Aminopyrazinamides: novel and specific GyrB inhibitors that kill replicating and non-replicating *Mycobacterium tuberculosis*

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SUPPORTING INFORMATION

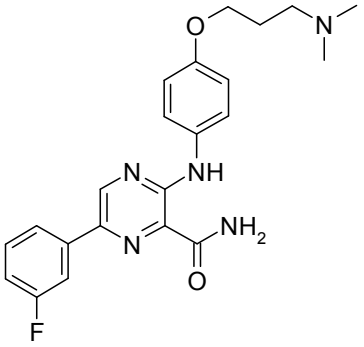
Experimental Section:

All anhydrous solvents, reagent grade solvents for chromatography and starting materials were purchased from either Sigma Aldrich Chemical Co. or Fisher Scientific. Water was distilled and purified through a Milli-Q water system (Millipore Corp., Bedford, MA). General methods of purification of compounds involved the use of silica cartridges purchased from Grace Purification systems. The reactions were monitored by TLC on precoated Merck 60 F254 silica gel plates and visualized using UV light (254 nm). All compounds were analyzed for purity by HPLC and characterized by ^{1}H NMR using Bruker 300 MHz NMR and/or Bruker 400 MHz NMR spectrometers. Chemical shifts are reported in ppm (δ) relative to the residual solvent peak in the corresponding spectra; chloroform δ 7.26, methanol δ 3.31, DMSO-d_6 δ 3.33 and coupling constants (*J*) are reported in hertz (Hz) (where s = singlet, bs = broad singlet, d = doublet, dd = double doublet, bd = broad doublet, ddd = double doublet of doublet, t = triplet, tt – triple triplet, q = quartet, m = multiplet) and analyzed using ACD NMR data processing software. Mass spectra values are reported as *m/z*.

All reactions were conducted under Nitrogen unless otherwise noted. Solvents were removed *in vacuo* on a rotary evaporator.

Abbreviations: NMP = N-methyl Pyrrolidine; HCl = hydrochloric acid; DMF = *N*,*N*-dimethylformamide; NaH = sodium hydride. EI = electrospray ionization; HRMS = high resolution mass spectrometry.

Preparation of Key Intermediates and Final Products:



1

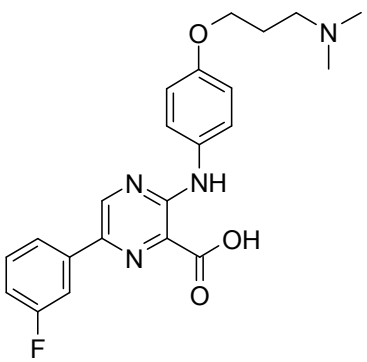
3-(4-(3-(dimethylamino)propoxy)phenylamino)-6-(3-fluorophenyl)pyrazine-2-carboxamide (1). In a Biotage microwave vial was methyl 3-(4-(3-(dimethylamino)propoxy)phenylamino)-6-(3-fluorophenyl)pyrazine-2-carboxylate (60 mg, 0.14 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was concentrated in vacuo. Purification of the resultant crude in silica gel flash column chromatography to afford the 3-(4-(3-(dimethylamino)propoxy)phenylamino)-6-(3-fluorophenyl)pyrazine-2-carboxamide (21 mg, 36 %) and the 3-(4-(3-(dimethylamino)propoxy)phenylamino)-6-(3-fluorophenyl)pyrazine-2-carboxylic acid (9 mg, 15 %) as a yellow solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.76 (s, 1H) 8.76 (s, 1H) 7.98 (s, 1H) 7.75-7.57 (m, 4 H) 7.54-7.40 (m, 1H) 7.13 (td, *J* = 8.4, 2.5 Hz, 1H) 6.94 (d, *J* = 8.8 Hz, 2H) 6.07 (s, 1H) 4.08 (t, *J* = 5.9 Hz, 2H) 3.04-2.90 (m, 2H) 2.62 (s, 6H) 2.26-2.13 (m, 2H)

LCMS (m/z) = 410 [M+1]

HPLC purity = 98%, tR = 5.07 min.

HRMS (EI), M+1 calcd. for $C_{22}H_{25}FN_5O_2$, 410.1987; found 410.1982



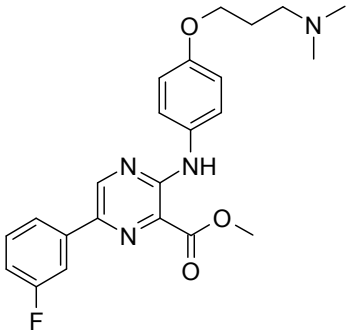
3

3-(4-(3-(Dimethylamino)propoxy)phenylamino)-6-(3-fluorophenyl)pyrazine-2-carboxylic acid (3). ^{1}H NMR (300 MHz, MeOH-d4): δ 7.10 (s, 1H) 6.52-6.36 (m, 2H) 6.07 (d, *J* = 8.9 Hz, 2 H) 6.04-5.97 (m, 1H) 5.68-5.58 (m, 1H) 5.35 (d, *J* = 9.0 Hz, 2H) 2.59 (t, *J* = 5.7 Hz, 2H) 1.84 (t, *J* = 7.3 Hz, 2H) 1.47 (s, 6H) 0.79-0.66 (m, 2H)

LCMS (m/z) = 411 [M+1]

HPLC purity = 97%, tR = 4.21 min.

HRMS (EI), M+1 calcd. for $C_{22}H_{24}FN_4O_3$, 411.1827; found 411.1833



2

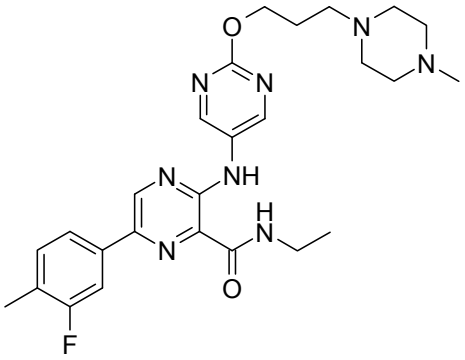
Methyl 3-(4-(3-(dimethylamino)propoxy)phenylamino)-6-(3-fluorophenyl)pyrazine-2-carboxylate (2). In a 10 ml of RB flask was added methyl 6-bromo-3-(4-(3-(dimethylamino)propoxy)phenylamino)pyrazine-2-carboxylate (250 mg, 0.61 mmol), 1,1'-bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex (24.94 mg, 0.03 mmol), 3-fluorophenylboronic acid (94 mg, 0.67 mmol) and K_3PO_4 (389 mg, 1.83 mmol) and treated with 1,4-dioxane (3 mL). The mixture was heated at 75 °C for 16 h. The reaction was mixed water (30 mL) and then extracted with dichloromethane (3 x 30 mL). The combined organic layer was dried over anhydrous sodium sulfate and evaporated under vacuo. Purification of the crude by silica gel column chromatography to afford methyl 3-(4-(3-(dimethylamino)propoxy)phenylamino)-6-(3-fluorophenyl)pyrazine-2-carboxylate (120 mg, 46 %) as a solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.05 (s, 1H) 8.76 (s, 1H) 7.78-7.68 (m, 2H) 7.56 (d, *J* = 8.8 Hz, 2H) 7.50-7.43 (m, 1H) 7.17-7.07 (m, 1H) 6.97 (d, *J* = 8.8 Hz, 2H) 4.12-4.03 (m, 5H) 2.53 (t, *J* = 7.2 Hz, 2H) 2.32 (s, 6H) 2.12-1.95 (m, 2H)

LCMS (m/z) = 425 [M+1]

HPLC purity = 96%, tR = 5.79 min.

HRMS (EI), M+1 calcd. for $C_{23}H_{26}FN_4O_3$, 425.1983; found 425.1979



4

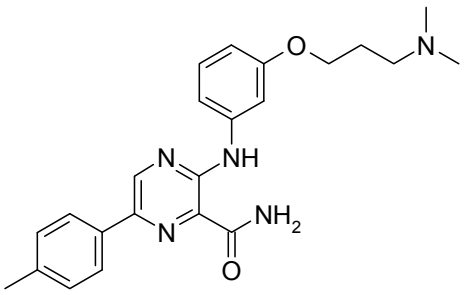
N-ethyl-6-(3-fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy) pyrimidin-5-ylamino)pyrazine-2-carboxamide (4). In a biotage microwave vial was added methyl 6-(3-fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxylate (120 mg, 0.24 mmol), ethylamine hydrochloride (197 mg, 2.42 mmol), N-ethyldiisopropylamine (0.419 mL, 2.42 mmol) and methanol (3 mL). Then the reaction mixture was sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was concentrated in vacuo. Purification of the resultant crude in silica gel flash column chromatography to afford N-ethyl-6-(3-fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide (90 mg, 73.1 %) as a solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.03 (s, 1H) 9.27 (s, 1H) 9.01 (s, 1H) 8.88 (s, 2H) 8.14 (d, *J* = 11.6 Hz, 1H) 7.97 (d, *J* = 7.9 Hz, 1H) 7.40 (t, *J* = 7.8 Hz, 1H) 4.34-4.30 (m, 2 H) 3.52-3.36 (m, 2H) 2.42-2.30 (m, 13H) 2.16 (s, 3H) 1.89 (t, *J* = 6.4 Hz, 2H) 1.21 (t, *J* = 6.7 Hz, 3H)

LCMS (m/z) = 509 [M+1]

HPLC purity = 97%, tR = 4.43 min.

HRMS (EI), M+1 calcd. for $C_{26}H_{34}FN_8O_2$, 509.2783; found 509.2800



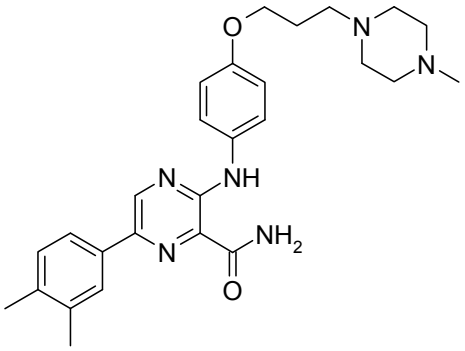
5

3-(3-(3-(dimethylamino)propoxy)phenylamino)-6-*p*-tolylpyrazine-2-carboxamide (5). In a biotage microwave vial was added methyl 3-(3-(3-(dimethylamino)propoxy)phenylamino)-6-p-tolylpyrazine-2-carboxylate (190 mg, 0.45 mmol) and 7N ammonia in methanol (4 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was evaporated in vacuo and was triturated with hexane to get yellow solid. The solid was filtrated out, washed with hexane and dried in vacuo to afford the 3-(3-(3-(dimethylamino)propoxy)phenylamino)-6-p-tolylpyrazine-2-carboxamide (160 mg, 87 %) as a solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.96 (s, 1H) 8.84 (s, 1H) 8.04 (s, 1H) 7.84 (d, *J* = 8.1 Hz, 2H) 7.51-7.48 (m, 1H) 7.34 (d, *J* = 8.1 Hz, 2H) 7.31-7.29 (m, 2H) 6.71-6.65 (m, 1H) 5.65 (s, 1H) 4.12 (t, *J* = 6.3 Hz, 2H) 2.66 (t, *J* = 7.2 Hz, 2H) 2.47 (s, 3H) 2.42 (s, 6H) 2.17-2.03 (m, 2H)

LCMS (m/z) = 406 [M+1]

HPLC purity = 98%, tR = 5.46 min.



6

6-(3,4-Dimethylphenyl)-3-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenylamino)pyrazine-2-carboxamide (6). In a biotage microwave vial was added methyl 6-(3,4-dimethylphenyl)-3-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenylamino)pyrazine-2-carboxylate (0.057 g, 0.12

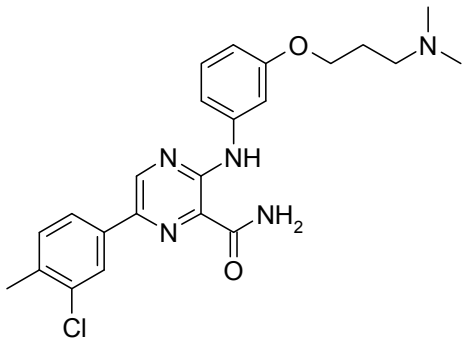
mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was evaporated in vacuo and was triturated with hexane to get yellow solid. The solid was filtrated out, washed with hexane and dried in vacuo to afford the 6-(3,4-dimethylphenyl)-3-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenylamino)pyrazine-2-carboxamide (0.050 g, 90%) as a yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.10 (s, 1H) 8.92 (s, 1H) 8.54 (s, 1H) 8.01-7.98 (m, 2 H) 7.90 (d, *J* = 7.5 Hz, 1H) 7.57 (d, *J* = 8.6 Hz, 2 H) 7.23 (d, *J* = 7.5 Hz, 1H) 6.92 (d, *J* = 8.7 Hz, 2 H) 3.99 (t, *J* = 6.1 Hz, 2H) 2.47-2.27 (m, 16H) 2.18 (s, 3H) 1.86 (t, *J* = 6.7 Hz, 2H)

LCMS (m/z) = 475 [M+1]

HPLC purity = 99%, tR = 5.32 min.

