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Biochemical and microbiological evaluation of *N*-aryl urea derivatives against mycobacteria and mycobacterial hydrolases†

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A focused library of 24 *N*-aryl urea derivatives was prepared and evaluated against serine esterases of *Mycobacterium tuberculosis* (*Mtb*) Rv3802c and *Mtb* Ag85C. The members of the library were evaluated for both selectivity and mode of inhibition. Furan-based urea derivative **6c** was found to be the most potent non-covalent inhibitor of Rv3802c with a K_i value of $5.2 \pm 0.7 \mu\text{M}$. On the other hand, triazole-based ureas **10a** and **10b** selectively inhibited Ag85C irreversibly with a k_{inact}/K_i value of 2.3 ± 0.3 and $5.5 \pm 0.4 \times 10^{-3} \mu\text{M}^{-1} \text{min}^{-1}$, respectively. The library was also evaluated for minimum inhibitory concentration (MIC) against two strains of *Mtb*, *Mycobacterium smegmatis*, and *Mycobacterium abscessus*. Compounds **4a** and **4c** were active against *Mtb* H37Rv mc²6206 with MIC values of 3.12 and 1.5 μM , respectively. Closely related **4e** showed similar activity against *Mtb* H37Rv mc²6206 but also possessed activity against *Mtb* H37Ra, *Mycobacterium smegmatis* and *Mycobacterium abscessus*. Compounds **4a**, **4c**, and **4e** all contained a common 1-(cyclohexylmethyl)-3-phenylurea motif. In summary, we identified a selective non-covalent inhibitor of Rv3802c and covalently irreversible inhibitors of Ag85C as well as the 1-(cyclohexylmethyl)-3-phenylurea motif which showed activity against a variety of mycobacteria.

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1. Introduction

The World Health Organization (WHO) declared tuberculosis (TB) a global health emergency in 1993.¹ The epidemic is the leading cause of death from a single infectious agent with an estimated 1.3 million deaths in 2017.¹ The emergence of multi-drug (MDR) and extensively drug resistant (XDR) TB strains has exacerbated the crisis. In 2017, half a million people developed TB that was resistant to the most effective first line drug, rifampicin.¹ The meeting held by the United Nations (UN) in September 2018 titled “United to End TB: An Urgent Global Response to a Global Epidemic” underlines the need for improved treatment against TB. The current standard TB treatment includes 2–6 months of first line and sec-

ond line drugs in different phases. The overwhelming global burden of the disease makes identifying new drug targets a high priority.

The complex cell wall structure of *Mycobacterium tuberculosis* (*Mtb*) plays a crucial role in the survival of the pathogen.^{2,3} The *Mtb* cell wall biosynthesis pathways have been under investigation as potential drug targets for decades.^{4,5} Specifically, mycolic acids are key components of the mycobacterial outer membrane/cell wall superstructure (mycolylarabinogalactan peptidoglycan), and the biosynthesis of these unique fatty acids has been targeted for drug discovery since researchers identified mycolic acids as a major biological barrier for drug entry and determined that mycolic acid biosynthesis is the target of the first-line TB drug isoniazid.^{6,7}

The enzyme Rv3802c has gained attention as a potential drug target.^{8–10} It is one of the enzymes encoded by an invariant mycobacterial gene cluster that encompasses the genes *Rv3799c–Rv3809c*. Each of these genes is responsible for various aspects of mycolic acid synthesis and the transfer of mycolic acids to trehalose or arabinogalactan.¹⁰ The location of the Rv3802c gene within the mycolic acid synthesis gene cluster suggests that the enzyme is involved in the cell wall biosynthesis.¹⁰ The enzyme is named cutinase-like because of sequence similarities with the cutinase family of enzymes

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and is retained in the periplasm following secretion because it possesses an N-terminal transmembrane helix that remains embedded in the plasma membrane.¹¹ Early Rv3802c studies indicated that the enzyme possesses thioesterase/phospholipase A activity.¹⁰ The essential role of *Corynebacterium glutamicum* ortholog NCgl2775 in regulation of cell wall lipid composition in response to stress was observed with elevated mycolate content.¹² Conditional disruption of the *M. smegmatis* ortholog MSMEG_6394 has shown the gene to be essential for mycobacterial viability.¹³ The structure of the MSMEG_6394 ortholog has revealed an α/β hydrolase fold with 68.8% sequence identity to the *Mtb* encoded Rv3802c. The more recent determination of the *Mtb* Rv3802c crystal structure identified the lipid binding site and showed that structural changes in the enzyme are required for substrate recognition and hydrolysis of the fatty acyl chains of phosphatidylinositol, which is the anchoring lipid for mycobacterial lipoarabinomannin.¹⁴ Combined with the study from Meniche *et al.*,¹² it seems clear that Rv3802c is an essential enzyme that hydrolyses phosphatidylinositol and plays a role in regulating mycomembrane lipid content.

