Supporting Information

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

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1) Synthesis of intermediates:

General procedure for synthesis of compounds ***55-58***. To a solution of 6-chloropyrimidine-4-carboxylic acid (300 mg, 1.892 mmol), HATU (1079 mg, 2.84 mmol) and DIPEA (0.991 ml, 5.68 mmol) dissolved in DMF (4 ml) was added corresponding aniline (2.271 mmol) and the reaction stirred for 4h at 25 °C. Water was then added and the precipitate filtered and dried to afford the appropriate amide which was either purified by column chromatography or used in the next step without further purification.

*6-Chloro-N-phenylpyrimidine-4-carboxamide (**55**)*. Yield 69%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.85 (s, 1H), 9.28 (d, J = 1.2 Hz, 1H), 8.21 (d, J = 1.2 Hz, 1H), 8.06 – 7.79 (m, 2H), 7.53 – 7.27 (m, 2H), 7.27 – 7.01 (m, 1H). LC-MS (ESI): m/z Not detected. HPLC purity 99%.

*6-Chloro-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (**56**).* Yield 63%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.82 (s, 1H), 9.31 (d, J = 1.1 Hz, 1H), 8.24 (d, J = 1.1 Hz, 1H), 8.04 – 7.77 (m, 2H), 7.17 – 6.93 (m, 2H), 3.82 (s, 3H). LC-MS (ESI): m/z 264.1. HPLC purity 100%.

*6-Chloro-N-(pyridin-4-yl)pyrimidine-4-carboxamide (**57**).* Yield 43%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.23 (s, 1H), 9.02 (d, J = 1.1 Hz, 1H), 8.62 – 8.42 (m, 2H), 8.28 (d, J = 1.1 Hz, 1H), 8.04 – 7.89 (m, 2H). LC-MS (ESI): Mass not detected.

*4-Chloro-N-phenylpicolinamide (**58**)*. Yield 69%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.66 (s, 1H), 8.73 (dd, J = 5.3, 0.6 Hz, 1H), 8.16 (dd, J = 2.1, 0.6 Hz, 1H), 7.95 – 7.81 (m, 3H), 7.53 – 7.27 (m, 2H), 7.27 – 7.04 (m, 1H). LC-MS (ESI): m/ z 233.2 [M + H]+. HPLC purity 100%.

*6-Chloro-N-(4-fluorobenzyl)-N-methylpyrazin-2-amine (**61**).* Yield 51%. A mixture of 500 mg (3.35 mmol) 2,6-dichloropyrazine, 500 mg (3.59 mmol) 1-(4-fluorophenyl)-*N*-methylmethanamine and 1g triethylamine in 6 ml dioxane was heated in a pressure tube to 60°C for 3 days. The mixture was diluted with 50 ml water and extracted with 2 x 50 ml EtOAc, dried over Na_2SO_4 and the solvent evaporated. Flash chromatography (Hexane/EtOAc 90/10 to 70/30)

yielded the product as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (s, 1H); 7.75 (s, 1H); 7.16-7.11 (m, 2H); 6.98-6.92 (m, 2H); 4.67 (s, 2H); 3.02 (s, 3H).

*4-Chloro-N-(4-fluorobenzyl)-N-methylpyrimidin-2-amine (**62**)*. Yield 21%. A mixture of 150 mg (1.01 mmol) 2,4-dichloropyrimidine, 150 mg (1.08 mmol) 1-(4-fluorophenyl)-*N*-methylmethanamine and 0.5g triethylamine in 3 ml dioxane was heated in a pressure tube to 90°C for 90 min. The mix was diluted with 50 ml water and extracted with 2 x 50 ml EtOAc, dried over Na_2SO_4 and the solvent evaporated. Flash chromatography (Hexane/EtOAc 90/10 to 70/30) yielded 54 mg (21%) 4-chloro-*N*-(4-fluorobenzyl)-*N*-methylpyrimidin-2-amine as colorless oil and 110 mg (43%) 2-chloro-*N*-(4-fluorobenzyl)-*N*-methylpyrimidin-4-amine as colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12 (d, J = 6.0 Hz, 1H); 7.20-7.11 (m, 2H); 6.96-6.90 (m, 2H); 6.48 (d, J = 6.0 Hz, 1H); 4.78 (s, 2H); 3.06 (s, 3H).

*6-Chloro-N-phenylpyridazine-4-carboxamide (**63**).* Yield 36%. In a 15 mL round-bottom flask, 6-oxo-1,6-dihydropyridazine-4-carboxylic acid (0.1 g, 0.714 mmol) was mixed with phosphorus(V) oxychloride (1 mL, 10.70 mmol) and the mixture was refluxed at 110 °C for 1 h. Phosphorus(V) oxychloride was then removed under vacuum and the dark residue was dissolved in CH_2Cl_2 (5 mL). This solution was cooled in ice-bath and added triethylamine (0.497 mL, 3.57 mmol) followed by the addition of aniline (0.072 mL, 0.785 mmol). The mixture was stirred for 1 h at RT. The reaction mixture was then diluted with DCM and washed with 1N HCl, water, saturated $NaHCO_3$ solution and brine, dried on anhydrous $MgSO_4$ and evaporated under vacuum. Residue was chromatographed on silica gel column using 30% ethyl acetate in hexane as the eluent. Pure fractions were combined and evaporated to afford 6-chloro-*N*-phenylpyridazine-4-carboxamide (60 mg, 0.257 mmol) as buff coloured crystalline solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25 -7.30 (m, 1H), 7.42-7.47 (m, 2H), 7.65-7.68 (m, 2H), 7.98-7.99 (m, 1H), 8.00-8.10 (m, 1H), 9.58 (br s, 1H). LC-MS (ESI): m/ z 234.1 [M + H]+. HPLC purity 100%

*Methyl 1-(4-fluorobenzyl)-6-oxo-1,6-dihydropyridine-3-carboxylate (**65**).* Yield 30%. In a 50 mL round-bottom flask, methyl 2-oxo-2H-pyran-5-carboxylate (500 mg, 3.24 mmol) was taken up in a mixture of water (15 mL) and ethanol (10 mL) after which (4-fluorophenyl)methanamine (0.550 mL, 4.81 mmol) was added and the mixture was stirred at RT for 5 days. The mixture was then extracted with ethyl acetate and washed with brine, dried on anhydrous $MgSO_4$ and evaporated to give a syrupy mass, which was subjected to flash chromatography on a biotage using a prepacked silica gel column (25 g). Solvent system 0-50% EtOAc in Hexane. Pure fractions were combined and evaporated to give methyl 1-(4-fluorobenzyl)-6-oxo-1,6-dihydropyridine-3-carboxylate (250 mg, 0.957 mmol) as light yellow coloured waxy solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.19 (d, J=3H, 1H), 7.85 (dd, J=12, 3 Hz, 1H), 7.33-7.38 (m, 2H), 7.04-7.09 (m, 2H), 6.60 (d, J=9 Hz, 1H), 5.15 (s, 2H), 3.86 (s, 3H). LC-MS (ESI): m/ z 262.1 [M + H]+. HPLC purity 96%

*Methyl 2-((4-fluorobenzyl)(methyl)amino)thiazole-4-carboxylate (**67**).* Yield 32% over 2 steps.

Step 1. In a 50 mL round-bottom flask, methyl 2-aminothiazole-4-carboxylate (0.2 g, 1.264 mmol), 4-fluorobenzaldehyde (0.161 mL, 1.517 mmol) were mixed in ethanol (10 mL). The mixture was heated to 110 °C and ethanol was slowly removed by distillation. The residue was heated further at 110 °C overnight. LCMS indicated formation of imine (M+1 = 265.1). This was dissolved in ethanol (10 mL) by heating at 60 °C. The solution was cooled to RT and added $NaBH_4$ (0.096 g, 2.53 mmol) to it. The mixture was stirred at RT for 1 h. LCMS indicated formation of desired product. The reaction was quenched by adding dilute HCl and extracted with dichloromethane. Dichloromethane was washed with brine and evaporated under vacuum to give crude methyl 2-((4-fluorobenzyl)amino)thiazole-4-carboxylate (0.170 g, 0.638 mmol, 50.5 % yield). This was used for next step without purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.42 (s, 1H), 7.33-7.40 (m, 2H), 7.02-7.10 (m, 2H), 6.8 (br.s, 1H), 4.51 (s, 2H), 3.90 (s, 3H),. LC-MS (ESI): m/ z 267.1 [M + H]+. HPLC purity 81%

Step 2. In a 10 mL screw-capped reaction vial, 2-((4-fluorobenzyl)amino)thiazole-4-carboxylate (150 mg, 0.563 mmol) methyl iodide (0.070 mL, 1.127 mmol), K_2CO_3 (234 mg, 1.690 mmol) were mixed in DMF (1 mL) and heated at 60 °C for 48 h. LCMS indicated formation of required mass along with impurities. The reaction mixture was cooled to RT and filtered. Filtrate was diluted with ethyl acetate and washed with water and brine, dried on anhydrous $MgSO_4$ and evaporated to afford an oily residue. This was purified on silica gel column using 1% MeOH in DCM as an eluent. Pure fractions were collected and evaporated to give an oily material. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.45 (s, 1H), 7.25 (t, J=9 Hz, 2H), 7.04 (t, J=9 Hz, 2H), 4.73 (s, 2H), 3.91 (s, 3H), 3.08 (s, 3H),. LC-MS (ESI): m/ z 281.1 [M + H]+. HPLC purity 80%. Product was used without further purification in the next step.

*Ethyl 6-hydroxy-5-methylpyrimidine-4-carboxylate (**70**).* Yield 15%. To a solution of formamidine acetate (6.0 g, 57.69 mmol) in ethanol (70 mL) in a screw capped reaction tube was added potassium hydroxide (8.0 g, 144.12 mmol) and diethyl-2-methyl-3-oxosuccinate 1 (11.6 g 57.69mmol) at 0 ^{O}C. The reaction mixture was stirred at 100 ^{O}C for 16 h. The reaction mixture was filtered and filtrate was acidified (pH = 2) with aqueous HCl solution at 0 ^{O}C. The crude reaction mixture was dried under vacuum, dissolved in methanol, stirred for 40 minutes and filtered. The filtrate was concentrated under vacuum and was purified by column chromatography on 230-400 mesh silica gel with 2% methanol in dichloromethane as eluent to yield the final compound. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (s, 1H), 5.95 (s, 1H), 4.42 (q, J =7.20 Hz, 2H), 2.27 (d, J =8.40 Hz, 3H), 1.38 (t, J =7.4 Hz, 3H). LC-MS (APCI): m/z 183.2 [M + H]+.

