contained a total of 11,492 protein sequences, 50 % forward, 50 % reverse. Protein identification and quantification was performed.³⁷ Proteins identified with >1 unique peptide matches were considered for further data analysis.

All commercial reagents were purchased from either Sigma-Aldrich, Combi-blocks, Waterstone or Fluorochem and were used without further purification. Solvents were used as received unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed on SiO₂ plates on aluminum backing. Visualization was accomplished by UV irradiation at 254 and 220 nm. Flash column chromatography was performed using a Teledyne ISCO flash purification

system with SiO₂ 60 (particle size 0.040–0.055 mm, 230–400 mesh). Purity of all final derivatives for biological testing was confirmed to be >95% as determined 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) mass spectrometer, equipped with APCI and ESI multimode ionisation source. Using a Kinetex Core C18 2.6 µm column (50 x 3 mm); Mobile Phase B: 0.4% acetic acid, 10 mM ammonium acetate in a 9:1 ratio of HPLC grade methanol and Type 1 water, Mobile Phase A: 0.4% acetic acid in 10 mM ammonium acetate in HPLC grade (Type 1) water; with flow rate = 0.9 mL/min; detector: diode array (DAD). Or an Agilent UPLC-MS: Agilent Technologies 6150 Quadrupole, ES ionization, coupled with an Agilent Technologies 1290 Infinity II series UPLC system Agilent 1290 Series HPLC at two wavelengths 254 and 290 nm using the following condition: Kinetex 1.7µm Evo C18 100A, LC column 50x2.1mm, Solvent A: 0.1% (formic acid) Water, Solvent B: 0.1% (formic acid) Acetonitrile. The structure of the intermediates and end products was confirmed by ¹H NMR and mass spectroscopy. Proton magnetic resonance spectra were determined in an appropriate deuterated solvent on a Varian Mercury spectrometer at 300 MHz or a Varian Unity spectrometer at 400 MHz.

General protocol for synthesis of Compounds 1-21, 28). The appropriate 6-chloro-*N*-phenylpyrimidine-4-carboxamide (1eq), *N*-methyl-benzylamine (1.2eq) and DIPEA (2eq) were added to a 50 mL round-bottomed flask containing *i*-PrOH (10 mL) and heated to reflux overnight. Compounds that precipitated upon cooling were filtered and washed with cold EtOH. Those that did not precipitate; the *i*-PrOH was removed under vacuum and the residue redissolved in EtOAc (20 mL) and washed with a saturated aqueous solution of NaHCO₃, the organic layer dried, and removed under reduced pressure. The resulting solid or residue was

either recrystallized from EtOH or purified by flash column chromatography to afford the appropriate 6-(benzyl(methyl)amino)-*N*-phenylpyrimidine-4-carboxamide.

6-(Benzyl(methyl)amino)-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (1). Yield 52%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.43 (s, 1H), 8.67 (s, 1H) 7.91 – 7.71 (m, 2H), 7.46 – 7.17 (m, 6H), 7.05 – 6.80 (m, 2H), 4.92 (s, 2H), 3.75 (s, 3H), 3.17 (s, 3H). LC-MS (ESI): *m/z* 349.2 [M + H]⁺. HPLC purity 100%.

6-(Benzyl(methyl)amino)-N-phenylpyrimidine-4-carboxamide (2). Yield 98%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.49 (s, 1H), 8.66 (s, 1H), 7.85 (d, *J* = 8.0 Hz, 2H), 7.36 - 7.21 (m, 8H), 7.11 (t, *J* = 7.2 Hz, 1H), 4.90 (s, 2H) and 3.15 (s, 3H). LC-MS (ESI): *m/z* 319.2 [M + H]⁺. HPLC purity 99%.

6-((Cyclohexylmethyl)(methyl)amino)-N-phenylpyrimidine-4-carboxamide (3). Yield 12.5%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.50 (s, 1H), 8.68 (s, 1H), 7.93 (d, *J* = 8.0 Hz, 2H), 7.40 (m, 2H), 7.27 (s, 1H), 7.16 (t, *J* = 8.0 Hz, 1H), 3.54 (s, 2H), 3.13 (s, 3H), 1.86 - 1.62 (m, 6H), 1.25 – 0.96 (m, 5H). LC-MS (ESI): *m/z* 325.2 [M + H]⁺. HPLC purity 99%.

6-(Benzyloxy)-N-phenylpyrimidine-4-carboxamide (4). Yield 86%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.64 (s, 1H), 8.99 (s, 1H), 7.87 (d, *J* = 8.0 Hz, 2H), 7.51 - 7.47 (m, 3H), 7.41 - 7.33 (m, 4H), 7.21 (m, 1H), 7.13 (t, *J* = 8.0 Hz, 1H) and 5.52 (s, 2H). LC-MS (ESI): *m/z* 306.1 [M + H]⁺. HPLC purity 98%.

6-(Benzylamino)-N-phenylpyrimidine-4-carboxamide (5). Yield 85%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.43 (s, 1H), 8.57 (d, *J* = 0.8 Hz, 1H), 8.39 (t, *J* = 8.0 Hz, 1H), 7.85 (d, *J* = 8.0 Hz, 2H), 7.36 - 7.24 (m, 8H), 7.11 (t, *J* = 7.2 Hz, 1H), 4.61 (s, 2H). LC-MS (ESI): *m/z* 305.2 [M + H]⁺. HPLC purity 99%.

6-(Methyl(phenethyl)amino)-N-phenylpyrimidine-4-carboxamide (**6**). Yield 97%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.47 (s, 1H), 8.62 (s, 1H), 7.86 (d, J = 8.0 Hz, 2H), 7.39 - 7.10 (m, 9H), 3.84 (m, 2H), 3.05 (s, 3H) and 2.87 (t, J = 7.2 Hz, 2H). LC-MS (ESI): m/z 333.2 [M + H]⁺. HPLC purity 99%.

6-(Methyl(phenyl)amino)-N-phenylpyrimidine-4-carboxamide (**7**). Yield 84%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.49 (s, 1H), 8.75 (d, J = 1.2 Hz, 1H), 7.81 (dd, J = 8.4, 1.2 Hz, 2H), 7.57 - 7.30 (m, 7H), 7.10 (t, J = 8.0 Hz, 1H), 6.96 (d, J = 1.2 Hz, 1H), 3.49 (s, 3H). LC-MS (ESI): m/z 305.2 [M + H]⁺. HPLC purity 99%.