HRMS (EI), M+1 calcd. for $C_{27}H_{35}N_6O_2$, 475.2816; found 475.2800



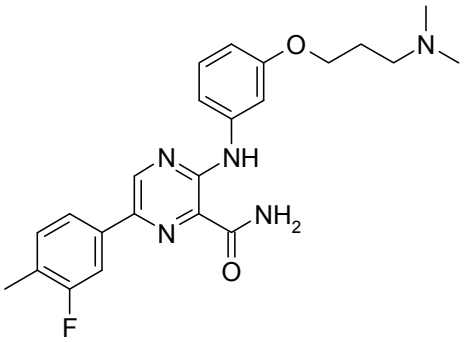
7

6-(3-chloro-4-methylphenyl)-3-(3-(3-(dimethylamino)propoxy)phenylamino)pyrazine-2-carboxamide (7). In a biotage microwave vial was added methyl 6-(3-chloro-4-methylphenyl)-3-(3-(3-(dimethylamino)propoxy)phenylamino)pyrazine-2-carboxylate (0.060 g, 0.13 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was concentrated in vacuo. Purification of the resultant crude in silica gel flash column chromatography to afford the 6-(3-chloro-4-methylphenyl)-3-(3-(3-(dimethylamino)propoxy)phenylamino)pyrazine-2-carboxamide (0.040 g, 65%) as a yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.46 (s, 1H) 9.06 (s, 1H) 8.74 (s, 1H) 8.35 (s, 1H) 8.10 (d, *J* = 8.2 Hz, 1H) 8.04 (s, 1H) 7.51-7.41 (m, 2H) 7.30-7.14 (m, 2H) 6.63 (d, *J* = 8.2 Hz, 1H) 4.03 (t, *J* = 6.2 Hz, 2H) 2.62-2.53 (m, 2H) 2.39 (s, 3H) 2.29 (s, 6H) 1.99-1.82 (m, 2H)

LCMS (m/z) = 440 [M+1]

HPLC purity = 95%, tR = 5.32 min.



8

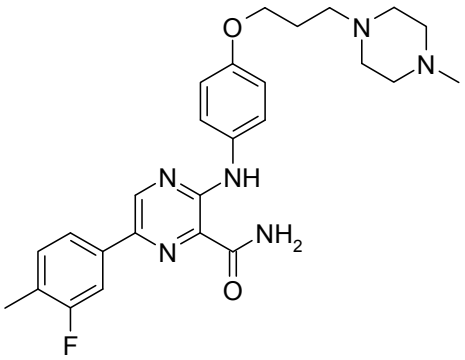
3-(3-(3-(Dimethylamino)propoxy)phenylamino)-6-(3-fluoro-4-methylphenyl)pyrazine-2-carboxamide (8). In a biotage microwave vial was added methyl 3-(3-(3-(dimethylamino)propoxy)phenylamino)-6-(3-fluoro-4-methylphenyl)pyrazine-2-carboxylate (0.070 g, 0.16 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was evaporated in vacuo and was triturated with methanol and diethyl ether mixture to get yellow solid. The solid was filtrated out, washed with hexane and dried in vacuo to afford the 3-(3-(3-(Dimethylamino)propoxy)phenylamino)-6-(3-fluoro-4-methylphenyl)pyrazine-2-carboxamide (0.045 g, 65%) as a solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.46 (s, 1H) 9.06 (s, 1H) 8.73 (s, 1H) 8.14 (d, *J* = 11.6 Hz, 1H) 8.04 (s, 1H) 7.97 (d, *J* = 8.2 Hz, 1H) 7.49-7.42 (m, 1H) 7.38 (t, *J* = 8.1 Hz, 1H) 7.31-7.16 (m, 2H) 6.70-6.59 (m, 1H) 4.04 (t, *J* = 6.2 Hz, 2H) 2.70 (t, *J* = 7.0 Hz, 2H) 2.41 (s, 6H) 2.29 (s, 3H) 2.03-1.87 (m, 2H)

LCMS (m/z) = 424 [M+1]

HPLC purity = 98%, tR = 5.58 min.

HRMS (EI), M+1 calcd. for $C_{23}H_{27}FN_5O_2$, 424.2143; found 424.2159



9

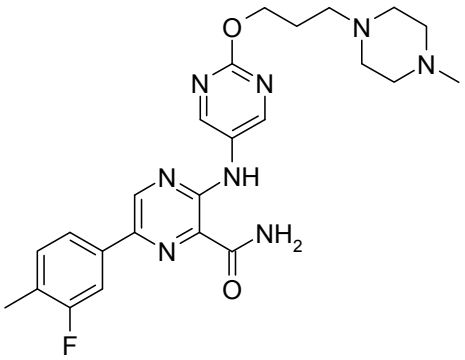
6-(3-Fluoro-4-methylphenyl)-3-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenylamino)-pyrazine-2-carboxamide (9). In a biotage microwave vial was taken methyl 6-(3-fluoro-4-methylphenyl)-3-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenylamino)pyrazine-2-carboxylate (200 mg, 0.41 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was concentrated in vacuo. The resultant crude was triturated with hexane (2 x 10ml) and diethyl ether (2 x 10ml). The solid was then filtrated and dried in vacuo to afford the 6-(3-fluoro-4-methylphenyl)-3-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenylamino)pyrazine-2-carboxamide (135 mg, 69 %) as a yellow solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.74 (s, 1H) 8.73 (s, 1H) 7.97 (s, 1H) 7.65-7.52 (m, 4H) 7.37-7.22 (m, 1H) 6.94 (d, *J* = 9.0 Hz, 2H) 4.07 (t, *J* = 6.0 Hz, 2H) 2.75-2.66 (m, 11H) 2.52 (s, 3H) 2.41-2.32 (m, 3H) 2.16-2.00 (m, 2H)

LCMS (m/z) = 479 [M+1]

HPLC purity = 98%, tR = 4.68 min.

HRMS (EI), M+1 calcd. for $C_{26}H_{32}FN_6O_2$, 479.2565; found 479.2577



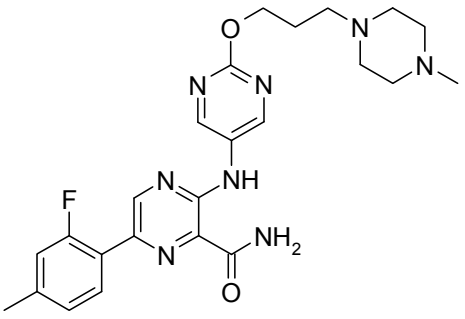
10

6-(3-Fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide(10). In a Biotage microwave vial was taken methyl 6-(3-fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxylate (70 mg, 0.14 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The yellow solid was precipitated out. The solid was filtered through funnel, washed with methanol and dried under in vacuo to afford 6-(3-fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide (50.0 mg, 73 %) as yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 10.98 (s, 1H) 9.00 (s, 1H) 8.87 (s, 2H) 8.70 (s, 1H) 8.13 (d, *J* = 11.6 Hz, 1H) 8.03 (s, 1H) 7.96 (d, *J* = 7.9 Hz, 1H) 7.38 (t, *J* = 8.0 Hz, 1H) 4.32 (t, *J* = 6.3 Hz, 2H) 2.46-2.29 (m, 13H) 2.15 (s, 3H) 1.89 (t, *J* = 6.5 Hz, 2H)

LCMS (m/z) = 481 [M+1]

HPLC purity = 96%, tR = 5.77 min.



11

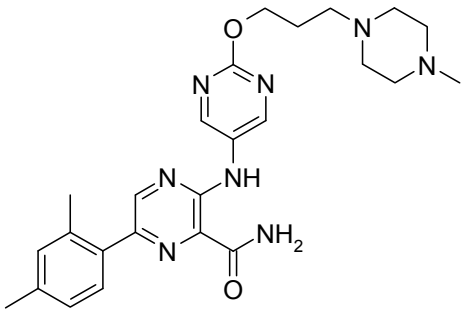
6-(2-Fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide (11). In a biotage microwave vial was added methyl 6-(2-fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxylate (3 g, 6.05 mmol) and 7N ammonia in methanol (10 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The yellow solid was precipitated out. The solid was filtered through funnel, washed with methanol and dried under in vacuo to afford 6-(2-fluoro-4-methylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide (2.70g, 93 %) as yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 10.94 (s, 1H) 8.88 (s, 2H) 8.75 (d, *J* = 2.2 Hz, 1H) 8.54 (s, 1H) 8.19 (t, *J* = 8.3 Hz, 1H) 8.10 (s, 1H) 7.26-7.10 (m, 2H) 4.31 (t, *J* = 6.5 Hz, 2H) 2.47-2.21 (m, 13H) 2.14 (s, 3H) 1.93-1.84 (m, 2H)

LCMS (m/z) = 481 [M+1]

HPLC purity = 98%, tR = 5.34 min.

HRMS (EI), M+1 calcd. for $C_{24}H_{30}FN_8O_2$, 481.2470; found 481.2493



12

6-(2,4-dimethylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)-pyrazine-2-carboxamide (12). In a biotage microwave vial was added methyl 6-(2,4-dimethylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxylate (1 g, 2.03 mmol) and 7N ammonia in methanol (15 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The yellow solid was precipitated out. The solid was filtered through funnel, washed with methanol and dried under in vacuo to afford 6-(2,4-dimethylphenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide (0.95 g, 98 %) as yellow solid.

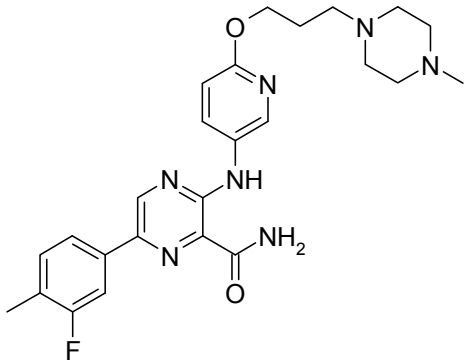
^{1}H NMR (300 MHz, DMSO-d6): δ 10.87 (s, 1H) 8.88 (s, 2H) 8.51 (s, 1H) 8.23 (s, 1H) 7.98 (s, 1H) 7.43 (d, *J* =7.5 Hz, 1H) 7.24-7.08 (m, 2H) 4.32 (t, *J* = 6.5 Hz, 2H) 2.48-2.31

(m, 16H) 2.20 (s, 3H) 1.90-1.80 (m, 2H)

LCMS (m/z) = 478 [M+1]

HPLC purity = 99%, tR = 6.73 min.