*6-Hydroxy-2-methylpyrimidine-4-carboxylic acid (**71**).* Yield 22%.To a suspension of 2.52 g (12.0 mmol) of diethyloxaloacetate sodium salt in 8 mL of water was added 1.9 mL of 6.25 M NaOH(aq.), dropwise over 1 min. The mixture was stirred at ambient temperature for 40 min to give an orange solution. Next, 2.1 g (26 mmol) of formamidine hydrochloride in 2 mL of water was added. The reaction was cooled with an ice bath, and with the aid of pH paper, the pH was maintained at 11, by the addition of 6.25 M NaOH as the reaction progressed over 40 min. The pH

was then adjusted to 1 by the addition of conc. HCl, giving a white precipitate. This was filtered and dried as a light tan solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.68 (s, 1H), 2.32 (s, 3H). LC-MS (ESI): m/ z 155.1 [M + H]+. HPLC purity 99%.

*Ethyl 6-((4-fluorobenzyl)(methyl)amino)-5-methylpyrimidine-4-carboxylate (**72**).* 22% over 2 steps

Step 1: To a cooled solution of ethyl 6-hydroxy -5-methyl pyrimidine-4-carboxylate 2 (1.6 g, 8.79 mmol) in thionyl chloride (16 mL) was added catalytic amount of DMF. The reaction mixture was stirred for 1 h at reflux temperature. The reaction mixture was concentrated under vacuum and then water added and extracted with ethyl acetate. The organic layer was washed with saturated sodium bicarbonate solution. The combined organic layer was washed with water and brine, dried over sodium sulfate and concentrated under vacuum to the product, *ethyl 6-chloro-5-methylpyrimidine-4-carboxylate.* Yield 67%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.92 (s, 1H), 4.51 (q, J =1.76 Hz, 2H), 2.56 (d, J =1.52 Hz, 3H), 1.46 (q, J =1.76 Hz, 3H). LC-MS (APCI): m/ z 201.0 [M + H]+.

Step 2: To a solution of Ethyl 6-Chloro-5-methyl pyrimidine-4-carboxylate (0.5 g, 2.5 mmol) dissolved in DMF (5 mL) was added potassium carbonate (0.517 g, 3.75mmol) and 1-(4-fluorophenyl)-*N*-methyl methanamine (0.347 g, 2.5mmol). The reaction mixture was stirred at 70 °C for 3 h. Water was added to the reaction mixture and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under vacuum to get crude product. The crude was purified by column chromatography on 60-120 mesh silica gel with 20% ethyl acetate in hexanes as eluent to yield the product. Yield 33%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.63 (s, 1H), 7.26 (q, J =7.16 Hz, 2H), 7.08 (t, J =2.80 Hz, 2H), 4.66 (s, 2H), 4.45 (q, J =9.52 Hz, 2H), 3.00 (s, 3H), 2.36 (s, 3H), 1.42 (t, J =9.2 Hz, 3H). LC-MS (APCI): m/ z 304.2 [M + H]+.

*6-Chloro-2-methyl-N-phenylpyrimidine-4-carboxamide (**73**).* Yield 47%. To a suspension of 6-hydroxy-2-methylpyrimidine-4-carboxylic acid (500mg, 3.24 mmol) in dry EtOAc (20 ml) under

a nitrogen atmosphere was added oxalyl chloride (1.420 ml, 16.22 mmol) and a drop of DMF (2.51 µl, 0.032 mmol). The reaction was then heated to reflux overnight after which the excess oxalyl chloride and solvent were removed under vacuum. The dark brown oil was then resuspended in dry EtOAc (20 ml) and cooled to 0 °C. A solution of triethylamine (0.904 ml, 6.49 mmol) and aniline (363 mg, 3.89 mmol) were then added slowly and the reaction stirred for 6h. Water was then added to the mixture carefully and extracted with EtOAc (2 x 20mL). The organic layer was separated, dried over MgSO4, adsorbed onto a minimum amount of silica and purified by column chromatogrphy (EtOAc:Hex, 40:60) to afford a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.63 (s, 1H), 7.99 (s, 1H), 7.86 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.9 Hz, 2H), 7.18 (t, J = 7.4 Hz, 1H), 2.79 (s, 3H). LC-MS (ESI): m/ z 248.1 [M + H]+. HPLC purity 97%.

General procedure for synthesis of compounds **74**-**77**. To a cooled solution of methyl 2,4-dichloropyrimidine-6-carboxylate (48.30 mmol) in dry THF (50 mL) was added mixture of the appropriate substituted phenyl-*N*-methylmethanamine (53.14 mmol) and Et_3N (4.88 g, 48.30 mmol) in THF (25 mL). The reaction mixture was stirred overnight at RT. Water was added to the reaction mixture and extracted with EtOAc. The combined organic layer was washed with water, brine, dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified via column chromatography using 2% MeOH in DCM to afford to appropriate product.

*Methyl 2-chloro-6-((4-chlorobenzyl)(methyl)amino)pyrimidine-4-carboxylate (**74**).* Yield 65%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.41 (d, J = 8.3 Hz, 2H), 7.28 (d, J = 8.1 Hz, 2H), 7.22 (s, 1H), 4.85 (s, 2H), 3.87 (s, 3H), 3.12 (s, 3H). LC-MS (ESI): m/ z 327.1 [M + H]+. HPLC purity 100%.

*Methyl 2-chloro-6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-carboxylate (**75**).* Yield 51%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.59 – 6.90 (m, 5H), 4.84 (s, 2H), 3.86 (s, 3H), 3.10 (s, 3H). LC-MS (ESI): m/z 310.1 [M + H]+. HPLC purity 100%.

*Methyl 2-chloro-6-(methyl(4-(trifluoromethyl)benzyl)amino)pyrimidine-4-carboxylate (**76**).* Yield 69.3%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.72 (d, J = 7.44 Hz, 2H), 7.46 (s, 2H), 7.25 (s, 1H), 4.97 (s, 2H), 3.89 (s, 3H), 3.14 (s, 3H). LC-MS (APCI): m/z [M+H]+ 360.0.

*Methyl 2-chloro-6-(methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)pyrimidine-4-carboxylate (**77**).* Yield 70.6%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.72 (s, 1H), 7.95 (s, 1H), 7.88 (d, J = 7.60 Hz, 1H), 7.27 (s, 1H), 5.01 (s, 2H), 3.88 (s, 3H), 3.20 (s, 3H). LC-MS (APCI): m/z [M+H]+ 361.0.

Synthesis of pyrimidine 4-carboxylic esters **78-80**

Step 1: General procedure for the dechlorohydrogenation of 2-chloro pyrimidine esters. A mixture of appropriate 2-chloro-4-substituted pyrimidine ester (16.18 mmol), Pd/C, 10 %, (0.5 g) and Et_3N (32.36 mmol) in MeOH was stirred under an atmosphere of hydrogen for 4 h. Solvent was then evaporated and the mixture was then filtered through celite. Water was added to the reaction mixture and extracted with ethyl acetate. The organic layer was washed with water, brine, dried over sodium sulphate and concentrated under vacuum to give the appropriate dehalogenated ester.

methyl 6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-carboxylate. Yield 88.6%. 1H NMR (400 MHz, DMSO-d6): 8.60 (s, 1H), 7.13-7.28 (m, 5H), 4.86 (s, 2H), 3.84 (s, 3H), 3.09 (s, 3H). LC-MS (APCI): m/z [M+H]+ 276.0.

Methyl 6-(methyl(4-(trifluoromethyl)benzyl)amino)pyrimidine-4-carboxylate. Yield 81.5%. 1H NMR (400 MHz, DMSO-d6: δ 8.61 (s, 1H), 7.70 (d, J = 7.96 Hz, 2H), 7.43 (d, J = 8.00 Hz, 2H), 7.24 (s, 1H), 4.99 (s, 2H), 3.86 (s, 3H), 3.15 (s, 3H). LC-MS (APCI): m/z [M+H]+ 326.0.

Methyl 6-(methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)pyrimidine-4-carboxylate: Yield 77.4%). 1H NMR (400 MHz, DMSO-d6): δ 8.69 (s, 1H), 8.62 (s, 1H), 7.85-7.92 (m, 2H), 7.27 (s, 1H), 5.04 (s, 2H), 3.87 (s, 3H), 3.18 (s, 3H). LC-MS (APCI): m/z [M+H]+ 327.0.

Step 2: Typical procedure for the hydrolysis of pyrimidine 4-carboxylic esters Methyl 6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-carboxylate (8.0 g, 29.0 mmol) was stirred in a mixture of THF (80 mL/H2O (40 mL) and LiOH (3.0 g, 72.6 mmol). The reaction mixture was stirred at RT for 3 h. Solvents were evaporated under vacuum and the residue dissolved in water washed with EtOAc. Aqueous layer pH was adjusted with aqueous citric acid. Solids formed were stirred for 15 minutes at 0°C and filtered, washed with EtOAc, dried under vacuum to give 6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-carboxylic acid.

*6-((4-Fluorobenzyl)(methyl)amino)pyrimidine-4-carboxylic acid (**78**).* Yield 80%. ^{1}H NMR (400 MHz, DMSO-d6): δ 8.61 (s, 1H), 7.26-7.30 (m, 2H), 7.14-7.19 (m, 3H), 4.88 (s, 2H), 3.12 (s, 3H). LC-MS (APCI): m/z [M+H]+ 262.0.

*6-(Methyl(4-(trifluoromethyl)benzyl)amino)pyrimidine-4-carboxylic acid (**79**).* Yield 79.2%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.65 (s, 1H), 7.71 (d, J = 8.00 Hz, 2H), 7.45 (d, J = 8.00 Hz, 2H), 7.27 (s, 1H), 5.04 (s, 2H), 3.20 (s, 3H). LC-MS (APCI): m/z [M+H]+ 312.0.

*6-(Methyl((6-(trifluoromethyl)pyridin-3-yl)methyl)amino)pyrimidine-4-carboxylic acid (**80**).* Yield 66.4%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.67 (s, 1H), 8.46 (s, 1H), 7.84-7.88 (m, 2H), 7.13 (s, 1H), 4.99 (s, 2H), 3.14 (s, 3H). LC-MS (APCI): m/z [M+H]+ 313.0.

*2-Chloro-6-((4-chlorobenzyl)(methyl)amino)-N-(4-methoxyphenyl)pyrimidine-4-carboxamide (**81**).* Yield 31% over 2 steps.