6-(3,4-Dihydroisoquinolin-2(1H)-yl)-N-phenylpyrimidine-4-carboxamide (**8**). Yield 40%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.50 (s, 1H), 8.69 (s, 1H), 7.88 (d, J = 8.0 Hz, 2H), 7.45 (s, 1H) 7.38 - 7.20 (m, 6H), 7.12 (t, J = 8.0 Hz, 1H), 4.84 (s, 2H), 3.92 (m, 2H) and 2.93 (t, J = 8.0 Hz, 2H). LC-MS (ESI): m/z 331.2 [M + H]⁺. HPLC purity 99%.

6-((4-Chlorobenzyl)(ethyl)amino)-N-phenylpyrimidine-4-carboxamide (**9**). Yield 15%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.48 (s, 1H), 8.66 (s, 1H), 7.85 (d, J = 8.0 Hz, 2H), 7.39 - 7.24 (m, 7H), 7.11 (t, J = 8.0 Hz, 1H), 4.84 (s, 2H), 3.62 (s, 2H) and 1.12 (t, J = 8.0 Hz, 3H). LC-MS (ESI): m/z 367.1 [M + H]⁺. HPLC purity 99%.

6-((4-Fluorobenzyl)(methyl)amino)-N-phenylpyrimidine-4-carboxamide (**10**). Yield 48%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.54 (s, 1H), 8.70 (s, 1H), 7.90 (d, J = 8.0 Hz, 2H), 7.40 - 7.12 (m, 8H), 4.93 (s, 2H) and 3.21 (s, 3H). LC-MS (ESI): m/z 337.2 [M + H]⁺. HPLC purity 99%.

6-((4-Chlorobenzyl)(methyl)amino)-N-phenylpyrimidine-4-carboxamide (**11**). Yield 81%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.50 (s, 1H), 8.66 (s, 1H), 7.85 (d, J = 8.0 Hz, 2H), 7.39 - 7.24 (m, 7H), 7.11 (t, 8.0 = Hz, 1H), 4.89 (s, 2H) and 3.15 (s, 3H). LC-MS (ESI): m/z 353.1 [M + H]⁺. HPLC purity 99%.

6-(Methyl(4-(trifluoromethyl)benzyl)amino)-N-phenylpyrimidine-4-carboxamide (**12**). Yield 92%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.40 (s, 1H), 8.55 (s, 1H), 7.85 (m, 3H), 7.34 (t, J = 8.0 Hz, 2H), 7.17 (s, 1H), 7.11 (t, J = 8.0 Hz, 1H), 4.10 (m, 1H), 3.86 (m, 2H), 3.41 (m, 2H), 1.87 (m, 2H) and 1.46 (m, 2H). LC-MS (ESI): m/z 387.1 [M + H]⁺. HPLC purity 99%.

6-((3-Fluorobenzyl)(methyl)amino)-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (**13**). Yield 58%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.41 (s, 1H), 8.64 (s, 1H), 7.77 (d, J = 9.0 Hz, 2H), 7.38 (m, 1H), 7.28 (s, 1H), 7.10 - 7.04 (m, 3H), 6.92 (d, J = 9.0 Hz, 2H), 4.91 (s, 2H), 3.73 (s, 3H) and 3.16 (s, 3H). LC-MS (ESI): m/z 367.2 [M + H]⁺. HPLC purity 99%.

6-((3-Chlorobenzyl)(methyl)amino)-N-phenylpyrimidine-4-carboxamide (**14**). Yield 91%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.50 (s, 1H), 8.67 (s, 1H), 7.86 (d, J = 8.4 Hz, 2H), 7.36 - 7.12 (m, 8H), 4.91 (s, 2H), and 3.16 (s, 3H). LC-MS (ESI): m/z 353.1 [M + H]⁺. HPLC purity 98%

6-((2-Chlorobenzyl)(methyl)amino)-N-phenylpyrimidine-4-carboxamide (**15**). Yield 91%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.50 (s, 1H), 8.65 (s, 1H), 7.85 (d, J = 8.4 Hz, 2H), 7.50 - 7.07 (m, 8H), 4.95 (s, 2H), and 3.20 (s, 3H). LC-MS (ESI): m/z 353.1 [M + H]⁺. HPLC purity 96%.

6-((4-Cyanobenzyl)(methyl)amino)-N-phenylpyrimidine-4-carboxamide (**16**). Yield 71%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.50 (s, 1H), 8.65 (s, 1H), 7.86 (d, J = 8.0 Hz, 2H), 7.79 (d, J = 8.0 Hz, 2H) 7.42 - 7.31 (m, 5H), 7.11 (t, J = 8.0 Hz, 1H), 4.99 (s, 2H) and 3.19 (s, 3H). LC-MS (ESI): m/z 344.2 [M + H]⁺. HPLC purity 99%.

6-(Methyl(4-(methylsulfonyl)benzyl)amino)-N-phenylpyrimidine-4-carboxamide (**17**). Yield 48%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.54 (s, 1H), 8.69 (s, 1H), 7.89 (dd, J = 8.6, 7.0 Hz, 4H), 7.61 - 7.44 (m, 2H), 7.44 - 7.25 (m, 3H), 7.25 - 7.06 (m, 1H), 5.05 (s, 2H), 3.21 (d, J = 7.0 Hz, 6H). LC-MS (ESI): m/z 397.1 [M + H]⁺. HPLC purity 100%.

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3 *6-(Methyl(pyridin-2-ylmethyl)amino)-N-phenylpyrimidine-4-carboxamide (18)*. Yield 93%. ¹H
4 NMR (400 MHz, DMSO-d₆) δ 10.48 (s, 1H), 8.63 (s, 1H), 8.51 (d, J = 4 Hz, 1H), 7.85 (d, J = 8.0
5 Hz, 2H), 7.74 (t, J = 8.0 Hz, 1H), 7.37 - 7.23 (m, 5H), 7.11 (t, J = 8.0 Hz, 1H), 4.97 (s, 2H) and
6 3.24 (s, 3H). LC-MS (ESI): m/z 320.2 [M + H]⁺. HPLC purity 97%.