HRMS (EI), M+1 calcd. for $C_{25}H_{33}N_8O_2$, 477.2721; found 477.2728



13

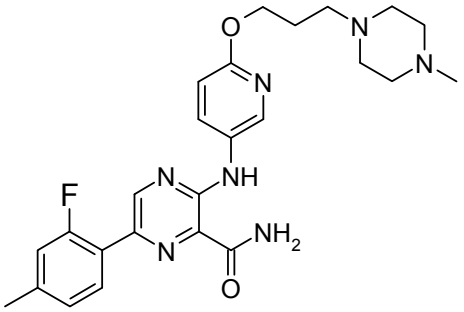
6-(3-fluoro-4-methylphenyl)-3-(6-(3-(4-methylpiperazin-1-yl)propoxy)pyridin-3-ylamino)pyrazine-2-carboxamide (13). In a biotage microwave vial was added methyl 6-(3-fluoro-4-methylphenyl)-3-(6-(3-(4-methylpiperazin-1-yl)propoxy)pyridin-3-ylamino)pyrazine-2-carboxylate (100 mg, 0.20 mmol) and 7N ammonia in methanol (4 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was concentrated in vacuo. Purification of the resultant crude in silica gel flash column chromatography to afford 6-(3-fluoro-4-methylphenyl)-3-(6-(3-(4-methylpiperazin-1-yl)propoxy)pyridin-3-ylamino)pyrazine-2-carboxamide (40.0 mg, 41.3 %) as a yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.06 (s, 1H) 8.98 (s, 1H) 8.68 (s, 1H) 8.40 (d, *J* = 2.6 Hz, 1H) 8.12 (d, *J* = 11.6 Hz, 1H) 8.06-7.88 (m, 3H) 7.37 (t, *J* = 8.1 Hz, 1H) 6.82 (d, *J* = 8.8 Hz, 1H) 4.26 (t, *J* = 6.5 Hz, 2H) 2.47-2.29 (m, 13H) 2.15 (s, 3H) 1.86 (q, *J* = 6.9 Hz, 2H)

LCMS (m/z) = 480 [M+1]

HPLC purity = 99%, tR = 6.17 min.

HRMS (EI), M+1 calcd. for $C_{25}H_{31}FN_7O_2$, 480.2518; found 480.2533



14

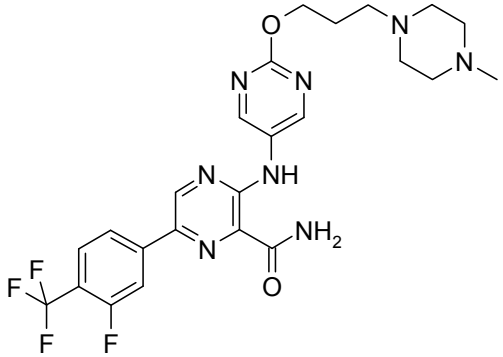
6-(2-fluoro-4-methylphenyl)-3-(6-(3-(4-methylpiperazin-1-yl)propoxy)pyridin-3-ylamino)pyrazine-2-carboxamide (14). In a biotage microwave vial was added methyl 6-(2-fluoro-4-methylphenyl)-3-(6-(3-(4-methylpiperazin-1-yl)propoxy)pyridin-3-ylamino)pyrazine-2-carboxylate (2.1 g, 4.25 mmol) and 7N ammonia in methanol (15 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The yellow solid was precipitated out. The solid was filtered through funnel, washed with methanol and dried under in vacuo to afford 6-(2-fluoro-4-methylphenyl)-3-(6-(3-(4-methylpiperazin-1-yl)propoxy)pyridin-3-ylamino)pyrazine-2-carboxamide (1.9 g, 93 %) as yellow solid.

^{1}H NMR (400 MHz, DMSO-d6): δ 11.03 (s, 1H) 8.73 (d, *J* = 2.0 Hz, 1H) 8.53 (s, 1H) 8.41 (d, *J* = 2.5 Hz, 1H) 8.18 (t, *J* = 8.2 Hz, 1H) 8.11-7.99 (m, 2H) 7.23-7.12 (m, 2H) 6.83 (d, *J* = 9.0 Hz, 1H) 4.25 (t, *J* = 6.5 Hz, 2H) 2.45-2.33 (m, 13H) 2.15 (s, 3H) 1.86 (t, *J* = 6.9 Hz, 2H)

LCMS (m/z) = 480 [M+1]

HPLC purity = 96%, tR = 5.94 min.

HRMS (EI), M+1 calcd. for $C_{25}H_{31}FN_7O_2$, 480.2518; found 480.2513



15

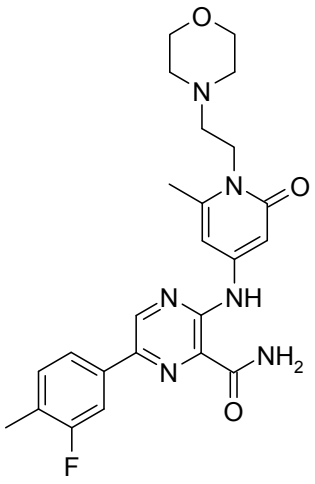
6-(3-Fluoro-4-(trifluoromethyl)phenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide (15). In a biotage microwave vial was added methyl 6-(3-fluoro-4-(trifluoromethyl)phenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxylate (180 mg, 0.33 mmol) and 7N ammonia in methanol (4 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was concentrated in vacuo. Purification of the resultant crude in silica gel flash column chromatography to afford 6-(3-fluoro-4-(trifluoromethyl)phenyl)-3-(2-(3-(4-methylpiperazin-1-yl)propoxy)pyrimidin-5-ylamino)pyrazine-2-carboxamide (160 mg, 91 %) as a yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.11 (s, 1H) 9.13 (s, 1H) 8.88 (s, 2H) 8.84 (s, 1H) 8.54 (d, *J* = 13.1 Hz, 1H) 8.27 (d, *J* = 8.2 Hz, 1H) 8.11 (s, 1H) 7.85 (t, *J* = 8.0 Hz, 1H) 4.32 (t, *J* = 6.5 Hz, 2H) 2.50-2.30 (m, 10H) 2.23 (s, 3H) 1.90 (p, *J* = 6.7 Hz, 2H)

LCMS (m/z) = 535 [M+1]

HPLC purity = 98%, tR = 5.98 min.

HRMS (EI), M+1 calcd. for $C_{24}H_{27}F_4N_8O_2$, 535.2188; found 535.2202



16

6-(3-Fluoro-4-methylphenyl)-3-(6-methyl-1-(2-morpholinoethyl)-2-oxo-1,2-dihydropyridin-4-ylamino)pyrazine-2-carboxamide (16). In a biotage microwave vial was added methyl 6-(3-fluoro-4-methylphenyl)-3-(6-methyl-1-(2-morpholinoethyl)-2-oxo-1,2-dihydropyridin-4-ylamino)pyrazine-2-carboxylate (150 mg, 0.31 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The yellow solid was precipitated out. The solid was filtered through funnel, washed with methanol and

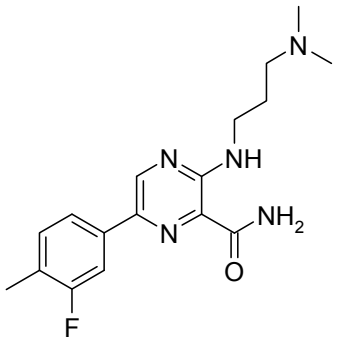
dried under in vacuo to afford 6-(3-fluoro-4-methylphenyl)-3-(6-methyl-1-(2-morpholinoethyl)-2-oxo-1,2-dihydropyridin-4-ylamino)pyrazine-2-carboxamide (145 mg, 98 %) as yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.50 (s, 1H) 9.16 (s, 1H) 8.81 (s, 1H) 8.23-8.10 (m, 2H) 8.01 (d, *J* = 8.2 Hz, 1H) 7.40 (t, *J* = 8.1 Hz, 1H) 7.14 (d, *J* = 2.0 Hz, 1H) 6.13 (s, 1H) 4.01 (t, *J* = 7.3 Hz, 2H) 3.63-3.52 (m, 4H) 2.48-2.36 (m, 9H) 2.30 (s, 3H)

LCMS (m/z) = 467 [M+1]

HPLC purity = 99%, tR = 6.53 min.

HRMS (EI), M+1 calcd. for $C_{24}H_{28}FN_6O_3$, 467.2201; found 467.2209



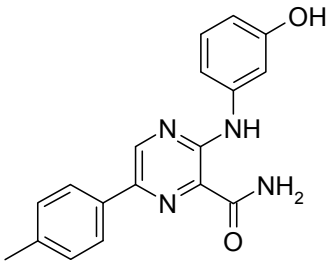
17

3-(3-(Dimethylamino)propylamino)-6-(3-fluoro-4-methylphenyl)pyrazine-2-carboxamide (17). In a biotage microwave vial was added methyl 3-(3-(dimethylamino)propylamino)-6-(3-fluoro-4-methylphenyl)pyrazine-2-carboxylate (0.045 g, 0.13 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was evaporated to dryness and was triturated with hexane to get yellow solid. The solid was filtered through funnel, washed with hexane and dried under in vacuo to afford 3-(3-(dimethylamino)propylamino)-6-(3-fluoro-4-methylphenyl)pyrazine-2-carboxamide (0.040 g, 93 %) as yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 8.93 (t, *J* = 5.6 Hz, 1H) 8.89 (s, 1H) 8.44 (s, 1H) 8.03 (dd, *J* = 11.7, 1.7 Hz, 1H) 7.87 (dd, *J* = 7.9, 1.7 Hz, 1H) 7.71 (s, 1H) 7.34 (t, *J* = 8.1 Hz, 1H) 3.52 (q, *J* = 6.7 Hz, 2H) 2.75-2.60 (m, 2H) 2.43 (s, 6H) 2.27 (s, 3H) 1.75-1.90 (m, 2 H)

LCMS (m/z) = 332 [M+1]

HPLC purity = 95%, tR = 5.24 min.



18

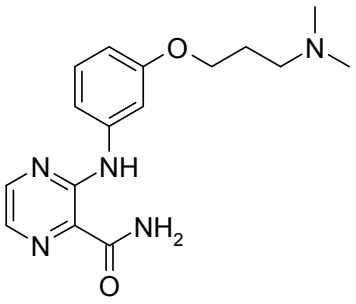
3-(3-hydroxyphenylamino)-6-p-tolylpyrazine-2-carboxamide (18). In a biotage microwave vial was added methyl 3-(3-hydroxyphenylamino)-6-p-tolylpyrazine-2-carboxylate (60 mg, 0.18 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was evaporated to dryness and was triturated with methanol and diethyl ether mixture to get yellow solid. The solid was filtered through funnel, washed with hexane and dried under in vacuo to afford 3-(3-hydroxyphenylamino)-6-p-tolylpyrazine-2-carboxamide (50.0 mg, 87 %) as yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.32 (s, 1H) 9.38 (s, 1H) 8.98 (s, 1H) 8.57 (s, 1H) 8.13 (d, *J* = 8.3 Hz, 2H) 8.02 (s, 1H) 7.35-7.21 (m, 3H) 7.12 (t, *J* = 8.0 Hz, 1H) 7.07-7.02 (m, 1H) 6.48-6.43 (m, 1H) 2.37 (s, 3H)

LCMS (m/z) = 321 [M+1]

HPLC purity = 98%, tR = 7.43 min.

HRMS (EI), M+1 calcd. for $C_{18}H_{17}N_4O_2$, 321.1346; found 321.1353



19

3-(3-(3-(dimethylamino)propoxy)phenylamino)pyrazine-2-carboxamide (19). In a biotage microwave vial was added methyl 3-(3-(3-

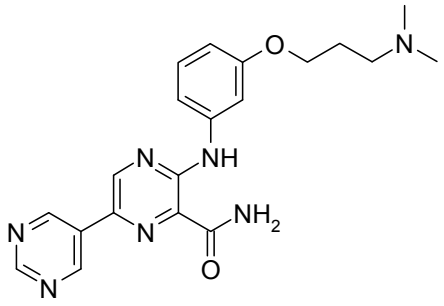
(dimethylamino)propoxy)phenylamino)pyrazine-2-carboxylate (240 mg, 0.73 mmol) and 7N ammonia in methanol (4 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was concentrated in vacuo. Purification of the resultant crude in silica gel flash column chromatography to afford 3-(3-(3-(dimethylamino)propoxy)phenylamino)pyrazine-2-carboxamide (120 mg, 52.4 %) as a solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.36 (s, 1H) 8.41 (d, *J* = 2.3 Hz, 2H) 8.05 (d, *J* = 2.3 Hz, 1H) 7.97 (s, 1H) 7.42 (s, 1H) 7.31-7.21 (m, 2H) 6.64 (d, *J* = 7.9 Hz, 1H) 4.07 (t, *J* = 5.8 Hz, 2H) 3.26-3.14 (m, 2H) 2.81 (s, 6H) 2.25-2.01 (m, 2 H)

LCMS (m/z) = 316 [M+1]

HPLC purity = 98%, tR = 3.59 min.