Step 1: To a suspension of methyl 2-chloro-6-((4-chlorobenzyl)(methyl)amino)pyrimidine-4-carboxylate (200mg , 0.613 mmol) in Water (5 ml) and THF (5.00 ml) was added lithium hydroxide (44.1 mg, 1.839 mmol) and the reaction heated to 50 °C for 2h. The pH of the reaction mixture was then acidified to pH 3 with 2M HCl and the THF removed under vacuum to afford a white precipitate which was filtered and dried to afford *2-chloro-6-((4-chlorobenzyl)(methyl)amino)pyrimidine-4-carboxylic acid* (171 mg, 0.548 mmol) as a white solid. *2-chloro-6-((4-chlorobenzyl)(methyl)amino)pyrimidine-4-carboxylic acid.* Yield 89%. ^{1}H NMR

(300 MHz, DMSO-d_6) δ 7.49 – 7.36 (m, 2H), 7.28 (d, J = 8.1 Hz, 2H), 7.19 (s, 1H), 4.84 (s, 2H), 3.11 (s, 3H). LC-MS (ESI): m/z 312.0 [M + H]+. HPLC purity 98%.

Step 2: A solution of 2-chloro-6-((4-chlorobenzyl)(methyl)amino)pyrimidine-4-carboxylic acid (113mg, 0.362 mmol), HATU (206 mg, 0.543 mmol)) and DIPEA (0.190 ml, 1.086 mmol) were stirred for 5 mins, after which 4-methoxyaniline (53.5 mg, 0.434 mmol) was added and the solution stirred at RT for 4h. Water was then added and the precipitate filtered and dried to afford a yellow solid. The yellow solid was then dissolved in DCM, adsorbed onto silica and purified by silica flash chromatography (EtOAc:Hex, 60:40) to afford a yellow gum-like substance which was recrytallised from EtOH to give a beige solid. Yield 35%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.21 (s, 1H), 7.75 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.34 – 7.14 (m, 3H), 6.94 (d, J = 8.9 Hz, 2H), 4.88 (s, 2H), 3.76 (s, 3H), 3.15 (s, 3H). LC-MS (ESI): m/z 417.0 [M + H]+. HPLC purity 97%

*2,2,2-Trifluoro-1-(6-((4-fluorobenzyl)(methyl)amino)pyrimidin-4-yl)ethan-1-one (**83**).* Yield 46% over 2 steps.

Step 1: *6-((4-fluorobenzyl)(methyl)amino)-N-methoxy-N-methylpyrimidine-4-carboxamide.* Yield 59%. To a solution of 6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-carboxylic acid (200mg, 0.766 mmol), HATU (437 mg, 1.148 mmol) and DIPEA (0.401 ml, 2.297 mmol), stirred for 5 mins in a round bottomed flask in DMF (4 ml), was added *N*,*O*-dimethylhydroxylamine hydrochloride (90 mg, 0.919 mmol) and the reaction stirred for a further 4h at 25 °C. A 10% aqueous solution of LiCl (30mL) was then added and the solution extracted with DCM (2 x 15mL). The organic layers were collected and dried with $MgSO_4$ and adsorbed onto silica. The silica residue was then purified by silica column chromatography (4:1, EtOAc:Hexane), to afford the product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.67 (s, 1H), 7.22 – 7.10 (m, 2H), 7.09 – 6.91 (m, 2H), 6.75 (s, 1H), 4.84 (s, 2H), 3.77 (s, 3H), 3.35 (s, 3H), 3.08 (s, 3H).

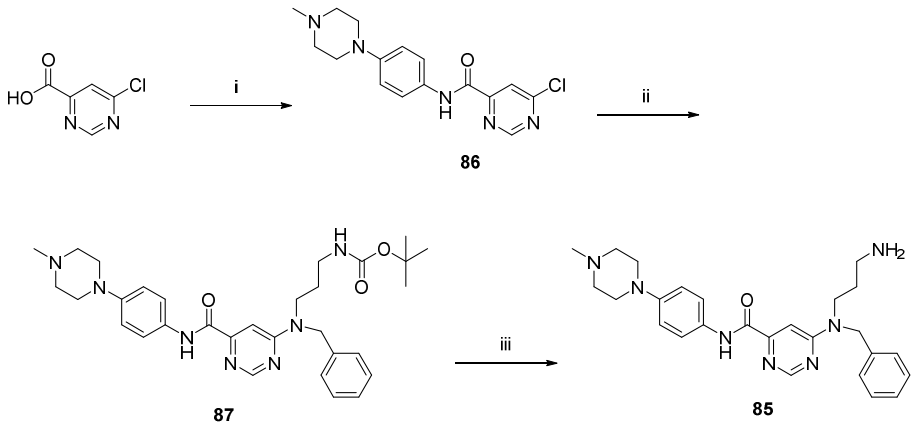
Step 2: *2,2,2-trifluoro-1-(6-((4-fluorobenzyl)(methyl)amino)pyrimidin-4-yl)ethan-1-one* ***(83)***. To a solution of 6-((4-fluorobenzyl)(methyl)amino)-*N*-methoxy-*N*-methylpyrimidine-4-carboxamide (100mg, 0.329 mmol) and cesium fluoride (9.98 mg, 0.066 mmol) in Toluene (2 ml) at 0 °C was added trimethyl(trifluoromethyl)silane (0.097 ml, 0.657 mmol) slowly over 10 mins and the reaction allowed to stir overnight at room temperature. On complete conversion to the silylated intermediate (monitered by LC-MS), Water (0.6 ml) and TBAF (1M in THF) (0.657 ml, 0.657 mmol) were added and the reaction warmed up to 50 oC for 2 hrs. The reaction was then cooled to room temperature and diluted with EtOAc (15mL) and the organic layer washed with brine (1 x 10mL) and water (1 x 10mL). The organics were then separated and dried over $MgSO_4$ and adsorbed onto silica. The silica residue was then purified by silica column chromatography (6:4, EtOAc:Hexane), to afford the product. Yield 78%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (s, 1H), 7.22 – 7.13 (m, 2H), 7.00 (t, J = 8.6 Hz, 2H), 6.82 (s, 1H), 4.84 (s, 2H), 3.06 (s, 3H). LC-MS (ESI): m/z 314.1 [M + H]+. HPLC purity 95%

6-((4-Fluorobenzyl)(methyl)amino)pyrimidine-4-carbohydrazide ***(84)***. Yield 71%. A solution of methyl 6-((4-fluorobenzyl)(methyl)amino)pyrimidine-4-carboxylate (100mg, 0.363 mmol) and hydrazine hydrate (36.4 mg, 0.727 mmol) was refluxed in MeOH (10 ml) overnight. The reaction was then cooled to room temperature, the MeOH removed and the residue dissolved with EtOAc (15mL). The organic layer was then washed with brine (1 x 10mL) and water (1 x 10mL). The organics were then separated and dried over $MgSO_4$ and removed under vacuum to afford the product which was used in the next step without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.87 (s, 1H), 8.55 (d, J = 1.1 Hz, 1H), 7.28 (dd, J = 8.6, 5.6 Hz, 2H), 7.16 (ddd, J = 8.8, 6.0, 3.3 Hz, 3H), 4.86 (s, 2H), 4.61 (s, 2H), 3.12 (s, 3H).

2) Chemoproteomics materials and methods

2.1 Synthesis of chemoproteomic biological probes.

Scheme S1. Synthesis of the linkable analogue 85.



Reagents and conditions: (i) 4-(4-methylpiperazin-1-yl)aniline, DIPEA, T_3P, isopropylacetate, RT (ii) tert-butyl (3-(benzylamino)propyl)carbamate, DIPEA, ACN, 100 ºC, MW (iii) TFA, DCM, RT.

The synthesis of **85** was performed in a three-step reaction as follows:

Under nitrogen atmosphere, 6-chloropyrimidine-4-carboxylic acid (300 mg, 1.892 mmol) was placed in isopropyl acetate (10 mL) and cooled to 0 ºC. *N*-ethyl-*N*-isopropylpropan-2-amine (0.991 mL, 5.68 mmol) and T_3P (50% in AcOEt) (2.253 mL, 3.78 mmol) were added dropwise and the yellow solution formed was stirred at 0 ºC for 30 min. 4-(4-methylpiperazin-1-yl)aniline (362 mg, 1.892 mmol) was added and the reaction was left to reach room temperature and stirred overnight. After 16 h the reaction was quenched with $NaHCO_3$ and AcOEt was added. The organic phase was separated and washed again with $NaHCO_3$ twice and then brine. It was dried over $MgSO_4$, filtered and concentrated. The crude was purified using a 9 g silica column (20 mL.min-1) on an Isco Brand auto column and was eluted with 50% EtOAc:EtOH(3:1)/cyclohexane to afford desired product 6-chloro-*N*-(4-(4-methylpiperazin-1-yl)phenyl)pyrimidine-4-carboxamide (**86**, 210 mg, 33.4% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.69 (s, 1H), 9.25 (d, *J*=1.0 Hz, 1H), 8.18 (d, *J*=1.3 Hz, 1H), 7.74 (d, *J*=9.1 Hz, 2H), 6.94 (d,

J=9.3 Hz, 2H), 3.06-3.18 (m, 4H), 2.40-2.47 (m, 4H), 2.22 ppm (s, 3H). LCMS (ES) [M]+ calculated for $C_{16}H_{18}ClN_5O$ 331.120, found 332.20.

tert-butyl (3-(benzylamino)propyl)carbamate (96 mg, 0.362 mmol) was dissolved in acetonitrile (2 mL) and 6-chloro-*N*-(4-(4-methylpiperazin-1-yl)phenyl)pyrimidine-4-carboxamide (**86**, 100 mg, 0.301 mmol) and *N*-ethyl-*N*-isopropylpropan-2-amine (0.158 mL, 0.904 mmol) were added. The reaction was heated for 1h 30min at 100 ºC under MW radiation. The reaction mixture was concentrated under reduced pressure and the crude obtained was triturated with t-BuMe ether. After filtration, solid was discarded and the filtrate was purified using a supercritical HPLC, affording tert-butyl (3-(benzyl(6-((4-(4-methylpiperazin-1-yl)phenyl)carbamoyl)pyrimidin-4-yl)amino)propyl)carbamate (**87**, 24 mg, 0.043 mmol, 14.23 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.82 (s, 1H), 8.62 (s, 1H), 7.65 (d, *J*=8.8 Hz, 2H), 7.29-7.42 (m, 3H), 7.19 (d, *J*=7.3 Hz, 2H), 6.95 (d, *J*=9.1 Hz, 2H), 4.61-5.01 (m, 2H), 3.49-3.90 (m, 2H), 3.06-3.26 (m, 6H), 2.52-2.66 (m, 4H), 2.36 (s, 3H), 1.83 (br. s., 2H), 1.46 ppm (s, 9H). LCMS (ES) [M]+ calculated for $C_{31}H_{41}N_7O_3$ 559.327, found 560.50.