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12 *6-(Methyl(pyridin-3-ylmethyl)amino)-N-phenylpyrimidine-4-carboxamide (19)*. Yield 84%. ¹H
13 NMR (300 MHz, DMSO-d₆) δ 10.53 (s, 1H), 8.69 (d, J = 1.1 Hz, 1H), 8.59 – 8.38 (m, 2H), 7.99
14 – 7.75 (m, 2H), 7.66 (dt, J = 7.9, 2.0 Hz, 1H), 7.50 – 7.25 (m, 4H), 7.25 – 6.97 (m, 1H), 4.96 (s,
15 2H), 3.19 (s, 3H). LC-MS (ESI): m/z 320.2 [M + H]⁺. HPLC purity 100%.

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21 *6-(Methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)-N-phenylpyrimidine-4-carboxamide*
22 *(20)*. Yield 35%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.52 (s, 1H), 8.69 (m, 2H), 7.86 (m, 4H),
23 7.35 (m, 3H), 7.13 (m, 1H), 5.05 (s, 2H) and 3.22 (s, 3H). LC-MS (ESI): m/z 388.2 [M + H]⁺.
24 HPLC purity 98%.

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31 *4-((4-Fluorobenzyl)(methyl)amino)-N-phenylpicolinamide (21)*. Yield 26%. ¹H NMR (300
32 MHz, CDCl₃): 10.40 (br s, 1H), 8.82 (d, J = 6 Hz, 1H), 7.82 (d, J = 6 Hz, 2H), 7.72 (s, 1H), 7.39
33 (d, J = 9 Hz, 2H), 7.15-7.25 (m, 3H), 7.1 (t, J = 9 Hz, 2H), 6.65-6.75 (m, 1H), 4.68 (s, 2H), δ
34 3.19 (s, 3H) . LC-MS (ESI): m/z 336.1 [M + H]⁺. HPLC purity 98%.

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40 *6-((4-Fluorobenzyl)(methyl)amino)-N-(4-fluorophenyl)pyrazine-2-carboxamide (22)*. Yield
41 30%. A mixture of 95 mg (0.37 mmol) 6-chloro-*N*-(4-fluorobenzyl)-*N*-methylpyrazin-2-amine,
42 100 mg (0.90 mmol) 4-fluoroaniline, 100 mg (0.38 mmol) molybdenumhexacarbonyl, 100 mg
43 tetrabutylammonium chloride and 500 mg triethylamine in 3 mL toluene and 2 mL tetraethylene
44 glycol dimethylether were heated under nitrogen in a pressure tube to 150 °C for 4h. The cooled
45 mixture was evaporated onto silica gel. Silica flash chromatography (EtOAc/Hex 30/70 to 50/50)
46 yielded 40 mg as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 9.24 (s, 1H), 8.65 (s, 1H), 8.22 (s,
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1H), 7.55–7.50 (m, 2H), 7.19–7.13 (m, 2H), 7.02–6.96 (m, 4H), 4.75 (s, 2H), 3.20 (s, 3H). LC-MS (ESI): m/z 355.1 [M + H]⁺. HPLC purity 99%.

2-((4-Fluorobenzyl)(methyl)amino)-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (23).

Yield 93%. A mixture of 50 mg (0.20 mmol) 4-chloro-*N*-(4-fluorobenzyl)-*N*-methylpyrimidin-2-amine, 100 mg (0.81 mmol) 4-methoxyaniline, 100 mg (0.38 mmol) molybdenumhexacarbonyl, 100 mg tetrabutylammonium chloride and 500 mg triethylamine in 3 mL toluene and 2 mL teraethylene glycol dimethylether was heated under nitrogen in a pressure tube to 150 °C for 4h. The cooled mix was diluted with 60 ml water and extracted with EtOAc (2x50 ml), dried over Na₂SO₄ and the solvent evaporated. Flash chromatography (EtOAc/Hex 20/80 to 40/60) yielded 67 mg (93%) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 9.51 (s, 1H), 8.63 (d, J = 6.0 Hz, 1H), 7.59 (d, J = 9.0 Hz, 2H), 7.44 (d, J = 6.0 Hz, 1H), 7.31–7.26 (m, 2H), 7.09–7.03 (m, 2H), 6.94 (d, J = 9.0 Hz, 2H), 4.95 (s, 2H), 3.84 (s, 3H), 3.30 (s, 3H). LC-MS (ESI): m/z 367.1 [M + H]⁺. HPLC purity 99%.

6-((4-Fluorobenzyl)(methyl)amino)-N-phenylpyridazine-4-carboxamide (24). Yield 28%. In a 10 mL CEM microwave tube, 6-chloro-*N*-phenylpyridazine-4-carboxamide (50 mg, 0.214 mmol), 1-(4-fluorophenyl)-*N*-methylmethanamine (32.8 mg, 0.235 mmol) and Et₃N (0.060 mL, 0.428 mmol) were mixed in 1,4-Dioxane (0.5 mL). The mixture was microwaved at 125 °C and 200W in CEM microwave for 6 h. Solvent was evaporated and the residue was purified by prep-HPLC to give a buff coloured powder. ¹H NMR (300 MHz, DMSO-d₆): δ 10.50 (br s., 1H), 8.91 (d, J = 3 Hz, 1H), 7.72–7.75 (m, 2H), 7.46 (d, J = 3 Hz, 1H), 7.30–7.42 (m, 4H), 7.14–7.20 (m, 3H), 4.95 (s, 2H), 3.18 (s, 3H) LC-MS (ESI): m/z 337.1 [M + H]⁺. HPLC purity 99%.

1-(4-Fluorobenzyl)-6-oxo-N-phenyl-1,6-dihydropyridine-3-carboxamide (25). Yield 44% over 2 steps.

Step 1: In a 7 mL reaction vial, methyl 1-(4-fluorobenzyl)-6-oxo-1,6-dihydropyridine-3-carboxylate (100 mg, 0.383 mmol) was dissolved in MeOH (4 mL) and LiOH (92 mg, 3.83 mmol) added to the solution and stirred at RT overnight. Methanol was removed under vacuum and the residue was dissolved in water. Aqueous layer was extracted with ethyl acetate. Aqueous layer was then cooled in ice-bath and acidified with concentrated HCl. Precipitated solid was extracted with ethyl acetate. Ethyl acetate layer was then dried on anhydrous MgSO₄ and concentrated under vacuum to give an off-white solid, *1-(4-fluorobenzyl)-6-oxo-1,6-dihydropyridine-3-carboxylic acid*. Yield 95%. ¹H NMR (300 MHz, DMSO-d₆) δ 12.33 (br s, 1H), 8.60 (d, J = 3 Hz, 1H), 7.80 (dd, J = 9, 3 Hz, 1H), 7.38-7.43 (m, 2H), 7.15-7.21 (m, 2H), 6.45 (d, J=9 Hz, 1H), 5.18 (s, 2H). LC-MS (ESI): m/z 248.1 [M - H]⁻. HPLC purity 94%.