Another α/β hydrolase encoded by a gene in the invariant Rv3799c–Rv3809c gene cluster is antigen 85A, which is one of the three highly-similar homologs composing what is termed as the antigen 85 (Ag85) complex.¹⁵ The three enzymes of the Ag85 complex: Ag85A, Ag85B and Ag85C, each possess a Ser-His-Glu catalytic triad essential for catalyzing mycolyl transfer to components of the mycobacterial cell wall.¹⁶ A variety of Ag85 inhibitors have been reported. Substrate analogs such as trehalose and AG derivatives^{17–21} along with compounds that mimic the tetrahedral intermediate such as phosphonates and sulfonates, have been synthesized and screened against these enzymes.^{22,23}

A variety of heterocyclic urea derivatives have been synthesized over the past few years against many hydrolases. Proschak *et al.* reported a library of substituted diaryl ureas against soluble epoxide hydrolase (sEH).²⁴ Cravatt *et al.* have also reported the use of 1,2,3-triazole ureas as a chemotype for serine hydrolase inhibitors.^{25,26} We report herein an evaluation of *N*-aryl urea derivatives for their ability to inhibit the serine hydrolases *Mtb* Ag85C and Rv3802c. In addition, the library was screened for minimal inhibitory concentration (MIC) activity against a variety of mycobacterial species and strains. To our surprise, the 1-(cyclohexylmethyl)-3-phenylurea motif emerged as an antimycobacterial motif.

2. Results and discussion

2.1 Synthesis and evaluation

We synthesized a small library of *N*-phenyl ureas 2a–g (Scheme 1) in order to evaluate their potential for serine hydrolase inhibition. Phenyl isocyanate was added to a solution of each respective amine in anhydrous toluene and refluxed. The desired *N*-phenyl urea derivatives were recrystallized after 1 h with excellent yields (Scheme 1).

Compounds	R–NH ₂	Yield (%)	Enzyme activity of Rv3802c at 500 μ M (%)
2a		92	43.0 $\pm$ 1.9
2b		77	53.0 $\pm$ 10.7
2c		92	38.2 $\pm$ 8.5
2d		96	50.5 $\pm$ 5.7
2e		95	73.1 $\pm$ 2.8
2f		93	84.3 $\pm$ 17.3
2g		73	56.2 $\pm$ 7.7

Scheme 1 Synthesis of *N*-phenyl ureas 2a–g.

The *N*-phenyl ureas 2a–g were screened for inhibitory activity against *Mtb* Ag85C and Rv3802c. *Mtb* Ag85C and Rv3802c were recombinantly expressed and purified as previously reported.²² Activity of Ag85C was assessed using a previously established assay.²² Briefly, reaction components consisted of 500 nM enzyme and 4 mM trehalose, reactions were initiated upon the titration of 100 μ M resorufin butyrate (RfB, Sigma Aldrich, 10 mM DMSO stock). Rv3802c activity was initially assessed by monitoring the hydrolysis of RfB; reactions consisted of 1 μ M enzyme and were initiated upon the titration of 100 μ M (K_m of RfB = 57.4 $\pm$ 15.5 μ M). Both Ag85C and Rv3802c assays were performed in triplicate. All assays were performed with respective compounds (500 μ M) in a sodium phosphate buffer at pH 7.5. Determination of covalent or non-covalent inhibition was assessed *via* reaction progress curves and time dependent inhibition (ESI[†]).

Compounds 2a–g were found to selectively inhibit Rv3802c while no inhibition of Ag85C was observed up to 500 μ M (Scheme 1). Linear progress curves indicated inhibition

to be non-covalent in nature. We suspect that the lack of irreversible interaction is due to an inadequate leaving group ability of the *N*-phenyl moiety and decided to include electron withdrawing substituents on the phenyl isocyanate (Scheme 2). Only furan-2-ylmethanamine and cyclohexylmethanamine were used for the next inhibitor series since these two amine derivatives exhibited the best inhibitory activity in round I screening.