HRMS (EI), M+1 calcd. for $C_{16}H_{22}N_5O_2$, 316.1768; found 316.1778



20

3-(3-(3-(dimethylamino)propoxy)phenylamino)-6-(pyrimidin-5-yl)pyrazine-2-carboxamide (20). In a biotage microwave vial was added methyl 3-(3-(3-(dimethylamino)propoxy)phenylamino)-6-(pyrimidin-5-yl)pyrazine-2-carboxylate (62 mg, 0.15 mmol) and 7N ammonia in methanol (3 ml) and then sub*J*ected to microwave reaction at 110 °C for 30 min. The reaction mixture was evaporated to dryness and was triturated with methanol and diethyl ether mixture to get yellow solid. The solid was filtered through funnel, washed with hexane and dried under in vacuo to afford 3-(3-(3-(dimethylamino)propoxy)phenylamino)-6-(pyrimidin-5-yl)pyrazine-2-carboxamide (35.0 mg, 47 %) as solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.55 (s, 1H) 9.66 (s, 2H) 9.20 (d, *J* = 6.2 Hz, 2H) 8.83 (s, 1H) 8.08 (s, 1H) 7.48 (s, 1H) 7.26 (t, *J* = 7.9 Hz, 1H) 7.21-7.16 (m, 1H) 6.69-6.63 (m, 1H) 4.02 (t, *J* = 6.5 Hz, 2H) 2.37 (t, *J* = 6.9 Hz, 2H) 2.16 (s, 6H) 1.91-1.82 (m, 2H)

LCMS (m/z) = 394 [M+1]

HPLC purity = 98%, tR = 3.65 min.

HRMS (EI), M+1 calcd. for $C_{20}H_{24}N_7O_2$, 394.1986; found 394.1988

Reagents for assay

All reagents and buffers were sourced from Sigma Aldrich Inc. (St. Louis, MO) and were of ACS reagent grade or higher purity unless specified otherwise. All buffer solutions were prepared with high purity water (≥18 MΩ•cm) obtained from a Milli-Q purifier (Millipore, Billerica, MA).

Msm GyrB ATPase assay

The assay was carried out in 30 µL volume for 100 min at 25°C in 60 mM HEPES-KOH pH 7.7, 250 mM potassium glutamate, 200 mM KCl, 2 mM magnesium chloride, 1 mM DTT, 2% Glycerol, 4% DMSO, 0.001% Bri*J*, 0.65 mM ATP, 15 nM Msm GyrB. To measure inhibition of ATPase activity by test compounds, two or three-fold dilutions of compounds were made in neat DMSO at 30x the final assay concentration. Assays were performed in 384-well polystyrene, untreated, Corning flat-bottomed plates (Corning, Tewksbury MA, USA). 1 µL of compound was placed in the assay well, followed by 15 µL of enzyme and substrate mix in 2x final assay buffer, and reactions were initiated by the addition of 14 µL of magnesium chloride solution. Reactions were allowed to progress 100 min at 25 °C prior to quenching with 30 µL of malachite green reagent (Innova Biosciences, Cambridge, U.K. (or variant thereof)) to measure the inorganic phosphate.[19] The absorbance at 635 nm was recorded 20min after the addition of the detection reagent.

It was important for us to develop a robust assay which is amenable to high throughput screening. As the the specific ATPase activity of Mtb protein was found to be low as compared to M. smegmatis (Msm) protein, it did not allow the development of a high throughput robust assay for monitoring ATPase activity following the inorganic phosphate detection by malachite green reagent. This is not unusual as Mtb is a slower growing organism as compared to Msm.

However, a set of representative compounds including a known Gyrase B ATPase inhibitor, novobiocin were screened in the low throughput gel-based Mtb gyrase supercoiling assay. A tight correlation (< 3-fold variation in IC_{50}) between the Msm ATPase activity and Mtb Supercoiling activity was established, giving us confidence to use Msm as a surrogate enzyme for the ATPase activity.

Compound	*Msm* GyrB IC_{50} (μM)	*Mtb* GyrAB IC_{50} (μM)
	ATPase	Supercoiling Activity
6	0.695	0.465
16	0.022	0.058
Novobiocin	0.008	0.02

Furthermore, sequence alignment between Msm and Mtb gyrase proteins indicates a high degree of conservation in the ATP binding pocket.

High Throughput Screening

1,190,372 compounds were tested at a single compound concentration of 10μM in 0.1% (v/v) DMSO, diluted in water prior to assay against Msm GyrB. The activity cut-off flag was set at >30% inhibition. The assay set-up and detection is as described above. 7717 compounds were tested in an 8 point concentration response with a two-fold compound dilution and a top concentration of 100 μM to determine the IC_{50} of the test compounds and confirm activity of compound samples scored as active in the primary screen. In parallel, an 8 point concentration response was performed in identical assay condition excluding the enzyme, in order to eliminate any artefacts arising from interference of the malachite green detection reagent with the test compounds. A chemical evaluation of hits was performed and the aminopyrazinamide scaffold was prioritized for further prosecution by synthetic chemistry.

Eco Gyrase ATPase assay

The assay was carried out for 60 min at 30°C in ATP solution (50mM HEPES.KOH pH 7.5, 5mM magnesium chloride, 5 mM DTT, 200mM potassium glutamate, 2 mM glycerol, 10 μg/mL DNA, 130 μM ATP, 0.001% Bri*J*-35). Purified recombinant Eco Gyrase A and Gyrase B proteins were reconstituted in the presence of sheared salmonella testes DNA (Sigma D-1626) at 10-fold the final working concentration and incubated on ice for 15 min before diluting in 2X reconstitution buffer (100mM HEPES.KOH pH 7.5, 400mM Potassium Glutamate, 10mM DTT, 2mM EDTA). 25μL of enzyme-substrate mix in 2X final assay buffer was added to assay wells containing 2uL of test compound and the reaction was initiated by addition of 24 μL of the ATP solution. The reaction was quenched with 50 μL of the malachite green reagent and absorbance at 635 nm measured after 30 min of incubation.

Protein crystallization, X-ray diffraction data collection and structure determination Crystals of the 22 kDa double loop-deletion mutant of the 27kDa N-terminal ATPase domain of MsmGyrB (MsmGyrB-EP8) in complex with compound **6** were grown at 20ºC by hanging drop vapour diffusion. Purified MsmGyrB-EP8 at 15 mg/mL in storage buffer (50mM Tris/HCl, 50mM NaCl, 1mM ethylene diamine tetraacetic acid (EDTA), 1mM dithiothreitol (DTT), 10% v/v glycerol) was incubated at 4ºC for 1 hour with compound **6** at a final concentration of 1mM compound **6** and 1% DMSO. Drops (0.4μL) contained equal volumes of protein-compound **6** and reservoir solution (100mM Sodium acetate pH 5.7, 200mM Calcium acetate, 19% PEG 8000). Crystals grew over a six week period and were harvested into reservoir solution supplemented with 20% 2,3-butanediol before cryo-cooling in a stream of nitrogen gas at 100K (Oxford CryoSystems). Diffraction data were collected to a resolution of 2.2Å from a single crystal using a Rigaku FRE CuKα rotating anode generator equipped with VariMaxHF Optics, an XStream cryo-cooling system and a Saturn944 CCD detector. Data were processed using DStarTrek[1]. Crystals of the MsmGyrB-EP8-compound **6** complex belong to the orthorhombic space group $C222_1$ with cell dimensions of a=43.45Å, b=82.25Å and c=191.94Å. The asymmetric unit contains two molecules of the MsmGyrB-EP8-compound **6** complex related by non-crystallographic symmetry (NCS dimer). The structure was determined by molecular replacement using a monomer from a previously determined structure of an MsmGyrB-EP8-inhibitor complex in a different space group (Madhavapeddi *et al*., to be published) and the program MolRep.[22] Initial maps and model were improved using ARP/wARP[2]. Coordinates

and initial refinement restraints for compound **6** were generated using Corina[3]. Model completion, including fitting of compound **6** to the difference electron density, was carried out manually in Coot[4] interspersed with rounds of refinement using Refmac5[5] and then autoBUSTER[6], applying Translation/Libration/Screw (TLS) restraints[7, 8]. The final model has Rcryst = 20.2% and Rfree = 25.4%. Model quality was assessed using the validation tools in Coot[4], Mogul[9], MolProbity[10] and Procheck[11, 12][13]. Coordinates were confirmed to have good stereochemistry as indicated by the Ramachandran plot (91.2% residues in the most favoured regions and 7.7% in the additionally allowed regions); r.m.s. bond and angle deviations (0.010Å and 1.07º respectively).

The final model comprises residues 36-86 and 95-255 of chain A and 37-255 of chain B, two molecules of compound **6**, 112 water molecules and two sodium ions. The region surrounding the active site loop deletion (residues 95-125) forms a helical structure which contributes to one face of the compound **6** binding site. The path followed by these residues and the degree of order in this region differs between the two chains in the NCS dimer. The helical structure also diverges from that observed for the equivalent region in structures of complexes of MsmGyrB-EP8 with other scaffolds (data not shown) and the *S. sureus* GyrB loop-deletion mutant structure (e.g. PDB ID 3U2K[14]). Despite these differences between the two binding sites, there appears to be little effect on the compound **6** binding mode. Access to solvent is restricted in both monomers of the NCS dimer by the close approach of either the other chain of the dimer (chain A) or a crystallographic symmetry-related molecule (chain B). The conformation observed for the methyl-piperazinyl-propoxy substituent may therefore be an artefact of these crystal contacts, since this moiety is observed to pack against the side-chains of residues Glu196, Phe199 and Tyr253 from a symmetry-related molecule. The conformation of the methyl piperazine moiety was not well defined in the electron density. The C-terminal loop-deletion and accompanying mutations (Δ214-245 [V213D V246G]) creates a β-turn equivalent to that observed for this region in *E. coli* GyrB ATPase domain structures, exactly as intended by the construct design.

Detailed statistics of the data collection and refinement are given in Table S3. Co-ordinates and structure factors have been deposited with the Protein Data Bank (PDB) under Accession Code 4B6C.

Bacterial Strains

M. tuberculosis: *M. tuberculosis* H37Rv ATCC 27294 was used for the studies included in this work and grown as reported earlier.[23] The inocula used for all the experiments were derived from a single seed lot that had been maintained at -70°C. Briefly, *M. tuberculosis* was grown in roller bottles at 37°C for 7 to 10 days in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma), and 10% albumin dextrose catalase (Difco Laboratories, Detroit, Mich.); referred to as 7H9 broth in the remainder of the manuscript. The cells were harvested by centrifugation, washed twice in 7H9 broth, and resuspended in fresh 7H9 broth. Aliquots of 0.5 mL were dispensed, and the seed-lot suspensions were stored at -70°C.

MIC Determination

The MICs of aminopyrazinamides were determined in 7H9 broth by a standard microdilution method (Franzblau et al 1998). The assay was performed in two sets of duplicates in a 96-well microtiter plate (catalog no. 900196; Tarsons, India), in which all the peripheral wells were filled with sterile distilled water. Serial two-fold dilutions of compound made in DMSO were placed in the remaining wells, with the concentrations ranging from 32 to 0.06 mg/liter. Column 11 contained no drug and served as culture controls. Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma), and 10% albumin dextrose catalase (Difco Laboratories, Detroit, Mich.), was used as the diluent. Each well was inoculated with a final inoculum of approximately 5 x 10^5 CFU/mL. The plates were packed in gas permeable polythene bags and incubated at 37°C for 7 days. In one set of duplicate plates, 40µl of a freshly prepared 1:1 mixture of 10X Alamar Blue (Accumed International, Westlake, Ohio), and 10% Tween 80 was added to all the wells. The plates were incubated for an additional 24 hours at 37°C, and the colours of all wells were recorded. A blue colour in the well was interpreted as no growth, and a pink colour was scored as growth. Minimum Inhibitory Concentration (MIC) was defined as the lowest drug concentration which prevented the colour change from blue to pink.

Killing kinetics in 7H9 Broth and human THP-1 Macrophages

The killing kinetics assay in 7H9 broth was performed in a 200 µL volume using 96-well plates with Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma), and 10% albumin dextrose catalase (Difco Laboratories, Detroit, Mich.). Serial two-fold dilutions of aminopyrazinamides were made in DMSO separately, with the concentrations

ranging from 128 to 0.25 mg/L. From each of these dilutions, 4µL were added to each of the 96-wells which also contain approximately 3 X 10^7 CFU/mL of *M. tuberculosis* H37Rv. The plates were incubated at 37°C and on days 0, 3, 7, 14 aliquots were plated for bacterial enumeration after making serial dilutions in Middlebrook 7H9 broth. Bacterial enumeration was performed on Middlebrook 7H11 agar plates after incubation of plates for 21-28 days at 37°C, 5% CO_2 in a humidified atmosphere. Data are expressed as the $\log_{10}$CFU for each drug concentration.