Step 2: In a 7 mL reaction vial, 1-(4-fluorobenzyl)-6-oxo-1,6-dihydropyridine-3-carboxylic acid (50 mg, 0.202 mmol) was dissolved in DMF (1 mL) and added Et₃N (0.056 mL, 0.404 mmol) and HATU (115 mg, 0.303 mmol) and stirred for 30 minutes at 25 °C. Aniline (0.022 mL, 0.243 mmol) was then added and the solution stirred at 25 °C for 24 h. Reaction mixture was diluted with ethyl acetate and washed with 10% LiCl solution (2x15mL), saturated aqueous NaHCO₃ solution and brine. The EtOAc layer was then dried and evaporated under vacuum. The residue was chromatographed on Biotage using 10g pre-packed silica gel column and 0-90% EtOAc in Hexane as eluent. Pure fractions were combined and evaporated under vacuum. The syrupy residue was triturated with a 3:1 DCM:Hexane mixture and precipitated solid was removed by filtration. Filtrate was evaporated and the residue was crystallized from ethanol to afford **25** as colorless crystalline needles. ¹H NMR (300 MHz, DMSO-d₆) δ 9.98 (br s, 1H), 8.62 (d, J = 3 Hz, 1H), 8.01 (dd, J = 9, 3 Hz, 1H), 7.68 (d, J = 6 Hz, 2H), 7.40-7.45 (m, 2H), 7.35 (t, J = 9H,

2H), 7.17-7.23 (m, 2H), 7.10 (t, J = 9 Hz, 1H), 6.52 (d, J = 12 Hz, 1H), 5.18 (s, 2H). LC-MS (ESI): m/z 323.1 [M + H]⁺. HPLC purity 95%.

2-((4-Fluorobenzyl)(methyl)amino)-N-phenylthiazole-4-carboxamide (26). 50% over 2-steps.

Step 1: *2-((4-Fluorobenzyl)(methyl)amino)thiazole-4-carboxylic acid*. Yield 81%. In a 7 mL glass vial, methyl 2-((4-fluorobenzyl)(methyl)amino)thiazole-4-carboxylate (150 mg, 0.535 mmol) was dissolved in Methanol (2 mL), added LiOH (128 mg, 5.35 mmol) to the solution and stirred at RT overnight. LCMS indicated complete hydrolysis. Methanol was removed under reduced pressure. Residue was taken-up in water and washed with ethyl acetate. Aqueous layer was cooled and acidified with dil. HCl. Precipitated acid was extracted with dichloromethane. Dichloromethane layer was washed with brine, dried on anhydrous MgSO₄ and concentrated under vacuum to give a buff coloured powder, 2-((4-fluorobenzyl)(methyl)amino)thiazole-4-carboxylic acid. Yield 81%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.52 (s, 1H), 7.25-7.29 (m, 2H), 7.07 (t, J = 9 Hz, 2H), 4.71 (s, 2H), 3.09 (s, 3H). LC-MS (ESI): m/z 267.1 [M + H]⁺. HPLC purity 88%. Product was used without further purification in the next step.

Step 2: In a 7 mL glass vial, 2-((4-fluorobenzyl)(methyl)amino)thiazole-4-carboxylic acid (50 mg, 0.188 mmol) was dissolved in dry DMF (2 mL). HATU (107 mg, 0.282 mmol) and Et₃N (0.052 mL, 0.376 mmol) were then added to the mixture and stirred at 25 °C for 30 min. Aniline was then (0.021 mL, 0.225 mmol) and stirred at 25 °C overnight. LCMS indicated complete reaction. Reaction mixture was diluted with ethyl acetate and washed successively with dil HCl, water, saturated NaHCO₃ solution and brine. Dried on anhydrous MgSO₄ and concentrated under vacuum. Residue was purified on Biotage using 10 g silica gel column and 0-50% ethyl acetate in hexane as eluent. Pure fractions were combined and concentrated to give **26** as a light yellow coloured solid. Yield 62%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.73 (br s, 1H), 7.77 (dd, J

= 9 Hz, 1 Hz, 2H), 7.52 (s, 1H), 7.33-7.44 (m, 4H), 7.17-7.23 (m, 2H), 7.08-7.13 (m, 1H), 4.83 (s, 2H), 3.08 (s, 3H). LC-MS (ESI): m/z 342.1 $[M + H]^+$. HPLC purity 95%.

6-((4-Fluorobenzyl)(methyl)amino)-N-(4-fluorophenyl)-5-methylpyrimidine-4-carboxamide (**27**). Yield 17% over 2 steps.

Step 1: To ethyl 6-((4-fluorobenzyl)(methyl)amino)-5-methylpyrimidine-4-carboxylate **4** (0.25 g, 0.82 mmol) in THF (3 mL) was added lithium hydroxide monohydrate (0.138 g, 3.3 mmol) and water (1.5 mL). The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated under vacuum, cooled to 0 °C and acidified with citric acid solution. The acidified mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum to yield the product, *6-((4-fluorobenzyl)(methyl)amino)-5-methylpyrimidine-4-carboxylic acid*. Yield 88.4%. ^1H NMR (400 MHz, DMSO- d_6): δ 12.48 (s, 1H), 8.45 (s, 1H), 7.32 (d, J = 5.60 Hz, 2H), 7.17 (d, J = 9.20 Hz, 2H), 4.71 (s, 2H), 3.17 (s, 3H). LC-MS (APCI): m/z 276.0 $[M + H]^+$.