A second library was synthesized using cyclohexylmethanamine and furfurylamine combined with various

isocyanates (Scheme 2). The new series of compounds (4a–e, 6a–c) was screened against *Mtb* Rv3802c and Ag85C. Again, a moderate level of Rv3802c inhibition was observed with the exception of 6c (Scheme 2). Compound 6c showed 88% inhibition of enzyme Rv3802c at 500 μ M concentration with a $K_i = 5.2 \pm 0.7$ μ M (Fig. S1†). All progress curves were linear, indicating equilibrium-based, non-covalent inhibition except for compound 4a which was found to be highly reactive to any free hydroxylate. No noticeable difference in inhibition levels were observed if compounds, except 4a, were allowed to incubate for 1 hour with respective enzyme prior to kinetic reads, suggesting no evidence of slow covalent inhibition.

To investigate the structure activity relationship (SAR) of 6c, we incorporated imidazole and benzimidazole moieties (8c and 8d, respectively) while retaining the *N*-3,5-difluorophenyl moiety. We also synthesized secondary amines using reductive amination of furfuraldehyde and 4-fluorobenzaldehyde in combination with furfurylamine.^{27,28} The respective secondary amines were reacted with 3,5-difluorophenyl isocyanates to afford 8a–b (Scheme 3).

Unfortunately, we did not observe any improvement in activity compared to the initial hit 6c. Use of secondary amine derivatives, 8a and 8b also eliminated the prior inhibitory activity. The loss of inhibition may be attributed to the increased steric demands of these derivatives.

Based on the reported success of triazole-ureas against some lipases,^{25,26} we decided to synthesize triazole-urea derivatives of cyclohexylmethanamine and furfurylamine as well. Both amines were converted into isocyanates using

Compounds	R-	Yield (%)	Enzyme activity of Rv3802c at 500 μ M (%)
4a		64	Too reactive
4b		79	100
4c		78	83.6 $\pm$ 10.9
4d		74	66.0 $\pm$ 0.2
4e		81	72.5 $\pm$ 3.5
6a		89	82.0 $\pm$ 3.8
6b		87	54.9 $\pm$ 2.9
6c		90	13.0 $\pm$ 1.6

Scheme 2 Synthesis of *N*-phenyl ureas 4a–e and 6a–c.

Compounds	R-	Yield (%)	Enzyme activity of Rv3802c at 200 μ M (%)
8a		70	100
8b		81	100
8c		58	20.0 $\pm$ 1.2
8d		54	100

Scheme 3 Synthesis of *N*-3,5-difluorophenyl ureas 8a–d.

Compounds	R	% yield	Inhibition against Rv3802c			Inhibition against Ag85C		
			Type of inhibition	Enzyme activity of Rv3802c at 200 μM (%)	K_{inact}/K_i ($\times 10^{-3} \mu\text{M}^{-1} \text{Min}^{-1}$)	Type of inhibition	Enzyme activity of Ag85C at 200 μM (%)	K_{inact}/K_i ($\times 10^{-3} \mu\text{M}^{-1} \text{Min}^{-1}$)
10a		78	non-covalent	43.0 $\pm$ 7.3	-	covalent	-	2.3 $\pm$ 0.3
10b		66	non-covalent	40.0 $\pm$ 4.1	-	covalent	-	5.5 $\pm$ 0.4
10c		70	covalent	-	1.6 $\pm$ 0.1	covalent	-	0.9 $\pm$ 0.1
12a		75	non-covalent	44.0 $\pm$ 6.6	-	non-covalent	80.0 $\pm$ 2.3	-
12b		82	non-covalent	72.5 $\pm$ 3.5	-	non-covalent	14.0 $\pm$ 2.9	-

Scheme 4 Synthesis of triazole ureas 10a–c and 12a–b.

triphosgene followed by the coupling with commercially available triazoles to afford compounds 10a–c and 12a–b (Scheme 4).