tert-Butyl (3-(benzyl(6-((4-(4-methylpiperazin-1-yl)phenyl)carbamoyl)pyrimidin-4-yl)amino) propyl)carbamate (**87**, 44 mg, 0.079 mmol) was dissolved in DCM (1 mL) and Trifluoroacetic acid (TFA) (0.060 mL, 0.786 mmol) was added. The reaction mixture was stirred at room temperature and monitored by TLC. After completion of the reaction, reaction mixture was evaporated to dryness and the crude obtained was dissolved in MeOH and loaded onto an Isolute SCX column. The material was washed with MeOH and then the desired compound was eluted off of the column using 7.0 N Ammonia in MeOH. The solvent was removed to afford the desired product, 6-((3-aminopropyl)(benzyl)amino)-*N*-(4-(4-methylpiperazin-1-yl)phenyl)pyrimidine-4-carboxamide (**85**, 30.4 mg, 0.066 mmol, 84 % yield), as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.83 (s, 1H), 8.61 (s, 1H), 7.65 (d, *J*=8.8 Hz, 2H), 7.41 (br. s., 1H), 7.28-7.36 (m, 2H), 7.20 (d, *J*=7.1 Hz, 2H), 6.94 (d, *J*=9.1 Hz, 2H), 4.86 (br. s., 2H), 3.38-4.07 (m, 2H), 3.10-3.28 (m, 4H), 2.75 (t, *J*=6.7 Hz, 2H),

2.52-2.65 (m, 4H), 2.36 (s, 3H), 1.74-1.86 ppm (m, 2H). LCMS (ES) [M]+ calculated for $C_{26}H_{33}N_7O$ 459.275, found 460.

2.2) Protein Lysates preparation

The *M. bovis* BCG extracts were prepared as follows: *M. bovis* BCG was cultured in 7H9 medium supplemented with 2% (w/v) glucose and 0.025% (v/v) tyloxapol at 37 ºC for 8–10 days to reach an OD600nm of 0.8–1.0. The culture was centrifuged and the pellet was washed with PBS and 0.025% (v/v) tyloxapol. The pellet was resuspended in lysis buffer (50mM Tris-HCl, pH 7.5, 0.8% (v/v) NP40, 1.5mM $MgCl_2$, 5% glycerol, 150mM NaCl, 25mM NaF, 1mM Na_3VO_4, 1mM dithiothreitol (DTT) and 1 tablet per 10 ml of Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Life Science ref. 05892791001)). Samples were sonicated for three cycles at 50% amplitude for 30s (VCX750 Sonics-VibracellTM) on ice. This lysate was ultracentrifuged (140.000 g) at 4 ºC for 60 min and the cellular debris was discarded. Protein lysates were snap frozen and stored at -80 ºC.

2.3) MIC evaluation BCG

The minimum inhibitory concentration (MIC) of the compounds against *M. bovis* BCG was determined using the Resazurin fluorescent method. Plates were dispensed with each compound performing 10 two-fold dilutions starting at 50 µM. Plates were inoculated with 200 µl of 10^5 CFU/ml of *M. bovis* BCG. Mycobacterium were grown in 7H9 media and supplemented with 0.025% Tyloxapol and 2% Glucose. Plates were incubated at 37ºC for 6 days. After that period, 25ul of Resazurin solution (Fisher Chemicals, R/0040/79) were added and plates were incubated for another 48 h. Finally, endpoint fluorescence was measured using M5 Spectramax (Molecular Devices) (settings: excitation 530nm, emission 590nm, cutoff 570nm).

2.4) Chemoproteomics:

Chemoproteomics experiments were performed as previously described.[1] Briefly, sepharose beads were derivatized with **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 $MgCl_2$, 5 % Glycerol, 150 mM NaCl, 25 mM NaF, 1 mM Na_3VO_4, 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 test compound or DMSO (vehicle control). Our experimental design employed isobaric tandem mass tags (TMT)[2], 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 IC_{50} 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 IC_{50} values by adding compound over a range of 6 concentrations (highest concentration 30 μM, then 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.[1] 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.[1] 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.tbdb.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.[2] Proteins identified with >1 unique peptide matches were considered for further data analysis. Raw data tables for the chemoproteomics experiments can be found in the Supplementary Excel spreadsheet provided.

3) Microbiology assays

3.1) GAST/Fe MIC_{90} determinations

The broth microdilution method[3,4] allows a range of antibiotic concentrations to be tested on a single 96-well microtitre plate in order to determine the minimum inhibitory concentration (MIC). Briefly, a 10 mL culture of *Mycobacterium tuberculosis* (H37RvMa)[5] is grown to an OD_{600} of 0.6 - 0.7. The culture is then diluted 1:100 in GAST/Fe medium. In a 96-well microtitre plate, 50 μL of GAST/Fe medium is added to all wells from Rows 2-12. The compounds to be tested are added to Row 1 in duplicate, at a final concentration of 640 μM (stocks are made up to a concentration of 12.8 mM in DMSO, and diluted to 640 μM in GAST/Fe medium). A two-fold serial dilution is prepared, by transferring 50 μL of the liquid in Row 1 to Row 2 and aspirating to mix. 50 μL of the liquid in Row 2 is then transferred to Row 3 and aspirated, and so on. This procedure is repeated until Row 12 is reached, from which 50 μL of the liquid is discarded so as to bring the final volume in all wells to 50 μL. Finally, 50 μL of the 1:100 diluted *M. tuberculosis* culture is added to all wells in Rows 2-12. Cells are not added to Row 1, as this serves as a contamination control. Controls include media only, 5% DMSO, Rifampicin and Kanamycin. The microtitre plate is stored is secondary container and incubated at 37°C with humidifier to prevent evaporation of liquid. The lowest concentration of drug that inhibits growth of more than 90% of the bacterial population is considered to be the MIC_{90}. MIC_{90} values are scored visually at 7-days and 14-days post inoculation, and digital images captured and stored.

3.2) GFP assay and activity of selected compounds against mutant *Mtb* strains.

The minimum inhibitory concentration (MIC) of compounds was determined in either 7H9 (Difco) medium supplemented with 0.05% Tween-80, 0.2% glycerol and albumin/NaCl/ glucose (ADC) complex, and/or in GAST-Fe (glycerol–alanine–salts) medium pH 6.6, supplemented with 0.05% Tween-80.[6] Compounds were dissolved in DMSO to a final stock concentration of 12.8mM. MICs against H37RvMa, Δ*cyd*KO and Δ*cyd*KO/A317T mutants[7] were determined using the standard broth microdilution method described previously.[8] MICs against the *Mtb*::*gfp* strain (H37RvMa::pMSP12GFP)[9] were determined as described previously[10], with some modifications. Briefly, a two-fold serial dilution of the compound was carried out across a 96-well plate from Row 2-11 (20-0.039µM) in 7H9 or GAST-Fe with a final DMSO concentration of 5% v/v. The minimum growth control (rifampicin at 2xMIC) was added to row 12 and the maximum growth control (5% DMSO in 7H9 or GAST-Fe) was added to row 1. A *Mtb*::*gfp* stock culture was grown to an OD_{600}-0.6, diluted (1:500 for 7H9 or 1:100 for GAST/Fe) and 50µL added to each well. Plates were incubated at 37°C and fluorescence (excitation 485nM; emission 520nM) measured using a plate reader (FLUOstar OPTIMA, BMG LABTECH) at day 7 and day 14. Data were normalised to the minimum and maximum inhibition controls to generate a dose response curve (% inhibition) from which the MIC_{90} was calculated. The raw data are archived and analysed using the CDD Vault from Collaborative Drug Discovery (https://support.collaborativedrug.com/forums/20625822-Dose-Response-Toolkit).

3.3) Microplate Alamar Blue Assay for MIC determination of clinical *M. tuberculosis* isolates.

The microdilution method described in 2.1 and 2.2 was adapted to determine the MICs of selected compounds against clinical strains of *M. tuberculosis* in 7H9 medium. The activity of the compounds was measured on days 7 and 14 by adding 10 µL Alamar Blue (Celtic Diagnostics) to all wells followed by reincubation of the plates at 37°C for 24 hr. Wells were then viewed for a colour change as blue indicated no growth as opposed to pink which suggested bacterial growth.

The MIC_{90} was defined as the lowest drug concentration where a colour change was not observed.[11]

3.4) Bioluminescence reporter assays

Reporter assays were set up as described previously[12], with minor modifications. Briefly, mutant *M. tuberculosis* strains carrying either P*iniB*-LUX or P*recA*-LUX reporter constructs were utilized in broth microdilution assays in which luminescence was recorded at specific intervals (24 hr, 48 hr, 72 hr, 144 hr) over a range of drug concentrations spanning the known MIC_{99} for each compound. Isoniazid and ciprofloxacin were utilized as the maximum luminescence controls for P*iniB*-LUX and P*recA*-LUX assays, respectively, and relative luminescence values (RLU) determined for each drug concentration at each time-point. Data were plotted as heat-maps, ranging from white (minimum) to red (maximum) as a function of the maximum RLU recorded for the control at each time-point.