Step 2: To a solution of *6-((4-fluorobenzyl)(methyl)amino)-5-methylpyrimidine-4-carboxylic acid* **5** (0.20 g, 0.72 mmol) in DMF (3 mL) was added diisopropylethyl amine (0.232 g, 1.8 mmol), HATU (0.41 g, 1.08 mmol) and 4-Fluoroaniline (0.08 g, 0.7 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. Water was added to the reaction mixture and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate, concentrated under vacuum to get a crude product. It was purified by column chromatography on 60-120 mesh silica gel with 10% ethyl acetate in hexanes as eluent to yield **27** (0.055 g). Yield 20%. ^1H NMR (400 MHz, CDCl_3): δ 10.20 (s, 1H), 8.56 (s, 1H), 7.73-7.70 (m, 2H), 7.30 – 7.26 (m, 2H), 7.10-7.04 (m, 4H), 4.69 (s, 2H), 3.01 (s, 3H), 2.63 (s, 3H). LC-MS (APCI): m/z 369.2 $[M + H]^+$. HPLC purity 100%.

6-((4-Chlorobenzyl)(methyl)amino)-2-methyl-N-phenylpyrimidine-4-carboxamide (28).

Reaction according to general synthesis final step of compounds **1-21**. Yield 95%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.31 (s, 1H), 7.82 (d, J = 8.0 Hz, 2H), 7.36 (m, 4H), 7.25 (d, J = 8.0 Hz, 2H), 7.12 (m, 2H), 4.87 (s, 2H), 3.11 (s, 3H) and 2.52 (s, 3H). LC-MS (ESI): 367.1 m/z [M + H]⁺. HPLC purity 98%.

Compounds **29** and **30** were both synthesized according to the general synthetic procedure below. ethyl 2-chloro-6-((4-chlorobenzyl)(methyl)amino)pyrimidine-4-carboxylate (1eq), DIPEA (2eq) and the appropriate amine (1.2eq) were added to a pressure tube containing *i*-PrOH and the tube sealed and heated to 120 °C overnight. The solution was then cooled and the resulting precipitate filtered off to afford the intended product as a white solid.

2-Amino-6-((4-chlorobenzyl)(methyl)amino)-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (29). Yield 68%. ¹H NMR (400 MHz, CDCl₃) δ 9.64 (s, 1H), 7.66 – 7.49 (m, 2H), 7.29 – 7.14 (m, 2H), 7.13 – 7.00 (m, 2H), 6.91 – 6.73 (m, 3H), 5.11 – 4.49 (m, 4H), 3.74 (s, 3H), 2.98 (s, 3H). LC-MS (ESI): m/z 398.1 [M + H]⁺. HPLC purity 96%.

6-((4-Chlorobenzyl)(methyl)amino)-2-(cyclopropylamino)-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (30). Yield 71%. ¹H NMR (400 MHz, CDCl₃) δ 9.81 (s, 1H), 7.67 – 7.45 (m, 2H), 7.26 – 7.17 (m, 2H), 7.10 (d, J = 8.1 Hz, 2H), 6.88 – 6.75 (m, 3H), 5.06 (s, 1H), 4.74 (s, 2H), 3.74 (d, J = 0.9 Hz, 3H), 3.03 (s, 3H), 2.69 (td, J = 5.8, 4.8, 2.6 Hz, 1H), 0.74 – 0.63 (m, 2H), 0.49 (m, 2H). LC-MS (ESI): m/z 438.2 [M + H]⁺. HPLC purity 95%.

General synthetic protocol for compounds **31** and **33-52** as follows: The appropriate 6-(benzyl(methyl)amino)pyrimidine-4-carboxylic acid (1eq), DIPEA (3eq), and HATU (1.5eq) were dissolved in DMF and stirred for 10 minutes. To the solution was then added the appropriate amine and stirred at RT overnight. Final compounds were either isolated by

precipitation with water and recrystallization with EtOH or extracted with EtOAc and washed successively with 10% LiCl, saturated aqueous NaHCO₃ and brine and then purified by silica flash chromatography.

6-(Benzyl(methyl)amino)-N-methyl-N-phenylpyrimidine-4-carboxamide (31). Yield 67%. ¹H NMR (400 MHz, DMSO-d₆) δ 8.25 (s, 1H), 7.34 - 7.06 (m, 10H), 6.68 (m, 1H), 4.72 (s, 2H), 2.97 (s, 3H) and 2.48 (s, 3H). LC-MS (ESI): m/z 333.2 [M + H]⁺. HPLC purity 99%.

N-(6-((4-Fluorobenzyl)(methyl)amino)pyrimidin-4-yl)benzamide (32). Yield 20%. To a stirred solution of 6-chloropyrimidin-4-amine (500 mg, 3.86 mmol) and DIPEA (1.348 mL, 7.72 mmol) in dry THF (20 mL) was added dropwise benzoyl chloride (0.672 mL, 5.79 mmol). The solution was heated at 50 °C for 12h. The crude reaction mixture was adsorbed onto silica and purification attempted on an ISCO flash purification system; solvent system gradient 0% to 70% EtOAc in Hex over 15 mins. Single peak isolated contained both product and impurity. LC-MS (ESI): m/z 234.1 [M + H]⁺. Purity 64%. Crude material from previous step was dissolved in *i*-PrOH and *N*-methyl-1-phenylmethanamine (349 mg, 2.88 mmol) and DIPEA (0.837 mL, 4.79 mmol) added and heated at 100 °C for 4h. The resulting solution was adsorbed directly onto silica gel and purified on ISCO purification system using a 4 g silica pre-packed cartridge and solvent system 0% to 100% EtOAc in Hexane over 15 mins to afford a pale cream solid.

¹H NMR (300 MHz, DMSO-d₆) δ 10.69 (s, 1H), 8.36 (d, J = 1.1 Hz, 1H), 8.05 – 7.95 (m, 2H), 7.65 – 7.57 (m, 1H), 7.57 – 7.46 (m, 3H), 7.40 – 7.30 (m, 2H), 7.30 – 7.19 (m, 2H), 4.85 (s, 2H), 3.07 (s, 3H). LC-MS (ESI): m/z 337.2 [M + H]⁺. HPLC purity 99%.

N-Isopropyl-6-(methyl(4-(trifluoromethyl)benzyl)amino)pyrimidine-4-carboxamide (33). Yield 20%. ¹H NMR (300 MHz, DMSO-d₆) δ 8.58 (s, 1H), 8.41 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 8.0

Hz, 2H), 7.43 (d, J = 8.0 Hz, 2H), 7.21 (s, 1H), 4.99 (s, 2H), 4.08 (h, J = 6.5 Hz, 1H), 3.18 (d, J = 4.3 Hz, 3H) and 1.18 (d, J = 6.7 Hz, 6H). LC-MS (ESI): m/z 353.2 [M + H]⁺. HPLC purity 98%.