These compounds (10a–c and 12a–b) were initially screened at 200 μM (20 mM DMSO stock) with no pre-incubation period. Percent enzymatic activity was determined for non-covalent inhibitors as previously described. Compounds that displayed progress curves that plateaued over time, indicating covalent inhibition were serially diluted with DMSO and screened in triplicate at 150, 75, 37.5, and 18.75 μM with no pre-incubation period. Following background subtraction, k_{obs} were determined by fitting the reaction progress curves with a one-phase association equation in PRISM 7. The k_{inact}/K_i was calculated by plotting the k_{obs} as a function of inhibitor concentration and fitting the

data with $k_{\text{obs}} = \left(\frac{k_{\text{inact}}}{1 + (K_i/[I])} \right)$ in PRISM 7 (Fig. S2 and S3†).

Triazole compounds were screened against Rv3802c using a different fluorometric substrate than the one used for the Ag85C RfB assay. Briefly, the previously published¹⁴ alternative assay uses 75 μM 4-methylumbelliferyl heptanoate (4MH, Sigma Aldrich) (7.5 mM DMSO stock) and 20 nM Rv3802c in 50 mM sodium phosphate buffer pH 7.5 at 37 °C. Kinetic reads were initiated upon titration of 4MH and obtained using $\lambda_{\text{ex}} = 360 \text{ nm}$ and λ_{em} at 450 nm on a Synergy H4 plate reader (Biotek). The 4MH assay provides su-

perior signal to noise required to accurately determine k_{inact}/K_i values.

An interesting trend of inhibition was observed when the *N*-3,5-difluorophenyl moiety was replaced with a series of triazoles (Scheme 4). With the sterically hindered triazoles and structurally rigid component such as a furan ring, non-covalent inhibition was observed against Rv3802c. But with the smaller triazole ring on one side and methylcyclohexanes on the other, compound 10c inhibited Rv3802c irreversibly with a determined k_{inact}/K_i value of $1.6 \pm 0.1 \times 10^{-3} \mu\text{M}^{-1} \text{min}^{-1}$. Additionally, the inhibitory activity against Ag85C was recovered with the triazole series of compounds. With the methylfuran substituent, non-covalent inhibition was observed against Ag85C while derivatives containing methylcyclohexanes ring showed irreversible inhibition (Fig. S2†). The covalent inhibition of Ag85C with sterically hindered triazoles 10a and 10b can be justified with the presence of a larger active site in Ag85C compared to Rv3802c. The k_{inact}/K_i values of 10a and 10b are 2.3 ± 0.3 and $5.5 \pm 0.4 \times 10^{-3} \mu\text{M}^{-1} \text{min}^{-1}$ respectively.

2.2 MIC determination

With a few active compounds in hand from enzyme inhibition studies, we decided to evaluate MIC values of the compound library against *M. abscessus* ATCC19977, *Mtb* H37Rv mc²6206, *Mtb* H37Ra ATCC25177, and *M. smegmatis* mc²155

(Tables SI1–SI3†). A colorimetric resazurin microtiter assay was used for *Mtb* and *M. smegmatis* while the *M. abscessus* 96 well-plate was visually scanned for cell growth after incubation.²⁹ Surprisingly, compounds **4a**, **4c** and **4e** had better MIC values compared to the hits from enzyme inhibition studies. The MIC values for each hit compound are shown in Scheme 1. The lack of activity of triazole-based covalent inhibitors (**10a–c**) of serine hydrolases in whole cell assays may suggest their inability to access the target enzyme. On the other hand, the activity of hit compounds (Table 1) must be a result of interactions with some other essential targets apart from or in addition to Ag85C and Rv3802c.

The cytotoxic effect of the active compound on L929 murine fibroblast cells was investigated by a MTT cell viability assays and are represented by the given IC₅₀ value.³⁰ The results indicated that all compounds tested in this study exhibited an IC₅₀ (against L929 indicator cells) close to 20 times higher than the MIC (against TB) suggesting a reasonable selectivity index as a starting point for further investigation (Table 1).

3. Experimental

3.1 General methods

All fine chemicals were obtained from Acros Organics, Alfa Aesar, Oakwood chemicals or Sigma-Aldrich. The solvents were purified using a PureSolv MD5 solvent purification system (SPS). Reactions were monitored using thin-layer chromatography (silica gel 60, f254) and spots were visualized under UV light. Flash column chromatography was performed on silica gel (230–400 mesh) obtained from Sorbent Technologies using solvents as received. ¹H-NMR and ¹³C NMR were carried out using a Bruker Avance III 600 MHz spectrometer using residual CHCl₃ or DMSO as internal references. High resolution mass spectroscopy (HRMS) was performed on a Micromass Q-TOF instrument.