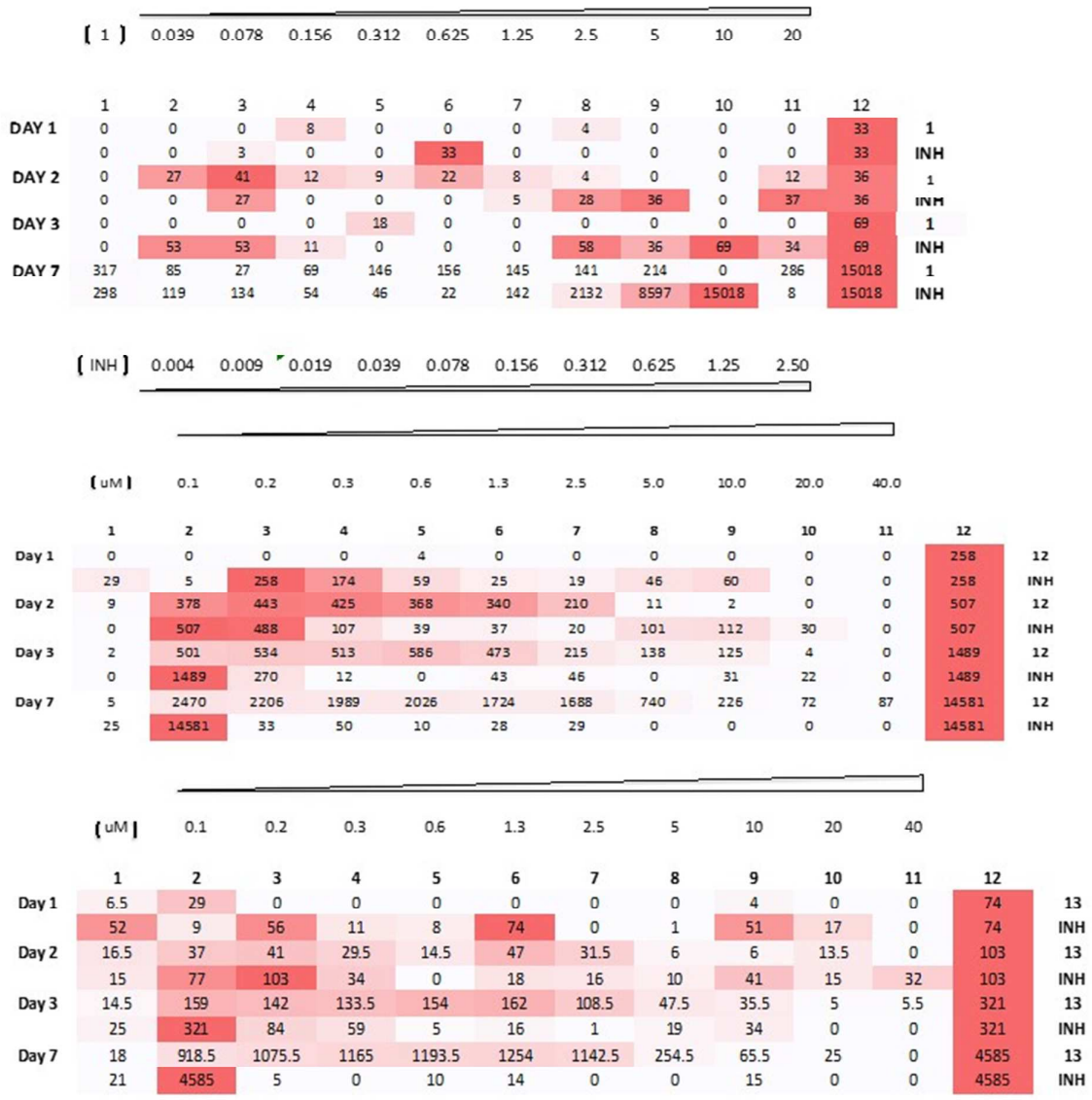


Figure S1: *M. tuberculosis* cell wall metabolism is not a direct target of the carboxamide compounds. Relative luminescence values (RLU) were determined for each drug concentration at each time-point, as indicated by the numerical values in each well. Data are plotted as heat-maps, ranging from white (minimum) to red (maximum) as a function of the maximum RLU recorded for the isoniazid (INH) control at the corresponding time-point. As reported previously[12], exposure to INH results in an immediate bioluminescent signal at day 1 and this is sustained over the full 7 days of the assay. In contrast, the carboxamide compounds either showed no response

(compound **1**) or a weak, delayed response (compounds **12**, **13**) suggesting that cell wall homeostasis is not the primary target of these compounds in *M. tuberculosis*.

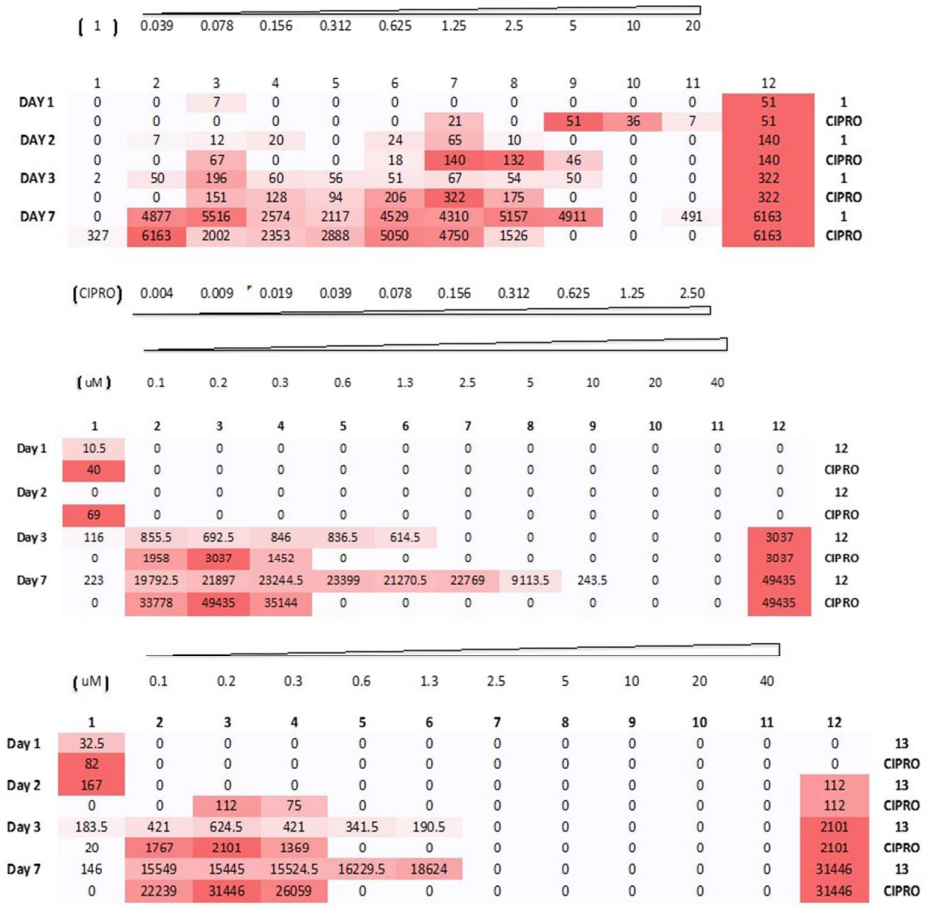


Figure S2: Exposure to the carboxamide compounds induces a slightly delayed DNA damage response in *M. tuberculosis*. Relative luminescence values (RLU) were determined for each drug concentration at each time-point, as indicated by the numerical values in each well. Data are plotted as heat-maps, ranging from white (minimum) to red (maximum) as a function of the maximum RLU recorded for the ciprofloxacin (CIPRO) control at the corresponding time-point. As reported previously[12], exposure to CIPRO results in an immediate bioluminescent signal at day 1 and this is sustained over the full 7 days of the assay. Notably, compounds **1, 12**, and **13** were all associated with a delayed (Day 3 onwards) induction of luminescence, suggesting that DNA metabolism may represent a downstream target of these compounds in *M. tuberculosis.*

4) *In vitro* ADME- assays

4.1) Kinetic solubility

The kinetic solubility assay was performed using a miniaturised shake flask method. 10 mM stock solutions of each of the test compounds were used to prepare calibration standards (10-220 μM) in DMSO, and to spike (1:50) duplicate aqueous samples of FaSSIF (simulating fasting state biorelevant media, pH 6.5), with a final DMSO concentration of 2%. After shaking for 2 hours at 25 °C, the solutions were filtered and analysed by means of HPLC-DAD (Agilent 1200 Rapid Resolution HPLC with a diode array detector). Best fit calibration curves were constructed using the calibration standards, which were used to determine the aqueous samples' solubility.[13]

4.2) Microsomal stability

The metabolic stability assay was performed in duplicate in a 96-well micro titre plate. The test compounds (0.1 μM) were incubated individually in mouse, rat and pooled human liver microsomes (0.4 mg/mL) at 37 °C for predetermined time points, in the presence and absence of the cofactor NADPH (1 mM). Reactions were quenched by adding 300 μL of ice cold acetonitrile containing internal standard (carbamazepine, 0.0236 μg/mL). Test compounds in the supernatant were analysed by means of LC-MS/MS (Agilent Rapid Resolution HPLC, AB SCIEX 4000 QTRAP MS) for the disappearance of parent compound. Metabolite searches were not conducted during the metabolic stability assay.[14]

4.3) Plasma protein binding (PPB) assay

The plasma protein binding assay was performed in a 96-well micro titre plate with pooled human plasma, spiked with test compound (5 μM). An aliquot was immediately removed and quenched using ice cold acetonitrile containing internal standard (carbamazepine, 0.0236 μg/mL), and placed in the freezer. This served as the total concentration sample. After pre-incubation (37 °C for 1 hour) duplicate aliquots of the spiked plasma were transferred to ultra-centrifugation tubes,

and ultracentrifuged for 4 hours (42000 rpm, 37 °C, Beckman Optima L-80XP). Analyte concentration of all compounds and sample types were determined by means of LC-MS/MS (Agilent Rapid Resolution HPLC, AB SCIEX 4000 QTRAP MS).

4.4) Lipophilicity ($LogD_{7.4}$)

The lipophilicity assay was performed in triplicate using a shake-flask procedure. 10 mM stock solutions of each test compound were used to spike (100 μM) a 1:1 mixture of phosphate buffer (pH 7.4) and *n*-octanol. The solutions were shaken vigorously (1500 rpm) on an orbital shaker for 3 hours at room temperature. Thereafter the samples were centrifuged in order to fully separate the two immiscible fluids. The samples were analysed by HPLC-DAD (Agilent 1200 Rapid Resolution HPLC with a diode array detector) and the amount of compound in the buffer and *n*-octanol were used to determine the partition coefficient, $LogD_{7.4}$.[15]

4.5) Parallel Artificial Membrane Permeability Assay (PAMPA):

The PAMPA assay was performed in triplicate in 96-well MultiScreen Filter plates (Millipore, 0.4 μM PCTE Membrane). Membrane filters were pre-coated with 5% hexadecane in hexane and allowed to dry prior to the assay. Membrane integrity marker, Lucifer yellow was added to the apical wells of the pre-coated MultiScreen plate donor/drug solutions containing test compound. Phosphate buffer (pH7.4) was added to the 96-well acceptor plate. 10 mM test compound was used to spike (1 μM) the donor buffer at physiologically relevant pH's (pH 4, 6.5 and 8), the donor plate slotted into the acceptor plate and incubated (4 hours at room temperature) with gentle shaking (40-50 rpm). Following the incubation, sample from the acceptor wells and theoretical equilibrium wells were transferred to the analysis plate and matrix matched with blank donor buffer. Acetonitrile containing internal standard (carbamazepine, 0.0236 μg/mL) was added to all samples and they were analysed by LC-MS/MS (Agilent Rapid Resolution HPLC, AB SCIEX 4500 MS). The normalised (analyte/internal) peak areas were used to calculate the apparent permeability (P_{app}). Membrane integrity was assessed by calculating the P_{app} of Lucifer Yellow

(acceptable values <50nm/s) using a Modulus microplate reader (Excitation 490nm/Emission 510-570nm).[16]

4.6) Cytotoxicity

Compounds were screened for *in vitro* cytotoxicity against VERO (kidney epithelial cells extracted from an African green monkey) or Chinese Hamster Ovarian (CHO) mammalian cell-lines, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays.[14] The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The tetrazolium ring is cleaved in active mitochondria. Thus only viable cells are able to reduce the water-soluble yellow coloured MTT to water-insoluble purple coloured formazan. Formazan crystals are dissolved in DMSO. The test samples were tested in triplicate on one occasion.