N-Cyclohexyl-6-(methyl(4-(trifluoromethyl)benzyl)amino)pyrimidine-4-carboxamide (34).

Yield 60%. ¹H NMR (300 MHz, DMSO-d₆) δ 8.58 (s, 1H), 8.38 (d, J = 8.5 Hz, 1H), 7.70 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 8.0 Hz, 2H), 7.20 (s, 1H), 4.99 (s, 2H), 3.93 – 3.53 (m, 1H), 3.17 (s, 3H), 1.90 – 1.51 (m, 5H), 1.51 – 1.00 (m, 5H). LC-MS (ESI): m/z 393.2 [M + H]⁺. HPLC purity 100%.

6-((4-Fluorobenzyl)(methyl)amino)-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (35).

Yield 68%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.40 (s, 1H), 8.65 (d, J = 1.2 Hz, 1H), 7.77 (d, J = 9.0 Hz, 2H), 7.32 – 7.24 (m, 3H), 7.15 (t, J = 8.8 Hz, 2H), 6.91 (d, J = 9.1 Hz, 2H), 4.88 (s, 2H), 3.73 (s, 3H), 3.13 (s, 3H). LC-MS (ESI): m/z 367.2 [M + H]⁺. HPLC purity 97%.

6-((4-Fluorobenzyl)(methyl)amino)-N-(4-isopropoxyphenyl)pyrimidine-4-carboxamide (36).

Yield 63%. ¹H NMR (400 MHz, CDCl₃) δ 9.92 – 9.74 (m, 1H), 8.61 (s, 1H), 7.63 (d, J = 8.9 Hz, 2H), 7.40 (dd, J = 1.2, 0.5 Hz, 1H), 7.18 (dd, J = 8.5, 5.4 Hz, 2H), 7.00 (t, J = 8.6 Hz, 2H), 6.89 (d, J = 8.9 Hz, 2H), 4.86 (s, 2H), 4.51 (hept, J = 6.1 Hz, 1H), 3.11 (s, 3H), 1.40 – 1.29 (m, 6H). LC-MS (ESI): m/z 395.2 [M + H]⁺. HPLC purity 99%.

6-((4-Fluorobenzyl)(methyl)amino)-N-(4-(2-methoxyethoxy)phenyl)pyrimidine-4-carboxamide

(37). Yield 45%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.44 (s, 1H), 8.67 (d, J = 1.1 Hz, 1H), 7.78 (d, J = 9.0 Hz, 2H), 7.36 – 7.24 (m, 3H), 7.17 (t, J = 8.8 Hz, 2H), 6.94 (d, J = 9.1 Hz, 2H), 4.90 (s, 2H), 4.25 – 3.96 (m, 2H), 3.73 – 3.53 (m, 2H), 3.30 (s, 3H), 3.16 (s, 3H). LC-MS (APCI): m/z 411.2 [M + H]⁺. HPLC purity 95%.

N-(4-(2-(Dimethylamino)ethoxy)phenyl)-6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-

carboxamide (38). Yield 25%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.45 (s, 1H), 8.68 (s, 1H),

7.79 (d, J = 8.6 Hz, 2H), 7.38 – 7.24 (m, 3H), 7.17 (t, J = 8.7 Hz, 2H), 6.96 (d, J = 8.6 Hz, 2H), 4.90 (s, 2H), 4.10 (t, J = 5.7 Hz, 2H), 3.16 (s, 3H), 2.81 (t, J = 5.7 Hz, 2H), 2.36 (s, 6H). LC-MS (APCI): m/z 424.2 [M + H]⁺. HPLC purity 95%.

6-((4-Chlorobenzyl)(methyl)amino)-N-(pyridin-4-yl)pyrimidine-4-carboxamide (39). Yield 35%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.88 (s, 1H), 8.68 (s, 1H), 8.48 (d, J = 5.6 Hz, 2H), 7.89 (d, J = 5.6 Hz, 2H), 7.38 (d, J = 8.1 Hz, 2H), 7.33 – 7.22 (m, 3H), 4.90 (s, 2H), 3.15 (s, 2H). LC-MS (ESI): m/z 354.1 [M + H]⁺. HPLC purity 100%.

6-(Methyl(4-(trifluoromethyl)benzyl)amino)-N-(4-(4-methylpiperazin-1-yl)phenyl)pyrimidine-4-carboxamide (40). Yield 74%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.75 (s, 1H), 8.74 (s, 1H), 7.79 (d, J = 9 Hz, 2H), 7.76 (s, 1H), 7.73 (d, J = 9 Hz, 2H), 7.50 (d, J = 9 Hz, 2H), 7.03 (d, J = 6 Hz, 2H), 5.11 (s, 2H), 3.80 (d, J = 9 Hz, 2H), 3.29 (s, 3H), 3.04-3.21 (m, 5H), 2.82 (m, 3H). LC-MS (ESI): m/z 485.1 [M + H]⁺. HPLC purity 98%.

6-(Methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)-N-(4-(4-methylpiperazin-1-yl)phenyl)pyrimidine-4-carboxamide (41). Yield 45%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.37 (s, 1H), 8.71 (d, J = 6 Hz, 2H), 7.94 (d, J = 9 Hz, 1H), 7.87 (d, J = 9 Hz, 1H), 7.77 (d, J = 9 Hz, 2H), 7.47 (s, 1H), 7.02 (d, J = 9 Hz, 2H), 5.09 (s, 2H), 3.80 (d, J = 15 Hz, 2H), 3.26 (s, 3H), 3.00-3.21 (m, 6H), 2.83 (d, J = 6 Hz, 3H). LC-MS (ESI): m/z 486.2 [M + H]⁺. HPLC purity 98%.

6-((4-Fluorobenzyl)(methyl)amino)-N-(3-(4-methylpiperazin-1-yl)phenyl)pyrimidine-4-carboxamide (42). Yield 12%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.34 (s, 1H), 8.68 (s, 1H), 7.51 (t, J = 2.2 Hz, 1H), 7.40 – 7.23 (m, 4H), 7.24 – 7.10 (m, 3H), 6.73 (dd, J = 8.2, 2.4 Hz, 1H), 4.90 (s, 2H), 3.38 (s, 4H), 3.16 (s, 7H), 2.26 (s, 3H). LC-MS (APCI): m/z 435.2 [M + H]⁺. HPLC purity 97%.