Intracellular efficacy of aminopyrazinamides in THP-1 macrophages

THP-1 cells (obtained from ATCC) were cultured in 75cm^2 flask to confluence using RPMI 1640 with 10% fetal calf serum (Sigma, St. Louis, Mo.) supplemented with 2mM L-Glutamine. The cells were grown till they reach a density of 500,000 cells/mL in a 37°C incubator with 5% CO_2 and 95% air. The cells are seeded at a density of 1-2 x 10^5 cells /mL and infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 1:10 (macrophage: bacteria) for 2 hours at 37°C (batch infection). After 2 hours, the cells were thoroughly washed twice with pre-warmed phosphate buffered saline to remove extracellular bacteria and replaced with complete RPMI1640. The cells were differentiated using Phorbol myristate acetate (Sigma) at a concentration of 40nm and allowed to adhere in a 96-well plate for 24 hours at 37°C. After 24 hours, varying concentrations of the test compounds are added to the monolayers and incubated for 7 days. The macrophage monolayers were periodically observed under a microscope to note any adverse changes in the cell morphology due to drug toxicity. At the start of drug treatment and at 7 days post-addition of drugs, the monolayers were gently washed and lysed with 0.04% SDS and plated on Middlebrook 7H11 agar plates. Bacterial colony formation was enumerated after incubation of plates for 21-28 days at 37°C under 5% CO_2 in a humidified incubator. Data were expressed as the $\log_{10}$CFU for each drug concentration.

Determination of Resistance Frequency

Spontaneous resistant mutants were raised against compound **11** using a single step selection method. Briefly, a mid-logarithmic phase culture of *M. tuberculosis* H37Rv was centrifuged and concentrated 100-fold to achieve a bacterial number of ~10^{10} CFU/mL. Varying dilutions of the bacterial culture were plated onto compound **11** containing plates (2 µg/mL to 8 µg/mL, corresponding to 4X, 8X and 16 X MIC concentrations of compound **11**). For comparison,

bacteria were plated on rifampicin containing plates (0.24µg/ml to 0.48µg/ml, corresponding to 8X and 16X MIC concentration of rifampicin). Appropriate dilutions of the bacterial culture were also plated on drug-free Middlebrook 7H11 agar to enumerate the bacterial numbers in the culture. Plates were incubated for 4 weeks at 37^{o}C and the CFUs in drug-free plates were enumerated. The drug-containing plates were incubated for up to 6 weeks at 37^{o}C to confirm the final number of spontaneously resistant colonies.

The spontaneous rate of resistance was calculated by dividing the number of colonies on drug - containing plates (at a given concentration) divided by the total number of viable bacteria estimated on drug-free plates. Resistant colonies were randomly picked from the drug containing plates and grown in complete 7H9 broth to determine their level of resistance against compound **11** as well as other standard TB drugs with different mechanisms of action. We were unable to obtain resistant mutants at 8µg/mL drug concentration indicating this is to be the mutant prevention concentration.

Genetic Mapping of mutations conferring resistance to aminopyrazinamides

To understand the genetic basis for reduced susceptibility to aminopyrazinamides, mutants were analyzed for genetic changes in the *gyrB* gene. Chromosomal DNA was isolated from well-characterized resistant clones by boiling the cultures for 20 minutes. The boiled supernatants were sub*J*ected to PCR analysis using specific *M. tuberculosis gyrB* primers to amplify the entire *gyrB* gene;

Forward 5'-GACGCACGGCGCGGTTAGA-3' and Reverse 5'-TTAGACATCCAGGAACCGAA-3'.

PCR was performed with cycling parameters of 94°C for 30 s, 67.5°C for 30 s, and 72°C for 2 min for 30 cycles in a DNA Engine Dyad cycler (Bio-Rad). PCR products were cleaned (PCR purification kit, Qiagen), quantitated and sequenced (Microsynth, Switzerland). The sequences from the resistant clones were aligned against the wild-type H37Rv *gyrB* gene using Vector NTI software to detect mutations in the target gene. The single resistant mutant revealed a point mutation resulting in a Glycine to Serine mutation at position 157 in the Mtb GyrB protein. The resistant clone displayed an 8-16 fold increase in MIC to the compound **11** and other

aminopyrazinamides, but lacked cross-resistance to novobiocin, rifampicin or isoniazid indicating the specificity of the resistance mechanism (data not shown).

Antimicrobial Activity against hypoxia induced non-replicating persistent (NRP) Mtb cells

M.tuberculosis H37Rv cultures were adapted to hypoxic conditions as described earlier (Wayne 1996) with minor modifications. Briefly, Mtb cells were grown in Dubos Tween broth in McCartney bottles with a magnetic bead using a defined head-space ratio (HSR) of 0.5. Methylene blue was added as a redox indicator (final concentration of 1.5µg/mL) to all bottles to monitor oxygen depletion. The MacCartney bottles are placed on a magnetic stirrer set at 180rpm, inside a 37^{o}C incubator. The methylene blue indicator started to fade by day 8 and completely decolorized by 12 days. The antimicrobial activity of various compounds against NRP Mtb cells was determined in 96-well microtiter plates using a 14-day old hypoxia adapted culture as described under the MIC determination section. The entire assay was performed in a hypoxic chamber (DuPoy) by exposing hypoxic cells to varying concentrations of compounds for 7 days at 37^{o}C. An anaerobic indicator strip was placed inside the chamber to visually confirm the removal of oxygen during the entire process. Bacterial enumeration was performed on Middlebrook 7H11 agar plates after incubation of plates for 21-28 days at 37^{o}C, 5% CO_2 in a humidified atmosphere. Isoniazid and nigericin were used as controls to check for hypoxic activity. Isoniazid showed no reduction in the bacterial CFU even at 10µg/mL concentration indicating a strict NRP state. Data are expressed as the $\log_{10}$CFU for each drug concentration.

SUPPORTING FIGURES AND TABLES:

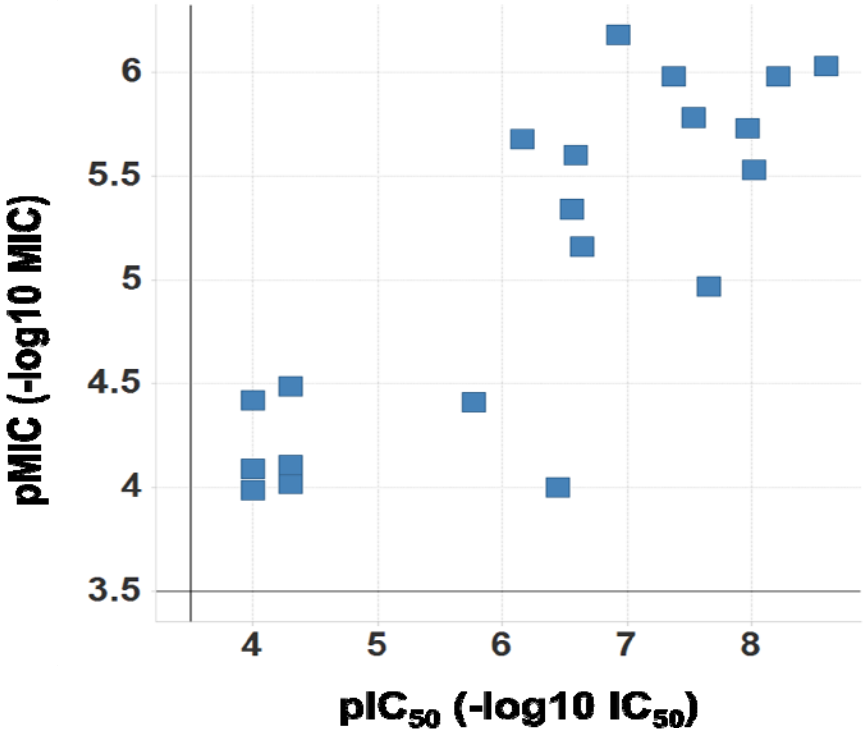


Figure S1. Correlation between GyrB pIC_{50} and Mtb pMIC

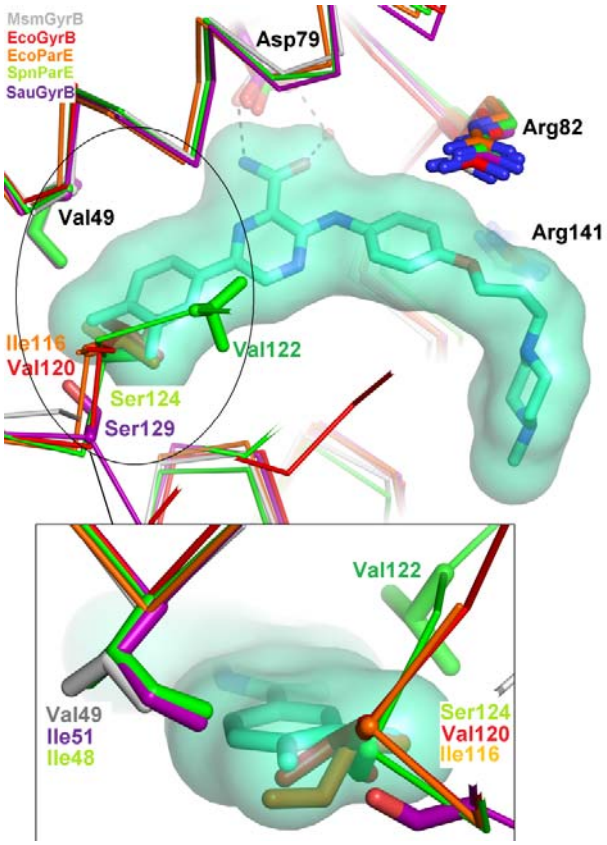


Figure S2a. Variations in sequence around the hydrophobic pocket account for the specificity of aminopyrazinamides for mycobacterial GyrB. The hydrophobic substituent of compound **6** clashes with several side-chains in other GyrB and ParE isozymes. The structure of MsmGyrB-EP8 (Cα trace and selected residues shown as sticks with carbon atoms coloured grey) in complex with compound **6** (carbon atoms in cyan) was superposed on the structures of *E. coli* GyrB (Cα trace and selected residues shown as sticks with carbon atoms coloured red, PDB ID 4DUH[15]), *E. coli* ParE (Cα trace and selected residues shown as sticks with carbon atoms coloured orange, PDB ID 3FV5[21]), *S. pneumoniae* ParE (Cα trace and selected residues shown as sticks with carbon atoms coloured green, PDB ID 4EMV[16]) and *S. aureus* GyrB (Cα trace and selected residues shown as sticks with carbon atoms coloured purple, PDB ID 3TTZ[17]) using the SSM Superpose option in Coot[4]. A semi-transparent molecular surface for compound **6** is shown. Hydrogen bonds between the carboxamide of compound **6** and the side-chain of MsmGyrB-EP8-Asp79 are shown as dotted lines. Figures prepared using PyMol (Schrödinger LLC).

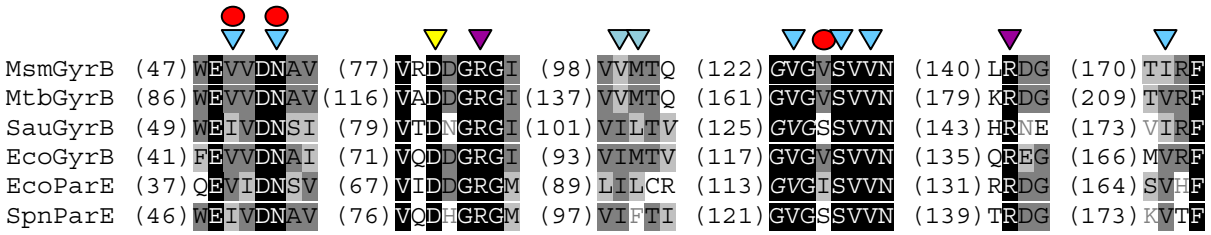


Figure S2b. Sequences for full length proteins were extracted from UniProt and aligned against the superposed structures in figure 2a using the Protein Align options in MOE (Chemical Computing Group). The alignment visualized, edited and annotated using Vector NTI (Invitrogen). Residues surrounding the hydrophobic pocket are less well conserved across isozymes. Residues which are disordered or missing from the structural models are highlighted in italics. Residues in site 1 (yellow), site 2 (purple) and the hydrophobic pocket (cyan) of MsmGyrB-EP8 are indicated with coloured triangles above the sequence. Residue positions showing steric conflict with the hydrophobic substituent of compound **6** upon superposition of other isozymes with the 4B6C (fig 2a) structures are indicated above the sequence with a red circle.