The test samples were prepared to a 20 mg/mL stock solution in 100% DMSO. Stock solutions were stored at -20 ºC. Further dilutions were prepared in complete medium on the day of the experiment. Samples were tested as a suspension if not completely dissolved. Emetine was used as the reference drug in all experiments. The initial concentration of emetine was 100 µg/mL, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/mL. The same dilution technique was applied to the all test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability (data not shown). The 50% inhibitory concentration ($IC_{50)}$ values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

4.7) hERG assay

Electrophysiological recordings were made from a Chinese Hamster Lung cell line stably expressing the full length hERG channel. Single cell ionic currents were measured in the

perforated patch clamp configuration (100μgml-1) amphoterocin) at room temperature (21-23^{0}C) using an Ion Works Quattro instrument. The internal solution contained (mM): 140 KCl, 1 $MgCl_2$, 1 EGTA, 20 HEPES and was buffered to pH 7.3. The external solution contained (mM): 138 NaCl, 2.7 KCl, 0.9 $CaCl_2$, 0.5 $MgCl_2$, 8 Na_2HPO_4, 1.5 KH_2PO_4 also buffered to pH 7.3. Cells were clamped at a holding potential of -70 mV for 30s and then stepped to +40 mV for 1s. This was followed by a hyperpolarising step of 1s to -30 mV to evoke the hERG tail current. This sequence was repeated 5 times at a frequency of 0.25Hz. Currents were measured from the tail step at the 5th pulse, and referenced to the holding current. Compounds were then incubated for 6-7 minutes prior to a second measurement of the hERG signal using an identical pulse train.

5) Metabolite identification studies

***In vitro* incubation with mouse liver microsomes (MLM)**

The test compounds **12** and **51** (10 μM) were incubated at 37 °C in a solution containing 1 mg/ml microsomes (MLM; mouse BALB, Xenotech), magnesium chloride (5 mM) and NADPH (1 mM) in phosphate buffer (100 mM, pH 7.4), for 1 hour while shaking. The samples were then prepared by ice-cold acetonitrile precipitation and filtered for LC-MS/MS analysis. Controls containing all the sample constituents (not incubated), and in which NADPH or microsomes were individually excluded were also prepared and handled similarly to the test sample. Propranolol (10 μM) was incubated concomitantly as a positive control.

Metabolite identification

Metabolites formed in microsomal incubations of **12** and **51** were identified by comparison of the T60 chromatogram with chromatograms at T0 and in the no NADPH control using Lightsight v2.3. The tentative identity of the metabolite was deduced by comparison of the product ion spectra of the $[M+H]^+$ ions of the metabolite with that of parent compound. The retention time and fragmentation pattern was also compared to that of synthetic standard, where available, to confirm the identity.

Analytical conditions:

HPLC system: Agilent 1200 Rapid Resolution (600 bar) HPLC

HPLC column: Supelco Ascentis C_{18}, 4.6 mm x 150 mm, 2.6 μm particles

Column temperature: 40 °C

Mobile phase A: 0.1% Formic acid 95/5 Mobile phase B: 95% 0.1% Formic acid in Acetonitrile 95/5

Mobile phase flow rate: 0.4 mL/min (gradient elution)

Injection volume: 10 μL

Sample tray temp.: 8 °C

MS system: AB SCIEX 4000 QTRAP MS, equipped with a Turbo V™ ion source. Analyst 1.5.1 software was used for instrument control and data acquisition. LightSight v2.3 software was used for metabolite identification data processing.

Ionization mode: ESI positive

Acquisition mode: Information dependent

Survey scan mode: Enhanced MS

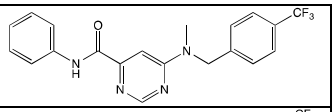
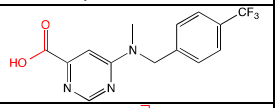
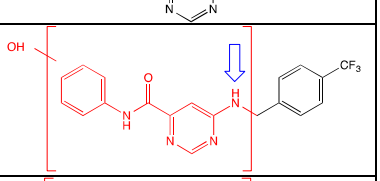
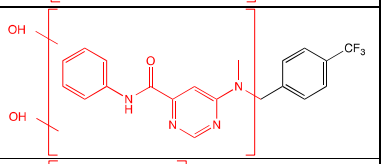
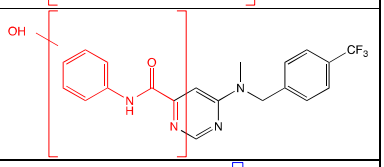
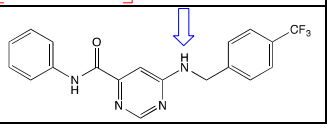
MS/MS scan mode: Enhanced product ion scan

Metabolite identification results of 12

The *in vitro* metabolism of **12** in mouse liver microsomes resulted in the formation of two main metabolites: the carboxylic acid derivative M1 and one monohydroxylation metabolite M4. Three additional metabolites resulting from dihydroxylation (M3), demethylation (M5) and demethylation + hydroxylation (M2) were also identified (Table S1).

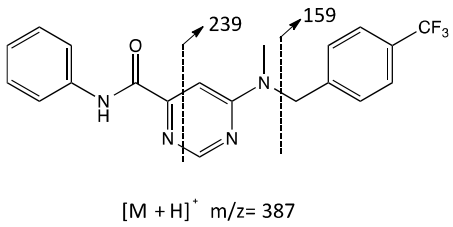
Table S1: Metabolites identified in MLM incubations of 12.

Drug and metabolites	Retention time (min)	$[M + H]^+$ (*m/z*)	Relative amounts (%)[b]	Biotransformation	Diagnostic ions (m/z)		Tentative Identity
					A	B	

12	9.72	387	56	-	239	159	
M1[a]	6.22	312	28	Conversion to COOH	239	159	
M2	7.52	389	<1	Hydroxylation + demethylation	-	159	
M3	7.74	419	<1	Dihydroxylation	-	159	
M4	8.44	403	12	Hydroxylation	239	159	
M5	8.87	373	2	N-demethylation	225	159	

[a]identification confirmed using a synthetic standard

[b]Relative amounts calculated by Lightsight as a percentage of total peak area (parent and metabolites), assuming similar MS response



$[M+H]^+$ m/z= 387

Figure S3: Diagnostic ions in the product ion spectra of **12** and its metabolites.

The formation of the carboxylic acid derivative was MLM dependent but not NADPH dependent, implying that hydrolases may contribute to its formation. The metabolism of the four other metabolites (M2-M5) was MLM and NADPH dependent.

The diagnostic ions i) showed that the hydroxylation site of the main metabolite M4 is on the phenyl ring and ii) confirmed that the demethylation occurred on the N- adjacent to the pyrimidine core (Figure S3).

The fragmentation of the compound, and the enhanced product ion spectra of the metabolites are presented in Figures S4.1-S4.6.