6-((4-Fluorobenzyl)(methyl)amino)-N-(6-(4-methylpiperazin-1-yl)pyridin-3-yl)pyrimidine-4-carboxamide (**43**). Yield 29%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.58 (s, 1H), 8.55 (s, 1H), 7.91 – 7.65 (m, 2H), 7.45 – 7.26 (m, 2H), 7.28 – 7.03 (m, 4H), 4.72 (s, 2H), 3.32 (s, 8H) 3.02 (s, 3H), 2.35 (s, 3H). LC-MS (APCI): m/z 436.2 [M + H]⁺. HPLC purity 97%.

N-(4-(4-Acetylpiperazin-1-yl)phenyl)-6-(methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)pyrimidine-4-carboxamide (**44**). Yield 38%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.39 (s, 1H), 8.71 (s, 1H), 8.67 (s, 1H), 7.96-7.86 (m, 2H), 7.75 (d, J = 9.1 Hz, 2H), 7.34 (s, 1H), 6.97 (d, J = 9.1 Hz, 2H), 5.07 (s, 2H), 3.62-3.50 (m, 4H), 3.23 (s, 3H), 3.18 – 3.04 (m, 4H), 2.05 (s, 3H). LC-MS (ESI): m/z 514.1 [M + H]⁺. HPLC purity 87.75%

6-((4-Fluorobenzyl)(methyl)amino)-N-(4-morpholinophenyl)pyrimidine-4-carboxamide (**45**). Yield 30%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.36 (s, 1H), 8.66 (s, 1H), 7.74 (d, J = 9.0 Hz, 2H), 7.35 – 7.10 (m, 5H), 6.94 (d, J = 9.1 Hz, 2H), 4.90 (s, 2H), 3.83 – 3.68 (m, 4H), 3.16 (s, 3H), 3.12 – 3.03 (m, 4H). LC-MS (ESI): m/z 422.1 [M + H]⁺. HPLC purity 100%

6-(Methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)-N-(5-morpholinopyridin-2-yl)pyrimidine-4-carboxamide (**46**). Yield 23%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.19 (s, 1H), 8.72 (d, J = 2.0 Hz, 1H), 8.68 (s, 1H), 8.12 (m, 2H), 7.93 (d, J = 8.1 Hz, 1H), 7.87 (d, J = 8.1 Hz, 1H), 7.52 (dd, J = 9.1, 3.1 Hz, 1H), 7.39 (d, J = 1.2 Hz, 1H), 5.08 (s, 2H), 3.76 (m, 4H), 3.25 (s, 3H), 3.17 (m, 4H). LC-MS (ESI): m/z 474.2 [M + H]⁺. HPLC purity 97%

6-((4-Fluorobenzyl)(methyl)amino)-N-(1-methyl-1H-pyrazol-3-yl)pyrimidine-4-carboxamide (**47**). Yield 54%. ¹H NMR (400 MHz, CDCl₃) δ 10.20 (s, 1H), 8.53 (d, J = 1.2 Hz, 1H), 7.30 (d, J = 1.2 Hz, 1H), 7.21 (d, J = 2.3 Hz, 1H), 7.15 – 7.08 (m, 2H), 6.93 (t, J = 8.7 Hz, 2H), 6.73 (d, J = 2.3 Hz, 1H), 4.78 (s, 2H), 3.76 (s, 3H), 3.03 (s, 3H). LC-MS (ESI): m/z 341.2 [M + H]⁺. HPLC purity 100%

6-(Methyl(4-(trifluoromethyl)benzyl)amino)-N-(1-methyl-1H-1,2,4-triazol-3-yl)pyrimidine-4-carboxamide (**48**). Yield 49%. ¹H NMR (300 MHz, DMSO-d₆) δ 10.46 (s, 1H), 8.66 (s, 1H), 8.39 (s, 1H), 7.71 (d, J = 8.0 Hz, 2H), 7.46 (d, J = 8.0 Hz, 2H), 7.28 (s, 1H), 5.02 (s, 2H), 3.85 (s, 3H), 3.20 (s, 3H). LC-MS (ESI): m/z 392.2 [M + H]⁺. HPLC purity 99%

6-((4-Fluorobenzyl)(methyl)amino)-N-(isoxazol-3-yl)pyrimidine-4-carboxamide (**49**). Yield 6%. ¹H NMR (400 MHz, DMSO-d₆) δ 10.95 (s, 1H), 8.86 (d, J = 1.8 Hz, 1H), 8.67 (d, J = 1.2 Hz, 1H), 7.33 – 7.24 (m, 3H), 7.16 (t, J = 8.8 Hz, 2H), 6.98 (d, J = 1.7 Hz, 1H), 4.89 (s, 2H), 3.30 (s, 3H). LC-MS (ESI): m/z 328.0 [M + H]⁺. HPLC purity 99%

6-((4-Fluorobenzyl)(methyl)amino)-N-(4-methylthiazol-2-yl)pyrimidine-4-carboxamide (**50**). Yield 6%. ¹H NMR (300 MHz, DMSO-d₆) δ 11.75 (s, 1H), 8.67 (d, J = 1.1 Hz, 1H), 7.36 – 7.23 (m, 3H), 7.24 – 7.09 (m, 2H), 6.91 (q, J = 0.9 Hz, 1H), 4.90 (s, 2H), 3.16 (s, 3H), 2.31 (d, J = 1.1 Hz, 3H). LC-MS (ESI): m/z 358.0 [M + H]⁺. HPLC purity 99%

6-((4-Fluorobenzyl)(methyl)amino)-N-(1,3,4-thiadiazol-2-yl)pyrimidine-4-carboxamide (**51**). Yield 47%. ¹H NMR (400 MHz, DMSO-d₆) δ 12.48 (s, 1H), 9.26 (d, J = 1.6 Hz, 1H), 8.68 (s, 1H), 7.33 (s, 1H), 7.28 (t, J = 6.2 Hz, 2H), 7.15 (t, J = 8.1 Hz, 2H), 4.88 (s, 2H), 3.13 (s, 3H). LC-MS (ESI): m/z 345.1 [M + H]⁺. HPLC purity 100%