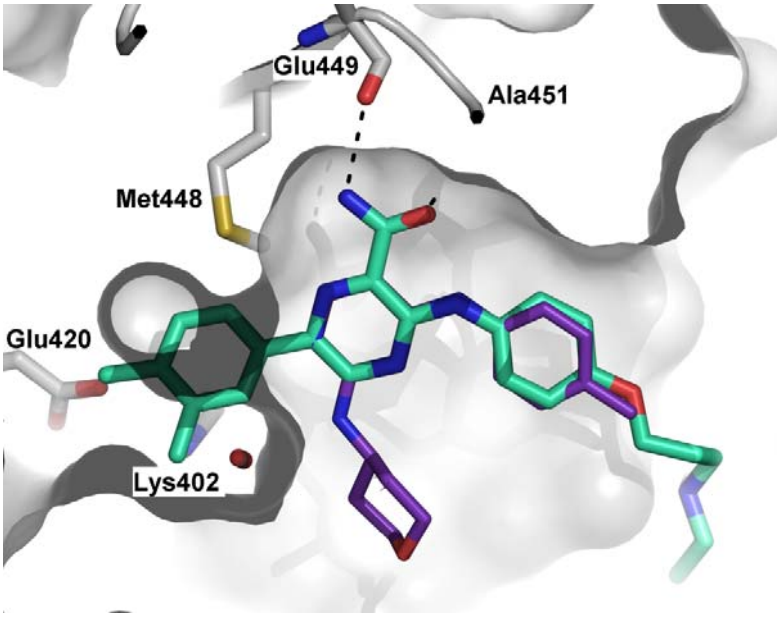


Figure S3. The structure of MsmGyrB-EP8 (not shown) in complex with compound **6** (carbon atoms in cyan) was superposed on the structure of Spleen Tyrosine Kinase (Syk, selected residues shown as sticks with carbon atoms coloured grey) in complex with 2-{[(3R,4R)-3-aminotetrahydro-2H-pyran-4-yl]amino}-4-[(4-methylphenyl)amino]pyrimidine-5-carboxamide (PDB ID 3SRV[15,18], GSK143, carbon atoms in purple) using the Superpose Ligands and Rotate/Translate Zone options in Coot[4]. The hydrophobic substituent of compound **6** clashes with side-chains from the back of the ATP pocket (Lys402 & Met448), αC helix (Glu420) and activation loop (Ser511 & Asp512, not shown) of Syk. A semi-transparent molecular surface for the Syk protein chain is shown. Hydrogen bonds between the carboxamide of

GSK143 and the backbone of Syk hinge residues Glu449 and Ala451 are shown as dotted lines. Figure prepared using PyMol (Schrödinger LLC).

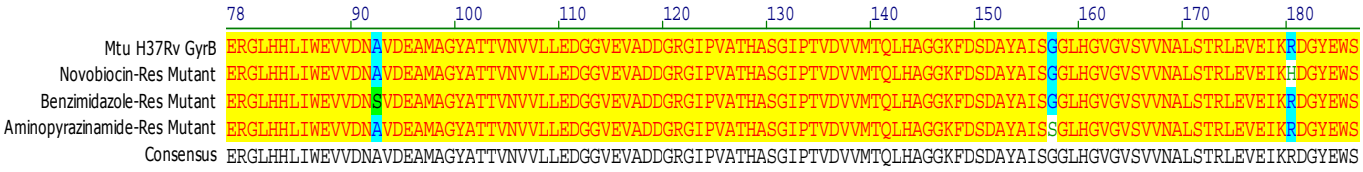
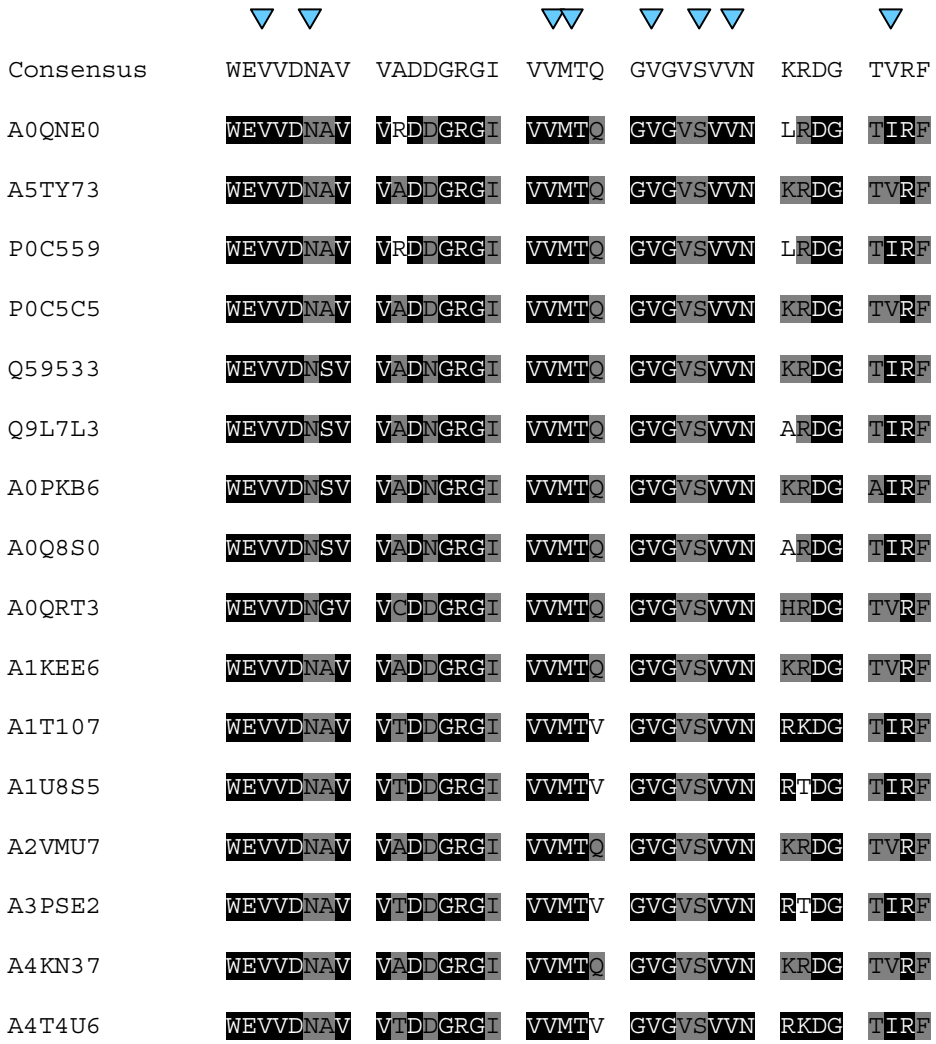
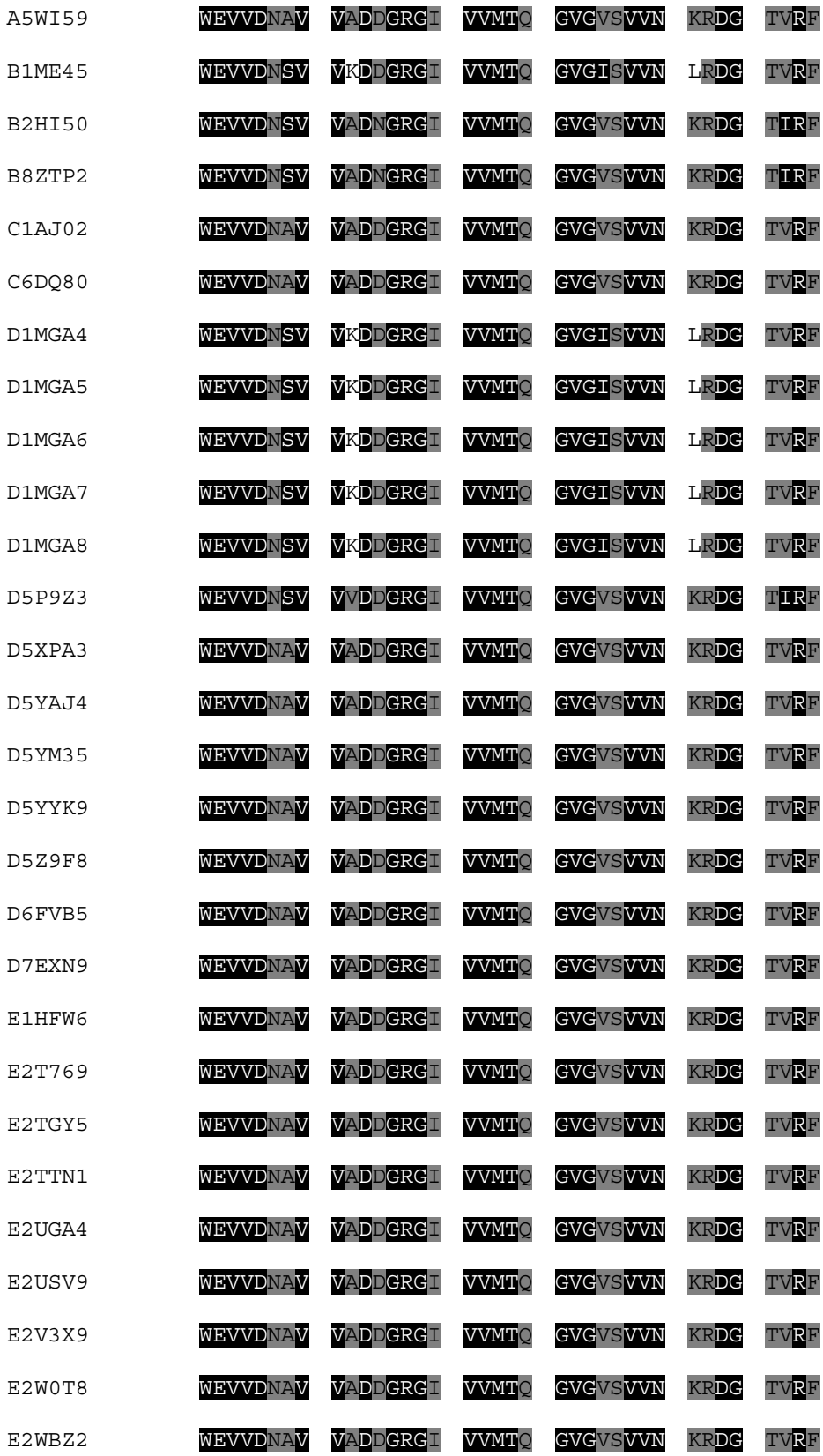


Figure S4. Multiple alignment of GyrB protein sequences from wild-type *M. tuberculosis* H37Rv against mutants resistant to novobiocin, benzimidazole[21] or compound **11** (aminopyrazinamide). All the designated mutations- Ala92Ser, Gly157Ser and Arg180His residue in the ATPase domain of GyrB.