3.2 General procedures for synthesis of urea derivatives (2a–g, 4a–e, 6a–c, 8a–d)

Respective isocyanate (1.1 equiv.) was added dropwise to a solution of amine in anhydrous toluene (15 mL). The reaction

mixture was refluxed at 100 °C for 1 h and lowered to room temperature (RT). The solvent was evaporated under pressure. The residual solid was recrystallized from toluene:hexanes (7:3). The crystals were filtered and dried under vacuum to afford desired compounds.

Compound characterization matched reported characterization for **2a–g**, **4a**, **6a–b**.^{31–35}

1-(4-bromophenyl)-3-(cyclohexylmethyl)urea (4b). Yield: 79% (712 mg); ¹H NMR (600 MHz, DMSO): δ 7.35 (m, 4H, Ar-H), 2.92 (t, *J* = 6.3 Hz, 2H, H-11), 1.6–1.70 (m, 5H, H-12, 13, 17), 0.84–1.38 (m, 6H, H-14, 15, 16). ¹³C NMR (600 MHz, DMSO): δ 155.05, 140.03, 131.34, 119.40, 112.11, 45.26, 37.94, 30.32, 26.06, 25.42. Melting point: 191–193 °C. HRMS data: [M + H] *m/z*: calcd for C₁₄H₂₀BrN₂O, 311.0759; found, 311.0770.

1-(cyclohexylmethyl)-3-(4-(trifluoromethyl)phenyl)urea (4c). Yield: 78% (170 mg); ¹H NMR (600 MHz, DMSO): δ 7.56 (q, *J* = 9.2 Hz, 4H, Ar-H), 2.94 (t, *J* = 6.3 Hz, 2H, H-11), 1.6–1.70 (m, 5H, H-12, 13, 17), 0.84–1.38 (m, 6H, H-14, 15, 16). ¹³C NMR (600 MHz, DMSO): δ 154.86, 144.32, 125.98, 125.96, 45.26, 37.90, 30.32, 26.08, 25.41. Melting point: 152–154 °C. HRMS data: [M + H] *m/z*: calcd for C₁₅H₂₀F₃N₂O, 301.1528; found, 301.1523.

1-(Cyclohexylmethyl)-3-(4-fluorophenyl)urea (4d). Yield: 74% (136 mg); ¹H NMR (600 MHz, DMSO): δ 7.37 (m, 2H, H-4, 6), 7.04 (t, *J* = 8.9 Hz, 2H, H-1, 3), 2.92 (t, *J* = 6.3 Hz, 2H, H-11), 1.6–1.70 (m, 5H, H-12, 13, 17), 0.84–1.38 (m, 6H, H-14, 15, 16). ¹³C NMR (600 MHz, DMSO): δ 157.56, 155.99, 155.30, 136.99, 119.00, 115.15, 45.29, 37.99, 30.34, 26.10, 25.43. Melting point: 149–151 °C. HRMS data: [M + H] *m/z*: calcd for C₁₄H₂₀FN₂O, 251.1560; found, 251.1566.

1-(Cyclohexylmethyl)-3-(3,5-difluorophenyl)urea (4e). Yield: 81% (186 mg); ¹H NMR (600 MHz, CDCl₃): δ 6.91 (dd, *J* = 9.1, 2.2 Hz, 2H, H-4, 6), 6.45 (m, 1H, H-2), 3.08 (m, 2H, H-11), 1.61–1.72 (m, 5H, H-12, 13, 17), 0.84–1.45 (m, 6H, H-14, 15, 16). ¹³C NMR (600 MHz, CDCl₃): δ 164.39, 162.77, 155.38, 141.56, 102.38, 98.19, 46.86, 38.49, 31.00, 26.55, 25.98. Melting point: 120–122 °C. HRMS data: [M + H] *m/z*: calcd for C₁₄H₁₉F₂N₂O, 269.1465; found, 269.1471.

1-(3,5-Difluorophenyl)-3-(furan-2-ylmethyl)urea (6c). Yield: 90% (186 mg); ¹H NMR (600 MHz, DMSO): δ 7.60 (dd, *J* = 1.7,

Table 1 MIC values (μM) of hits against *Mycobacterium* sp.