6-(Methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)-N-(1,3,4-thiadiazol-2-yl)pyrimidine-4-carboxamide (**52**). Yield 30%. ¹H NMR (400 MHz, DMSO-d₆) δ 12.61 (s, 1H), 9.29 (s, 1H), 8.72 (s, 2H), 7.95 - 7.86 (m, 2H), 7.42 (s, 1H), 5.08 (s, 2H) and 3.24 (s, 3H). LC-MS (ESI): m/z 396.1 [M + H]⁺. HPLC purity 96%

N-(4-Fluorobenzyl)-N-methyl-6-(2,2,2-trifluoro-1-((4-fluorophenyl)amino)ethyl)pyrimidin-4-amine (**53**). A solution of 4-fluoroaniline (42.6 mg, 0.383 mmol), Et₃N (0.133 mL, 0.958 mmol), titanium tetrachloride (0.035 mL, 0.319 mmol) and 2,2,2-trifluoro-1-(6-((4-

fluorobenzyl)(methyl)amino)pyrimidin-4-yl)ethan-1-one (100mg, 0.319 mmol) in dry DCM (10 ml) under nitrogen was stirred at RT for 18h. sodium cyanoborohydride (80 mg, 1.277 mmol) was then added portion wise and the reaction stirred for a further 30 minutes. The reaction was then quenched with water (10 mL) and washed with brine (10 mL). The organic layer was separated, dried and adsorbed onto silica and purified by silica flash chromatography to afford a clear oil. Yield 37%. ¹H NMR (300 MHz, CDCl₃) δ 8.68 (d, J = 1.1 Hz, 1H), 7.25 – 7.13 (m, 2H), 7.03 (t, J = 8.6 Hz, 2H), 6.92 (t, J = 8.7 Hz, 2H), 6.81 – 6.66 (m, 2H), 6.51 (s, 1H), 5.40 (s, 1H), 5.06 – 4.55 (m, 3H), 3.08 (s, 3H). LC-MS (ESI): m/z 409.1 [M + H]⁺. HPLC purity 97%

N-(4-Fluorobenzyl)-N-methyl-6-(5-phenyl-4H-1,2,4-triazol-3-yl)pyrimidin-4-amine (54). A solution of potassium carbonate, benzonitrile and 6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-carbohydrazide in n-butanol (4 mL) was heated in a sealed tube overnight. The resulting mixture was concentrated onto silica and purified by silica flash column chromatography, EtOAc:Hex (1:1) to afford an off white solid. Yield 61%. ¹H NMR (300 MHz, DMSO-d₆) δ 8.66 (d, J = 1.1 Hz, 1H), 8.14 – 8.00 (m, 2H), 7.60 – 7.41 (m, 3H), 7.41 – 7.25 (m, 3H), 7.18 (t, J = 8.9 Hz, 2H), 4.92 (s, 2H), 3.16 (s, 3H). LC-MS (ESI): m/z 361.2 [M + H]⁺. HPLC 97%

AUTHOR INFORMATION

Corresponding Authors

*Phone: +27 21 650 1250. E-mail: Sandeep.ghorpade@uct.ac.za

*Phone: +27 21 650 2553. E-mail: kelly.chibale@uct.ac.za

Author Contributions

Kelly Chibale and Richard Gordon came up with the original idea of screening the SoftFocus Library against *Mycobacterium tuberculosis* to identify hits for medicinal chemistry progression. Helena I. M. Boshoff, Clifton E. Barry III facilitated the screening of the SoftFocus library and analysed the data. Chemical synthesis for hit evaluation and SAR expansion was conducted by Richard K. Gessner, Colin R. Wilson, Candice Soares de Melo, Sandile B. Simelane, Aloysius Nchinda, Rudolf Mueller and Sandeep R. Ghorpade. SAR analysis and compound design was conducted by Leslie Street, Sandeep R. Ghorpade, Colin R. Wilson, Richard K. Gessner, Rudolf Muller, C John Harris and Kelly Chibale. Biology triage and MIC assays were performed by Ronnett Seldon. Digby F. Warner and Valerie Mizrahi were involved in the analysis of biology triage results and provided intellectual input into the overall biology of the project. Atica Moosa was responsible for mutant generation and cross-screening of compounds against the mutants. Frederick A. Sirgel and Paul van Helden conducted and analysed screening data against the clinical isolates. Efreem Abay, Dale Taylor, Mathew Njoroge, Christel Brunschwig, Nina Lawrence performed and analysed DMPK experiments. Beatriz Urones, Carolyn Selenski, Matthew Axtman performed the synthesis of derivatives for chemoproteomics experiments. Olalla Sanz, Gracia Santos, Maria José Rebollo-Lopez, Maria Jose Lafuente-Monasterio, Hannah Pflaumer, Sonja Ghidelli-Disse, Markus Boesche, and Gerard Drewes performed the chemoproteomics and related biology experiments. Joël Lelièvre, Lluís Ballell provided the leadership and intellectual inputs into the chemoproteomics experiments. Colin R. Wilson and Sandeep R. Ghorpade wrote the manuscript which was further edited by Leslie Street, Helena I. M. Boshoff, Clifton E. Barry III, Frederick A. Sirgel, Digby F. Warner, Christel Brunschwig, and was finally reviewed and edited by Kelly Chibale. The chemoproteomics section of the manuscript was written up by the personnel involved in the performing the experiments.

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Notes

Any additional relevant notes should be placed here.

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ABBREVIATIONS

TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; MIC, minimum inhibitory concentration; GFP, green fluorescent protein; SAR, structure-activity relationship; AUC, area under the curve; fu , fraction unbound; CFU, colony-forming units; RT, room temperature; EtOH, ethanol; EtOAc: ethyl acetate; DIPEA, diisopropylethylamine; THF, tetrahydrofuran; DMF, N,N-

dimethylformamide; BuOH, butanol; HATU: 1-[Bis(dimethyl amino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; TBAC, tetrabutylammonium chloride; POCl₃, Phosphorus(V) oxychloride; SOCl₂, thionyl chloride; NaBH₄, sodium borohydride; NaCNBH₃, Sodium cyanoborohydride, TiCl₄, Titanium(IV) chloride; TMT, Tandem Mass Tag

Supporting Information. Synthesis and analytical data of intermediates, Chemoproteomics materials and methods, Microbiology assays, *In vitro* ADMET assays, Metabolite identification studies, Mouse pharmacokinetic studies, Molecular formula strings

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