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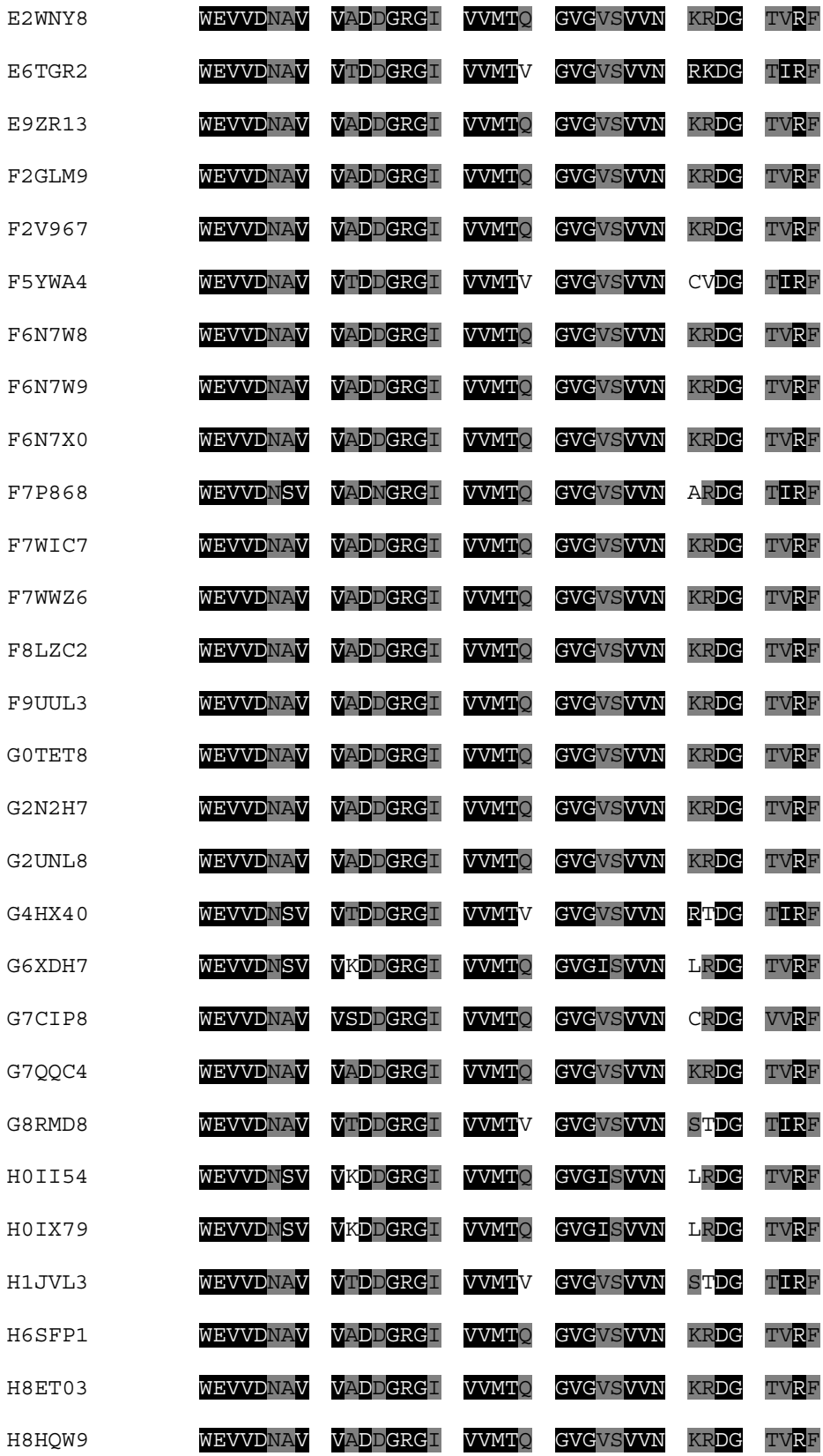
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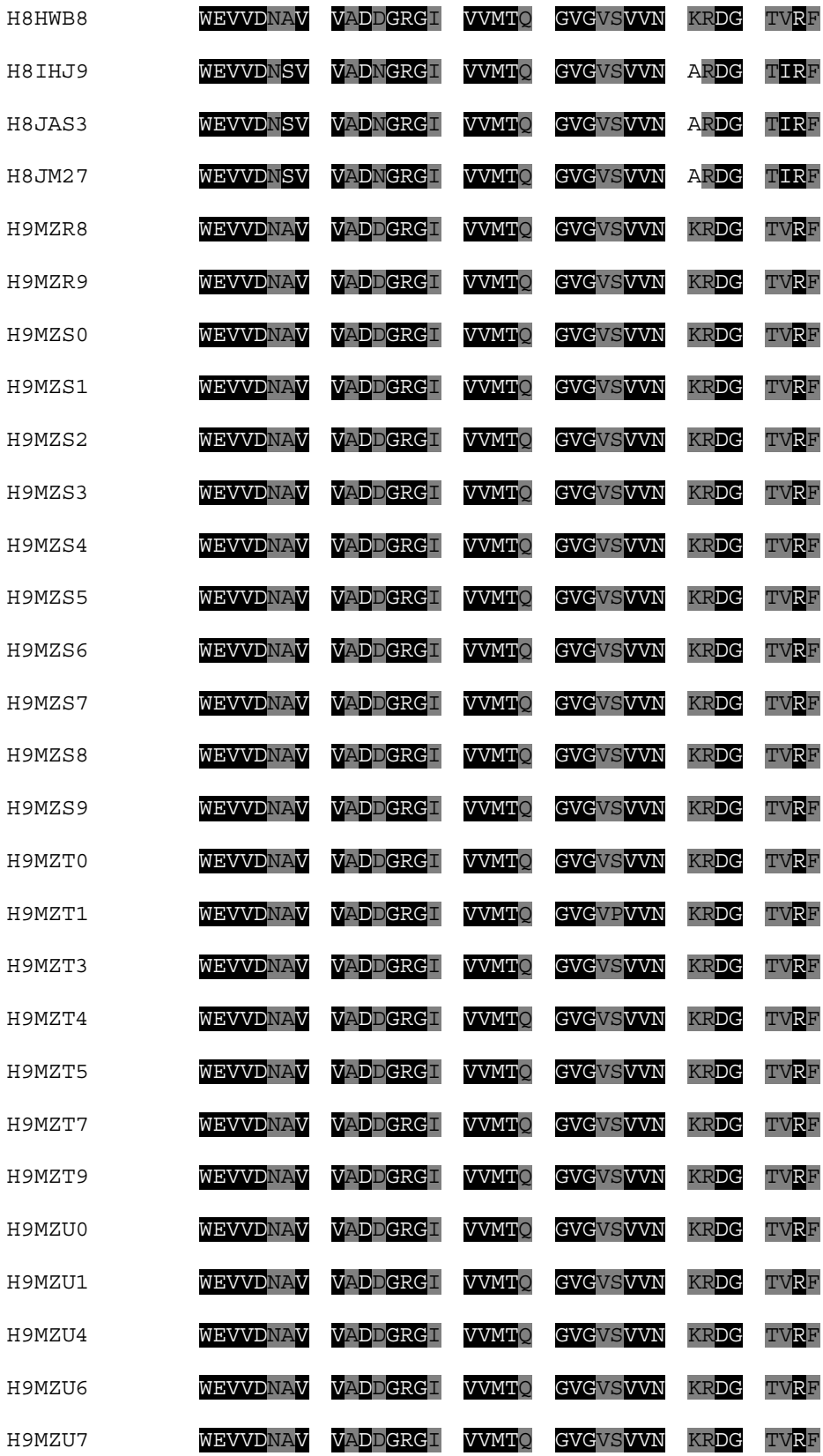
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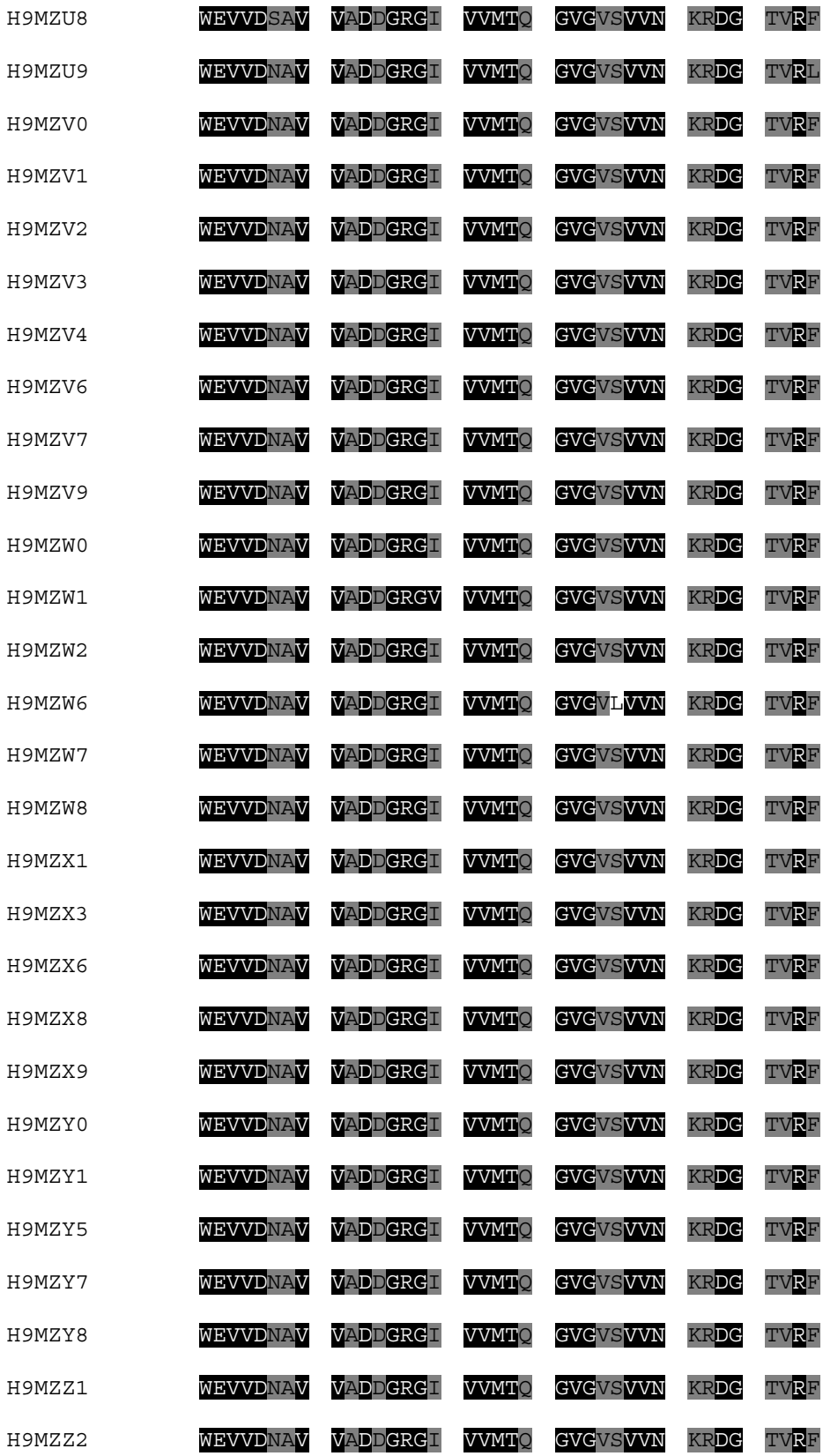
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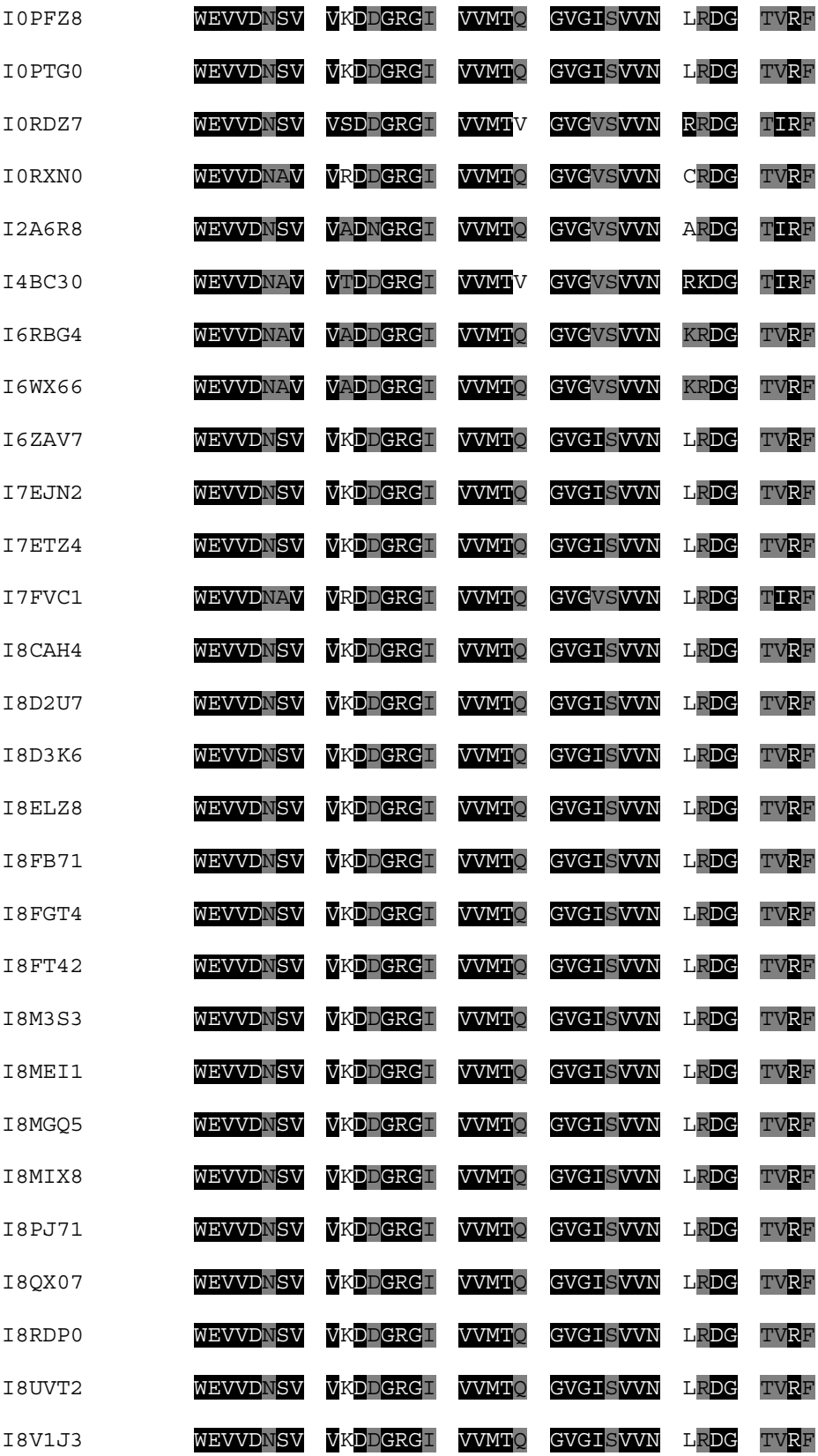
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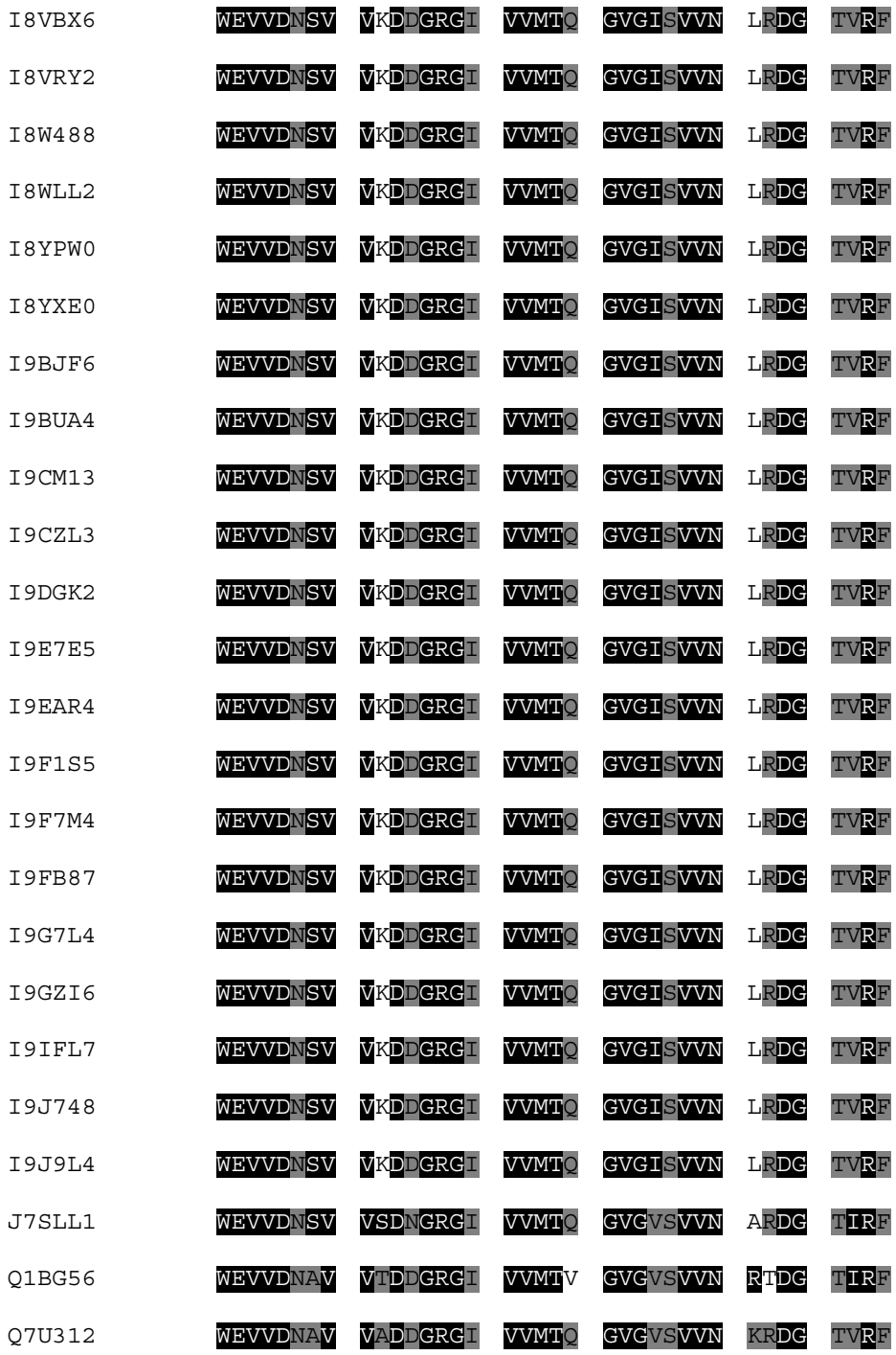