Compounds	<i>Mtb</i> H37Rv mc ² 6206 (<i>Mtb</i> H37Ra ATCC 25177)	<i>Mabs</i> ATCC 19977	<i>Msmeg</i> mc ² 155	IC ₅₀ against L929 cells (μM)
4a	3.12 (25)	>100	15.6	57
4c	1.5 (12.5)	>100	>100	56
4e	3.12 (25)	25	62.5	70

0.7 Hz, 1H, H-17), 7.14 (dd, $J = 10.1, 2.2$ Hz, 1H, H-2), 6.79 (m, 2H, H-4, 6), 6.41 (dd, $J = 3.1, 1.7$ Hz, 1H, H-16), 6.27 (dd, $J = 3.1, 0.7$ Hz, 1H, H-15), 4.30 (d, $J = 5.7$ Hz, 2H, H-11). ^{13}C NMR (600 MHz, DMSO): δ 163.46, 161.86, 154.50, 152.79, 143.09, 142.13, 110.49, 106.64, 100.48, 96.1, 36.1. Melting point: 123–125 °C. HRMS data: $[\text{M} + \text{H}]^+ m/z$: calcd for $\text{C}_{12}\text{H}_{11}\text{F}_2\text{N}_2\text{O}_2$, 253.0789; found, 253.0778.

3-(3,5-Difluorophenyl)-1,1-bis(furan-2-ylmethyl)urea (8a). Yield: 70% (68 mg); ^1H NMR (600 MHz, CDCl_3): δ 7.43 (dd, $J = 1.8, 0.7$ Hz, 2H, H-23, 16), 7.00 (dd, $J = 9.2, 2.2$ Hz, 2 H, H-4, 6), 6.47 (m, 1H, H-2), 6.38 (dd, $J = 3.2, 1.8$ Hz, 2H, H-15, 22), 6.27 (dd, $J = 3.1, 0.7$ Hz, 2H, H-14, 21), 4.53 (s, 4H, H-11, 19). ^{13}C NMR (600 MHz, CDCl_3): δ 164.25, 164.15, 162.62, 162.52, 154.87, 150.60, 142.97, 110.91, 109.03, 102.61, 102.41, 98.24, 43.48. Melting point: 98–99 °C. HRMS data: $[\text{M} + \text{H}]^+ m/z$: calcd for $\text{C}_{17}\text{H}_{15}\text{F}_2\text{N}_2\text{O}_3$, 333.1051; found, 333.1049.

3-(3,5-Difluorophenyl)-1-(4-fluorobenzyl)-1-(furan-2-ylmethyl)urea (8b). Yield: 81% (72 mg); ^1H NMR (600 MHz, CDCl_3): δ 7.44 (dd, $J = 1.8, 0.7$ Hz, 1H, H-16), 6.46–7.35 (m, 7H, H-2, 4, 6, 21–25) 6.37 (dd, $J = 3.2, 1.8$ Hz, 1H, H-15), 6.23 (dd, $J = 3.2, 0.7$ Hz, 1H, H-14), 4.57 (s, 2H, H-19), 4.44 (s, 2H, H-11). ^{13}C NMR (600 MHz, CDCl_3): δ 164.28, 163.45, 162.65, 162.55, 161.81, 155.14, 150.36, 143.16, 141.58, 129.59, 129.54, 116.15, 116.01, 110.98, 109.10, 102.65, 102.45, 98.36, 50.11, 43.74. Melting point: 142–143 °C. HRMS data: $[\text{M} + \text{H}]^+ m/z$: calcd for $\text{C}_{19}\text{H}_{16}\text{F}_3\text{N}_2\text{O}_2$, 361.1164; found, 361.1157.

1-((1H-Benzimidazol-2-yl)methyl)-3-(3,5-difluorophenyl)-urea (8c). Yield: 58% (355 mg); ^1H NMR (600 MHz, DMSO): δ 7.76 (m, 2H, H-19, 22), 7.51 (dd, $J = 9.2, 2.2$ Hz, 2 H, H-4, 6), 7.30 (m, 1H, H-2), 7.16 (m, 2H, H-20, 21), 4.79 (d, 5.52, 2H, H-11). ^{13}C NMR (600 MHz, DMSO): δ 163.48, 163.38, 161.88, 161.77, 155.17, 153.23, 142.78, 131.40, 125.45, 114.03, 100.67, 100.47, 96.59, 79.49, 36.56. Melting point: 92–94 °C. HRMS data: $[\text{M} + \text{H}]^+ m/z$: calcd for $\text{C}_{15}\text{H}_{13}\text{F}_2\text{N}_4\text{O}$, 303.1057; found, 303.1055.