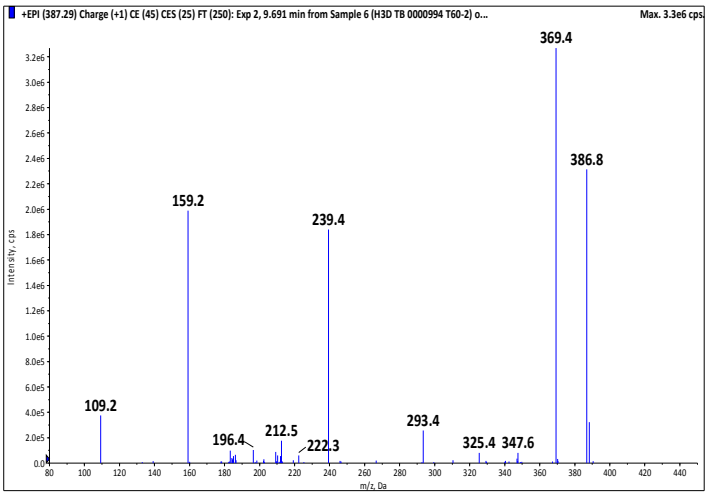


Figure S4.1: Product ion spectrum of the $[M + H]^+$ ion of **12**

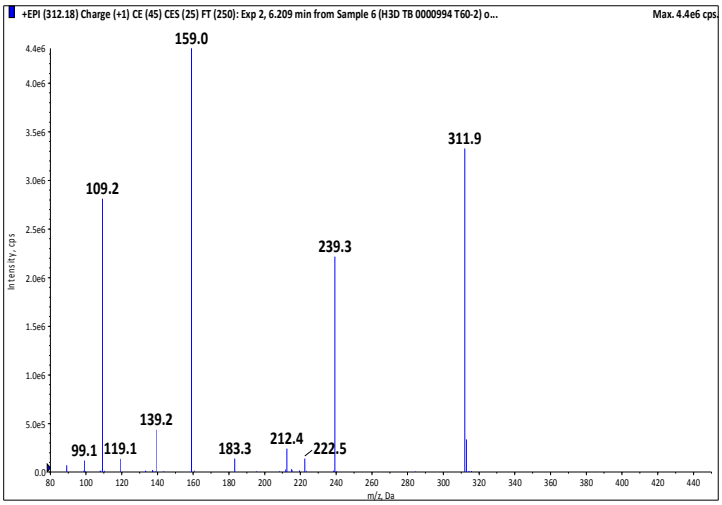


Figure S4.2: Product ion spectrum of the $[M + H]^+$ ion of M1

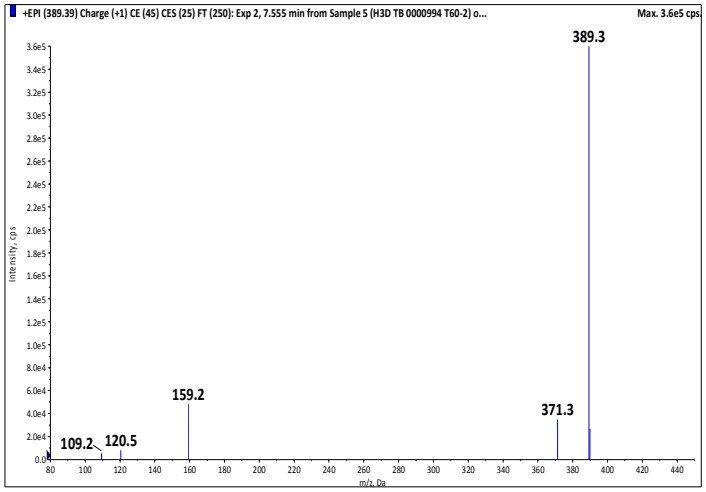


Figure S4.3: Product ion spectrum of the $[M + H]^+$ ion of M2

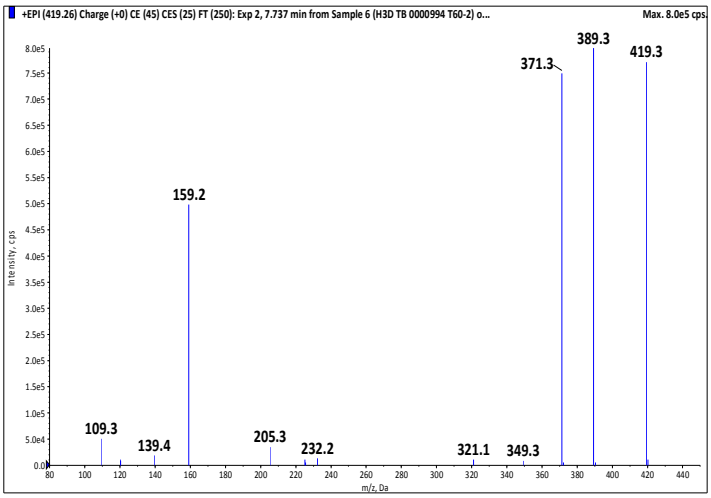


Figure S4.4: Product ion spectrum of the $[M + H]^+$ ion of M3

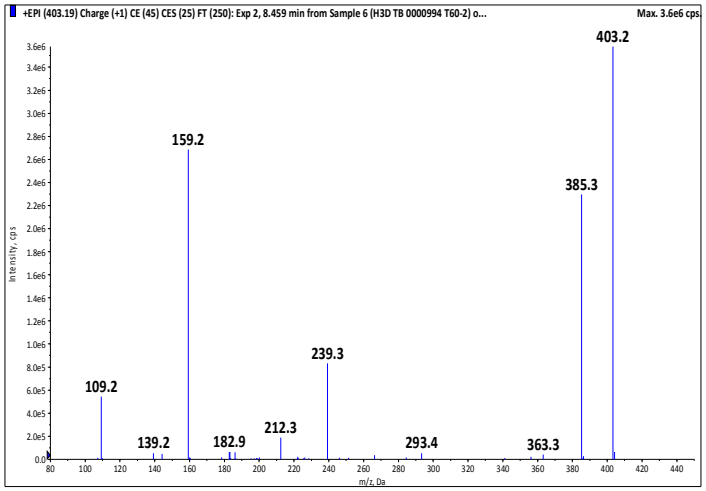


Figure S4.5: Product ion spectrum of the $[M + H]^+$ ion of M4

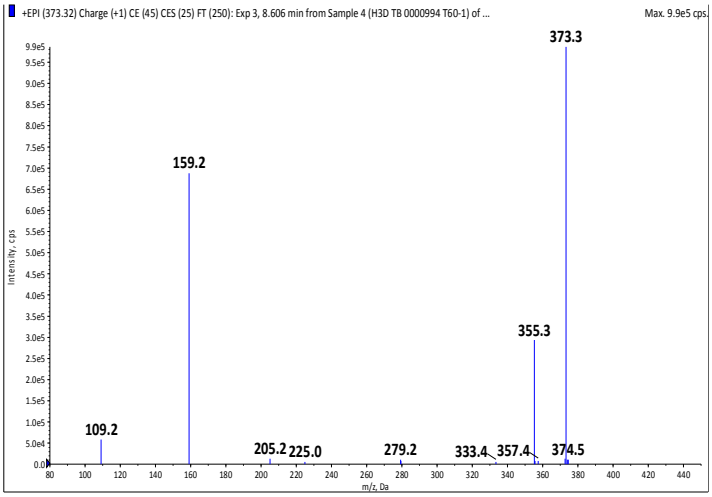
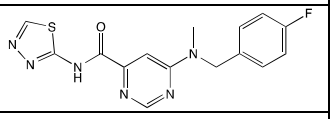
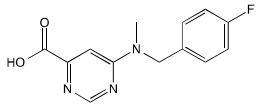
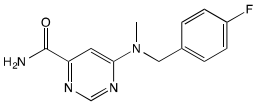
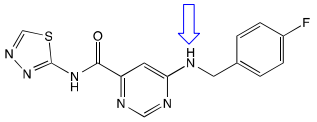
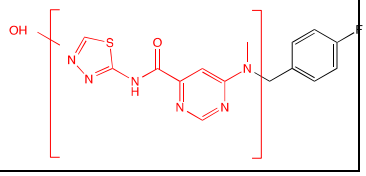


Figure S4.6: Product ion spectrum of the $[M + H]^+$ ion of M5

Metabolite identification results of 51

The *in vitro* metabolism of **51** in mouse liver microsomes resulted mainly in the formation of the carboxylic acid and a N-demethylated metabolite. The amide derivative and one hydroxylation metabolite were also detected (Table S2).

Table S2: Metabolites identified in MLM incubations of 51

Drug and metabolites	Retention time (min)	$[M + H]^+$ (*m/z*)	Relative amounts (%)[b]	Biotransformation	Diagnostic ions (m/z)			Tentative identity
					A	B	C	
51	7.65	345	64	-	303	189	109	
M1[a]	5.55	262	11	Conversion to COOH	-	189	109	
M2	6.60	261	6	Conversion to $CONH_2$	-	189	109	
M3	7.03	331	18	N-demethylation	289	175	109	
M4	7.58	361	1	Hydroxylation	-	-	109	

[a]identification confirmed using a synthetic standard

[b]Relative amounts calculated by Lightsight as a percentage of total peak area (parent and metabolites), assuming similar MS response

The formation of the carboxylic acid derivative was MLM dependent but not NADPH dependent, implying that hydrolases also contribute to its formation. The formation of the other metabolites was MLM and NADPH-dependent.

The diagnostic ions i) showed that the hydroxylation site is on the left side of the molecule, excluding the F-substituted phenyl ring and ii) confirmed that the demethylation occurred on the N- adjacent to the pyrimidine core (Figure S5).

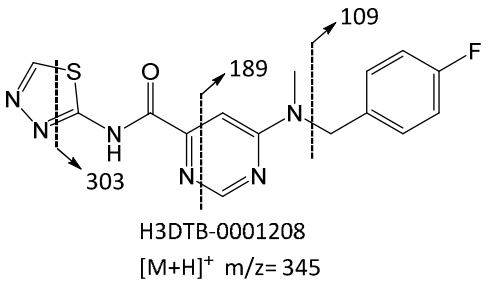


Figure S5: Diagnostic ions in the product ion spectra of 51 and its metabolites.

The fragmentation of the compound, together with the chromatograms of the microsomal incubations and the enhanced product ion spectra of the metabolites are presented in Figures S5.1 to S5.5.