Figure S5 – Conservation of hydrophobic pocket residues across Mycobacterial GyrB sequences. Full length Mycobacterial GyrB sequences were retrieved from UniProt using a BLAST search against the entry P0C5C5 and aligned using the Protein Align options in MOE (Chemical Computing Group). The alignment was edited to remove regions outside of the ligand binding pocket in MOE, and then coloured to show conservation using VectorNTi (Invitrogen). Residues in the hydrophobic pocket are indicated with cyan triangles above the sequence

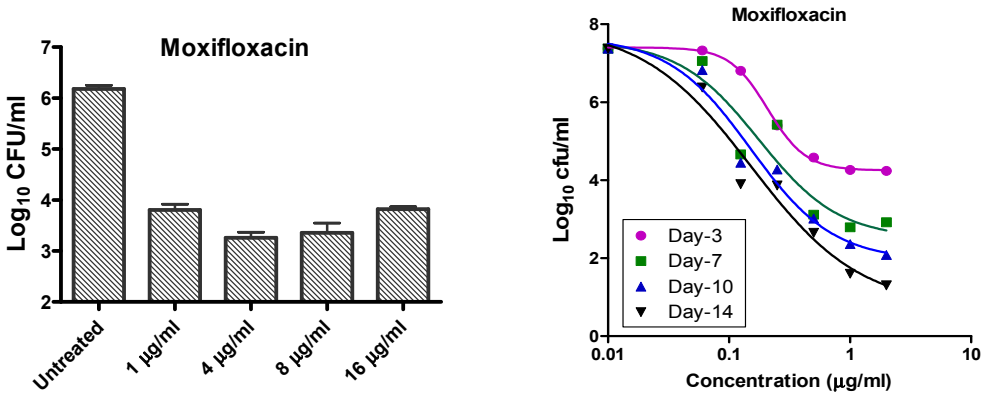

Figure S6 – The cidality of Moxifloxacin in the *in vitro* assay as well as in the THP-1 macrophage assay. Moxifloxacin displays excellent kill under in vitro conditions in a concentration as well as time dependent manner resulting in >6-logs of kill in 14 days following drug exposure. Similarly, Moxifloxacin exhibits 2 logs of kill against intracellular mycobacteria residing in THP-1 macrophages following 7 days of drug exposure.

Table S1. Kinase inhibition data for compound **11**

Kinase	**Compound 11 at 1 µM % Inhib**	**Kinase**	**Compound 11 at 1 µM % Inhib**	**Kinase**	**Compound 11 at 1 µM % Inhib**
Aurora B	-11	IKK epsilon(DU14231)	7	MST2	15
BTK	3	IKKb	6	NEK2a	5
CAMK-1	29	IRAK4	7	NEK6	-10
CAMKKb	8	IR-HIS	2	P70s6k	-6
CDK2/cyclinA2	6	JAK2	-3	PAK4	4
CHK2	9	JNK/SAPK1c	10	PBK	-8
CK1(hum)	-6	JNK2	5	PDK1	21

CK2a	-3	LCK (pur)	-2	PIM3	15
CSK	-7	LKB1	-29	PKA (rec)	-4
DYRK1a	-3	MAPKAP-K1b	1	PKBb	16
DYRK3	4	MAPKAP-K2	8	PKC zeta	-5
EF2K	7	MARK2	3	PKCa	17
EGFR	2	MARK3	-2	PKD1	18
EPH A2	11	MARK4	20	PLK1 (okadaic acid)	-8
EPHB3	7	MEKK1	15	PRAK	5
ERK2(hum)	15	MELK	26	PRK2	4
FGF-R1	19	MET	-6	RIPK2	-4
FYN	-20	MKK1 (hum)	28	ROCK-I	14
GCK	5	MLK3	-3	ROCK-II	-9
GSK3b	-7	MNK1	25	SAPK2A/p38	-39
HIPK2	1	MSK1	7	SAPK2b/P38b2	-23
SAPK4/p38d	7	SGK	25	Src (hum)	-1
TAK1	8	TTK	8	SRPK1	12
TBK1 (DU12569)	1	VEG-FR	25	SYK	-15
YES 1	15				

Table S2. Frequency of spontaneous resistance in *M. tuberculosis* H37Rv arising to compound **11** or rifampicin.

Compound	**Concentration (µg/mL)**	**Frequency**
Compound **11**	8 (16x MIC)	Mutant Prevention Concentration

Compound **11**	4 (8x MIC)	2.1×10^{-11}
Compound **11**	2 (4x MIC)	2.5×10^{-8}
Rifampicin	0.48 (16x MIC)	9.3×10^{-9}
Rifampicin	0.24 (8x MIC)	1.6×10^{-8}

Table S3. X-ray Diffraction Data-Processing and Refinement Statistics

PDB ID	**4B6C**
Space group	$C222_1$
Cell constants	a=43.45, b=82.25, c=191.94Å
Resolution range (Å) [1]	28.06-2.20 (2.28- 2.20)
Completeness (%)[1]	89.8 (51.9)
Reflections, unique	16097
Multiplicity [1]	5.26 (3.64)
*R*merge $_{overall}$ [1,2]	0.152 (0.409)
Resolution range for refinement (Å) [1]	28.06-2.20 (2.35- 2.20)
*R*value $_{overall}$ (%)[1, 3]	20.2 (20.3)
*R*value $_{free}$ (%)[1]	25.4 (27.5)
Non-hydrogen protein atoms	2591
Non-hydrogen ligand atoms	70
Solvent molecules	114
R.m.s. deviations from ideal values	
Bond lengths (Å)	0.010
Bond angles (°)	1.07
Average *B* values (Å^2)	
Protein main chain atoms	38.3

Protein all atoms	40.2
Ligand	35.9
Solvent	38.4
Sodium ions	44.0
Φ, Ψ angle distribution for residues [4]	
In most favoured regions (%)	91.2
In additional allowed regions (%)	7.7
In generously regions (%)	0.4
In disallowed regions (%)	0.7

1 values in parentheses correspond to the outer resolution shell

2 $R_{merge} = \Sigma_{hkl} [(\Sigma_i |I_i - \langle I \rangle|)/ \Sigma_i I_i]$

3 $R_{value} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$

R_{free} is the cross-validation R factor computed for the test set of 5 % of unique reflections

4 Ramachandran statistics as defined by PROCHECK [11-13]

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