1-((1H-Imidazol-2-yl)methyl)-3-(3,5-difluorophenyl)urea (8d). Yield: 54% (280 mg); ^1H NMR (600 MHz, DMSO): δ 7.13 (dd, $J = 9.2, 2.2$ Hz, 2 H, H-4, 6), 6.75 (m, 1H, H-2), 6.70 (m, 2H, H-15, 16), 4.29 (d, $J = 5.7$ Hz, 2H, H-11). ^{13}C NMR (600 MHz, DMSO): δ 163.49, 161.89, 154.68, 145.10, 143.14, 100.43, 100.23, 96.08, 37.13. Melting point: 204–206 °C. HRMS data: $[\text{M} + \text{H}]^+ m/z$: calcd for $\text{C}_{11}\text{H}_{11}\text{F}_2\text{N}_4\text{O}$, 253.0901; found, 253.0898.

3.3 General procedures for synthesis of urea derivatives (10a–c, 12a–b)

Triphosgene (1.1 equiv.) was added to a solution of amine in anhydrous toluene at 0 °C. The reaction was refluxed overnight and appeared complete by TLC. The solvent was evaporated and the residue was dissolved in EtOAc (20 mL). The reaction mixture was then washed with H_2O ($\times 2$, 15 mL) and organic layer was collected, dried and used without further purification. Isocyanate intermediates were dissolved in dry DCM (10 mL) followed by addition of the respective secondary amines. The reactions were stirred under N_2 at RT. The

reactions were monitored by TLC and appeared complete after 15 h. The solvent was evaporated and the residue was subjected to flash column chromatography on silica gel (hexanes:EtOAc) to afford the desired products.

Compound characterization matched reported characterization for 10a, 12a–b.^{36–38}

N-(Cyclohexylmethyl)-3-(methylthio)-4H-1,2,4-triazole-4-carboxamide (10b). Yield: 66% (35 mg); ^1H NMR (600 MHz, CDCl_3): δ 8.76 (s, 1H, H-10), 3.26 (t, $J = 6.6$ Hz, 2H, H-4), 2.63 (s, 3H, H-12), 1.61–1.72 (m, 5H, H-5, 13, 17), 0.84–1.45 (m, 6H, H-14, 15, 16). ^{13}C NMR (600 MHz, CDCl_3): δ 164.28, 144.38, 46.76, 38.14, 30.85, 26.44, 25.90, 14.50. Melting point: 102–104 °C. HRMS data: $[\text{M} + \text{Na}]^+ m/z$: calcd for $\text{C}_{11}\text{H}_{18}\text{NaN}_4\text{OS}$, 277.1099; found, 277.1090.

N-(Cyclohexylmethyl)-1H-1,2,3-triazole-1-carboxamide (10c). Yield: 70% (42 mg); ^1H NMR (600 MHz, CDCl_3): δ 8.29 (d, $J = 1.2$ Hz, 1H, H-1), 7.77 (d, $J = 1.2$ Hz, 1H, H-2), 3.35 (t, $J = 6.6$ Hz, 2H, H-9), 1.61–1.72 (m, 5H, H-5, 10, 11, 15), 0.84–1.45 (m, 6H, H-12, 13, 14). ^{13}C NMR (600 MHz, CDCl_3): δ 147.56, 134.58, 122.57, 47.09, 38.09, 30.84, 26.43, 25.88. Melting point: 115–117 °C. HRMS data: $[\text{M} + \text{H}]^+ m/z$: calcd for $\text{C}_{10}\text{H}_{17}\text{N}_4\text{O}$, 209.1402; found, 209.1410.