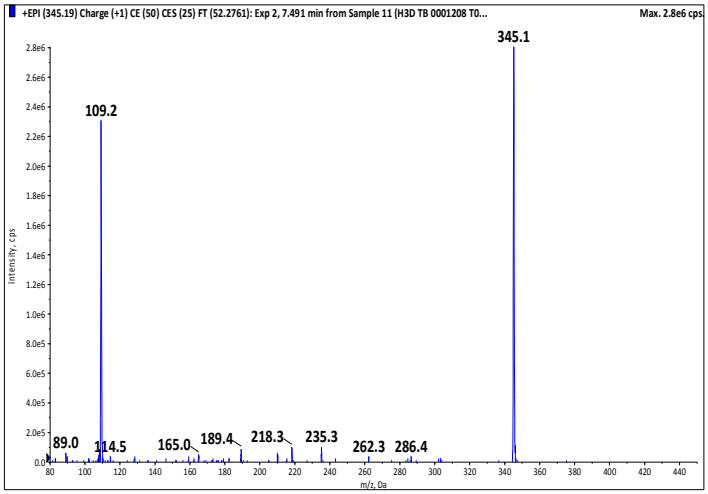


Figure S5.1: Product ion spectrum of the $[M + H]^+$ ion of **51**

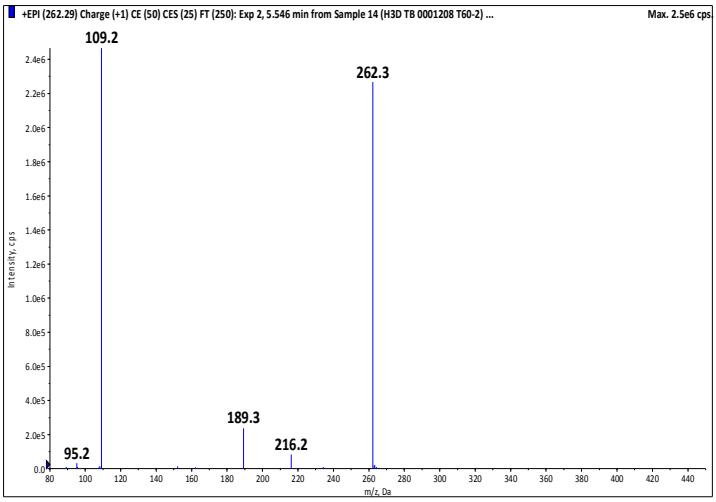


Figure S5.2: Product ion spectrum of the $[M + H]^+$ ion of M1

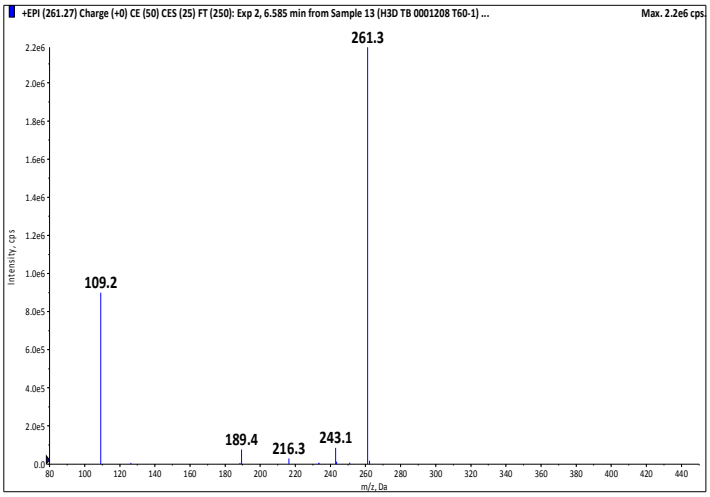


Figure S5.3: Product ion spectrum of the $[M + H]^+$ ion of M2

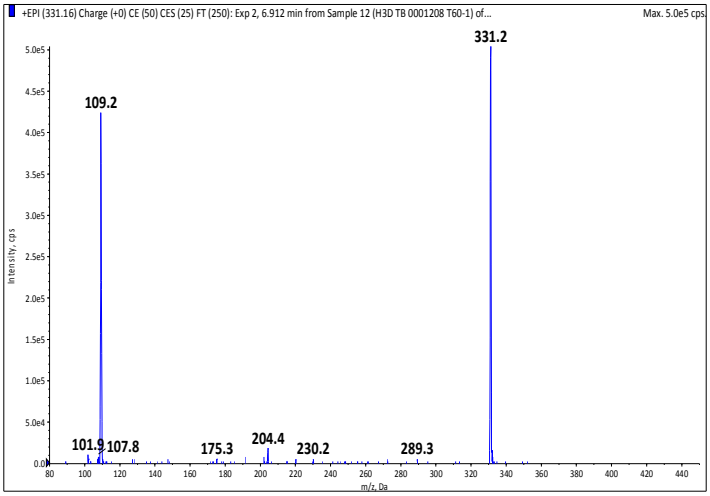


Figure S5.4: Product ion spectrum of the $[M + H]^+$ ion of M3

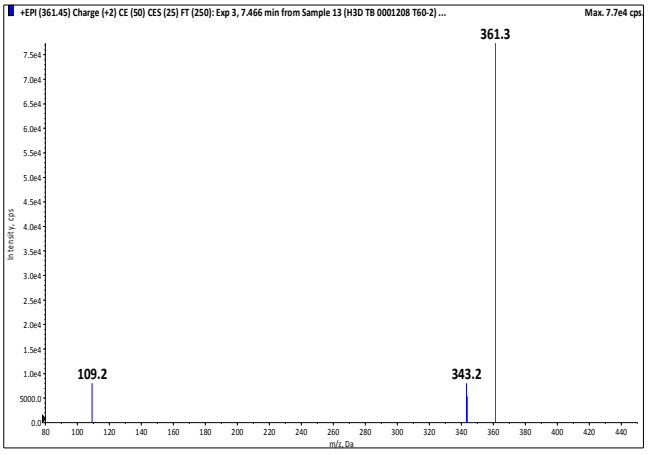


Figure S5.5: Product ion spectrum of the $[M + H]^+$ ion of M4

5) Mouse pharmacokinetic studies

5.1 Animal studies

Male CD57/B6 mice were part of the Animal Unit located at the Division of Clinical Pharmacology, University of Cape Town, South Africa. The compound was administered intravenously to 3 animals as a bolus of 10% (v/v) DMSO, 50% (v/v) propylene glycol/ethanol: 4/1; 40% (v/v) polyethylene glycol or water depending on the solubility of the compounds. The oral dose was administered to 3 animals as an aqueous suspension containing 0.5% (w/v) hydroxypropyl methylcellulose and 0.4% (v/v) Tween 80. Mice were not fasted overnight. Animals were permitted access *ad libitum* to water.

5.2 Sample analysis

Blood samples were collected from mice into heparinised microcentrifugation tubes at 0.17 (IV only), 0.5, 1, 3, 5, 8, 12, 24 hours after dosing and stored frozen (< -15°C) until analysis.

5.3 Bioanalytical method

The compound concentration was determined using an LC-MS/MS (AB Sciex API3200 triple quadrupole instrument). Chromatographic separation was conducted using an Agilent 1100 series HPLC with a Phenomenex Hydro-RP column (2.5 µm particle size; 50 x 2.1mm internal diameter), maintained at 30 °C. The mobile phase consisted of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile mixed using a linear gradient. Sample concentrations were calculated by comparison to calibration standards prepared and assayed under the same conditions. Elution of analytes was confirmed by multiple-reaction monitoring using internal standards. Blood samples and calibration standards (prepared in drug-free whole mice blood) were prepared using protein precipitation with ice-cold acetonitrile, followed by centrifugation and analysis of the supernatant fraction. The accuracy, precision, and recovery for each compound were within acceptable limits.

5.4 Calculation of pharmacokinetic parameters.

Pharmacokinetic parameters were calculated by non-compartmental analysis using PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA) using a method based on curve stripping.

6) References

(1) Bantscheff, M.; Hopf, C.; Savitski, M. M.; Dittmann, A.; Grandi, P.; Michon, A.-M.; Schlegl, J.; Abraham, Y.; Becher, I.; Bergamini, G.; Boesche, M.; Delling, M.; Dümpelfeld, B.; Eberhard, D.; Huthmacher, C.; Mathieson, T.; Poeckel, D.; Reader, V.; Strunk, K.; Sweetman, G.; Kruse, U.; Neubauer, G.; Ramsden, N. G.; Drewes, G. Chemoproteomics Profiling of HDAC Inhibitors Reveals Selective Targeting of HDAC Complexes. *Nat. Biotechnol.* **2011**, *29*, 255–265.

(2) Werner, T.; Sweetman, G.; Savitski, M. F.; Mathieson, T.; Bantscheff, M.; Savitski, M. M. Ion Coalescence of Neutron Encoded TMT 10-Plex Reporter Ions. *Anal. Chem.* **2014**, *86*, 3594–3601.

(3) Collins, L. A.; Torrero, M. N.; Franzblau, S. G. Green Fluorescent Protein Reporter Microplate Assay for High-Throughput Screening of Compounds against Mycobacterium Tuberculosis. *Antimicrob. Agents Chemother.* **1998**, *42*, 344–347.

(4) Collins, L. A.; Franzblau, S. G. Microplate Alamar Blue Assay versus BACTEC 460 System for High-Throughput Screening of Compounds against Mycobacterium Tuberculosis and Mycobacterium Avium. *Antimicrob. Agents Chemother.* **1997**, *41*, 1004–1009.

(5) Ioerger, T. R.; Feng, Y.; Ganesula, K.; Chen, X.; Dobos, K. M.; Fortune, S.; Jacobs, W. R.; Mizrahi, V.; Parish, T.; Rubin, E.; Sassetti, C.; Sacchettini, J. C. Variation among Genome Sequences of H37Rv Strains of Mycobacterium Tuberculosis from Multiple Laboratories. *J. Bacteriol.* **2010**, *192*, 3645–3653.

(6) De Voss, J. J.; Rutter, K.; Schroeder, B. G.; Su, H.; Zhu, Y.; Barry III, C. E. The Salicylate-

Derived Mycobactin Siderophores of Mycobacterium Tuberculosis Are Essential for Growth in Macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 1252–1257.

(7) Arora, K.; Ochoa-Montano, B.; Tsang, P. S.; Blundell, T. L.; Dawes, S. S.; Mizrahi, V.; Bayliss, T.; Mackenzie, C. J.; Cleghorn, L. A. T.; Ray, P. C.; Wyatt, P. G.; Uh, E.; Lee, J.; Barry III, C. E.; Boshoff, H. I. Respiratory Flexibility in Response to Inhibition of Cytochrome C Oxidase in Mycobacterium Tuberculosis. *Antimicrob. Agents Chemother.* **2014**, *58*, 6962–6965.

(8) Tukulula, M.; Sharma, R. K.; Meurillon, M.; Mahajan, A.; Naran, K.; Warner, D.; Huang, J.; Mekonnen, B.; Chibale, K. Synthesis and Antiplasmodial and Antimycobacterial Evaluation of New Nitroimidazole and Nitroimidazooxazine Derivatives. *ACS Med. Chem. Lett.* **2013**, *4*, 128–131.

(9) Abrahams, G. L.; Kumar, A.; Savvi, S.; Hung, A. W.; Wen, S.; Abell, C.; Barry III, C. E.; Sherman, D. R.; Boshoff, H. I. M.; Mizrahi, V. Pathway-Selective Sensitization of Mycobacterium Tuberculosis for Target-Based Whole-Cell Screening. *Chem. Biol.* **2012**, *19*, 844–854.

(10) Ollinger, J.; Bailey, M. A.; Moraski, G. C.; Casey, A.; Florio, S.; Alling, T.; Miller, M. J.; Parish, T. A Dual Read-out Assay to Evaluate the Potency of Compounds Active against Mycobacterium Tuberculosis. *PLoS One* **2013**, *8*, 1–9.

(11) Franzblau, S. G.; Witzig, R. S.; Mclaughlin, J. C.; Torres, P.; Madico, G.; Hernandez, A.; Degnan, M. T.; Cook, M. B.; Quenzer, V. K.; Ferguson, R. M.; Gilman, R. H. Rapid, Low-Technology MIC Determination with Clinical Mycobacterium Tuberculosis Isolates by Using the Microplate Alamar Blue Assay. *J. Clin. Microbiol.* **1998**, *36*, 362–366.

(12) Naran, K.; Moosa, A.; Barry III, C. E.; Boshoff, H. I. M.; Mizrahi, V.; Warner, D. F. Bioluminescent Reporters for Rapid Mechanism of Action Assessment in Tuberculosis Drug Discovery. *Antimicrob. Agents Chemother.* **2016**, *60*, 6748–6757.

(13) Hill, A. P.; Young, R. J. Getting Physical in Drug Discovery: A Contemporary Perspective on Solubility and Hydrophobicity. *Drug Discov. Today* **2010**, *15*, 648–655.

(14) Obach, R. S. Prediction of Human Clearance of Twenty-Nine Drugs from Hepatic Microsomal Intrinsic Clearance Data: An Examination of in Vitro Half-Life Approach and Nonspecific Binding to Microsomes. *Drug Metab. Dispos.* **1999**, *27*, 1350–1359.

(15) Alelyunas, Y. W.; Pelosi-Kilby, L.; Turcotte, P.; Kary, M. B.; Spreen, R. C. A High Throughput Dried DMSO Log D Lipophilicity Measurement Based on 96-Well Shake-Flask and Atmospheric Pressure Photoionization Mass Spectrometry Detection. *J. Chromatogr. A* **2010**, *1217* (12), 1950–1955.

(16) Wohnsland, F.; Faller, B. High-Throughput Permeability pH Profile and High-Throughput Alkane/water Log P with Artificial Membranes. *J. Med. Chem.* **2001**, *44*, 923–930.