3.4 In vitro inhibition assays

Mtb Ag85C and Rv3802c were recombinantly expressed and purified as previously reported.³⁹ Following purification, both enzymes were dialyzed into a 50 mM sodium phosphate pH 7.5 buffer for all inhibition assays. Activity of Ag85C was assessed using a previously established assay.³⁹ Briefly, reaction components consisted of 500 nM enzyme and 4 mM trehalose, reactions were initiated upon the addition of 100 μM resorufin butyrate (RfB, Sigma Aldrich) (10 mM DMSO stock). Rv3802c activity was initially assessed by monitoring the hydrolysis of RfB; reactions consisted of 1 μM enzyme and were initiated upon the titration of 100 μM (K_m of RfB = 57.4 ± 15.5 μM). Both Ag85C and Rv3802c assays were performed in triplicate in 50 mM sodium phosphate buffer pH 7.5 at 37 °C. Determination of covalent or non-covalent inhibition was assessed *via* reaction progress curves and time dependent inhibition.⁴⁰

Compound series 2, 4, and 6 were dissolved in DMSO for a stock concentration of 50 mM for initial inhibition screening at 500 μM in triplicate. The tested compounds were incubated with respective enzyme for 15 minutes at room temperature prior to kinetic reads, which were initiated upon addition of resorufin butyrate (RfB). Kinetic reads were obtained using $\lambda_{\text{ex}} = 500$ nm and $\lambda_{\text{em}} = 590$ nm on a Synergy H4 plate reader (Biotek). Reaction rates were calculated using a linear fit between time points 2 to 6 minutes, with reads every 30 seconds. Following background subtraction, percent enzyme activity was determined by $(V_o/V'_o) \times 100$ where V_o is inhibited reaction and V'_o is uninhibited reaction. All progress curves were linear, indicating equilibrium based, non-covalent inhibition except for compound 4a which was found

to be highly reactive to any free hydroxylate. No noticeable difference in inhibition levels were observed if compounds, except **4a**, were allowed to incubate for 1 hour with respective enzyme prior to kinetic reads, suggesting no evidence of slow covalent inhibition.

Compound **6c** was serially diluted from 50 mM to 156 μ M in DMSO for K_i determination. The respective concentrations of **6c** were added to Rv3802c (1% v/v final) in triplicate and allowed to incubate for 15 minutes at RT prior to addition of RfB and initiation of the kinetic read. The reaction rates were determined as previously stated with the background subtracted. The resulting rates were plotted as a function of inhibitor concentration and the K_i determined by fitting the data with the Morrison K_i equation in PRISM 7 (Fig. S1†).

The series 8 compounds were screened in triplicate at 200 μ M (20 mM DMSO stock) and allowed to incubate with enzyme 15 minutes at room temperature prior to initiation of the reaction by adding RfB. Data were acquired and processed in an identical fashion to that of compound series 2, 4, and 6. All progress curves were linear, indicating equilibrium based, non-covalent inhibition. No noticeable difference in inhibition levels were observed if compounds were allowed to incubate for 1 hour with respective enzyme prior to initiation of the kinetic reads, suggesting no evidence of slow covalent inhibition.

Conclusions

We synthesized a library of *N*-aryl urea derivatives that was evaluated against two mycobacterial hydrolases. The furan-based urea derivative **6c** inhibited Rv3802c selectively, exhibiting a K_i value of $5.2 \pm 0.7 \mu$ M in a non-covalent fashion. On the other hand the 1,2,3-triazole derivative **10c** showed irreversible inhibition of Ag85C with a k_{inact}/K_i value of $1.6 \pm 0.1 \times 10^{-3} \mu\text{M}^{-1} \text{min}^{-1}$. The presence of a 2-methylfuran or methylcyclohexane ring on the triazole ureas affected the mode of inhibition against Ag85C, where the methylcyclohexane-containing derivatives bind Ag85C irreversibly. The MIC values of compounds **4a**, **4c**, and **4e** against *Mtb* H37Rv mc²6206 are observed to be in the range of 1.5–6.25 μ M. The structures all share a common 1-(cyclohexylmethyl)-3-phenylurea motif which may represent the active pharmacophore. However, the antibacterial activity of **4a**, **4c** and **4e** appears to be largely independent of the Rv3802c and Ag85C enzyme inhibitory activity. This distinction can be indicative of a different target and mechanism of action of these compounds. The hit compounds were evaluated for the cytotoxicity against a mammalian cell line and demonstrated good selectivity. These hits can potentially be converted into probes for mycobacterial target identification and used as tools in chemical genomics-based experiments.

Author contributions

SJS and AV designed and synthesized the compounds and wrote that portion of the paper. CG, CMS, and DRR

performed Ag85C and Rv3802c isolation and enzyme inhibition studies and wrote that portion of the paper. MJ, AV, and ADL performed MIC studies and edited key aspects of the paper. BL and JD performed the MTT assay.

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Conflicts of interest

There are no conflicts to declare.

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