Supporting Information

Identification of ML251, a potent inhibitor of *T. brucei and T. cruzi* phosphofructokinase

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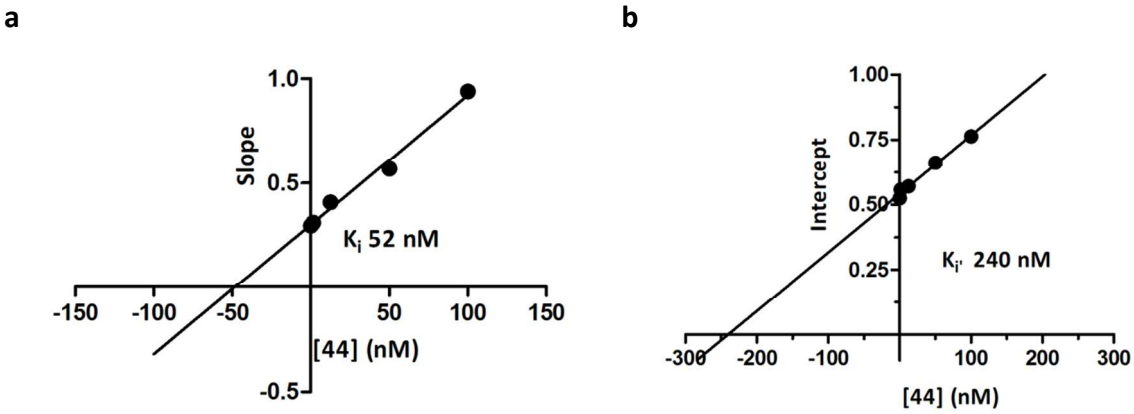
Sequence	Parameter	Value	Description
1	Reagent	3 µL	ATP/F6P substrate buffer (0.1 & 0.5mM final conc., resp.)
2	Compound	23 nL	Compound Library
3	Reagent	1 µL	*Tb* PFK (1.25nM final conc.) or buffer-only control
4	Time	45 min	RT incubation
5	Reagent	2.5 µL	ADP-Glo reagent I
6	Time	10 min	RT incubation
7	Reagent	5 µL	ADP-Glo reagent II (Kinase Detection reagent)
8	Time	10 min	RT incubation
9	Detector	Luminescence	ViewLux in end-point mode: 1 second exposure

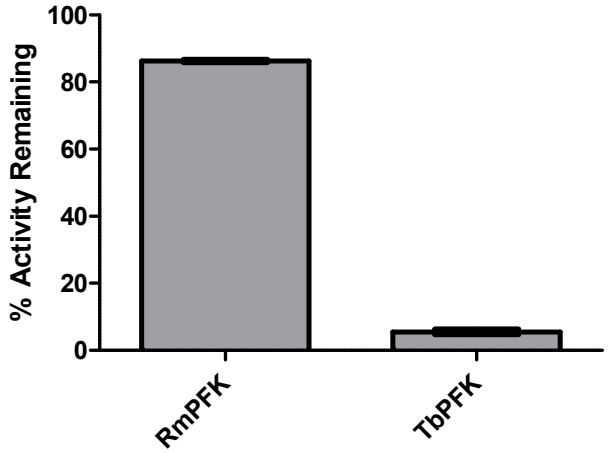
Supplemental Table 1. ADP-Glo *Tb* PFK qHTS assay protocol.

Sequence	Parameter	Value	Description
1	Reagent	3 µL	ATP/F6P substrate buffer (0.1 & 0.5mM final conc., resp.)
2	Compound	23 nL	Compound Library
3	Reagent	1 µL	*Tb* PFK (1.25nM final conc.) or buffer-only control
4	Time	45 min	RT incubation
5	Reagent	2 µL	Kinase-Glo Plus reagent
6	Time	10 min	RT incubation
7	Detector	Luminescence	ViewLux in end-point mode: 1 second exposure

Supplemental Table 2. Kinase-Glo Plus *Tb* PFK qHTS assay protocol.



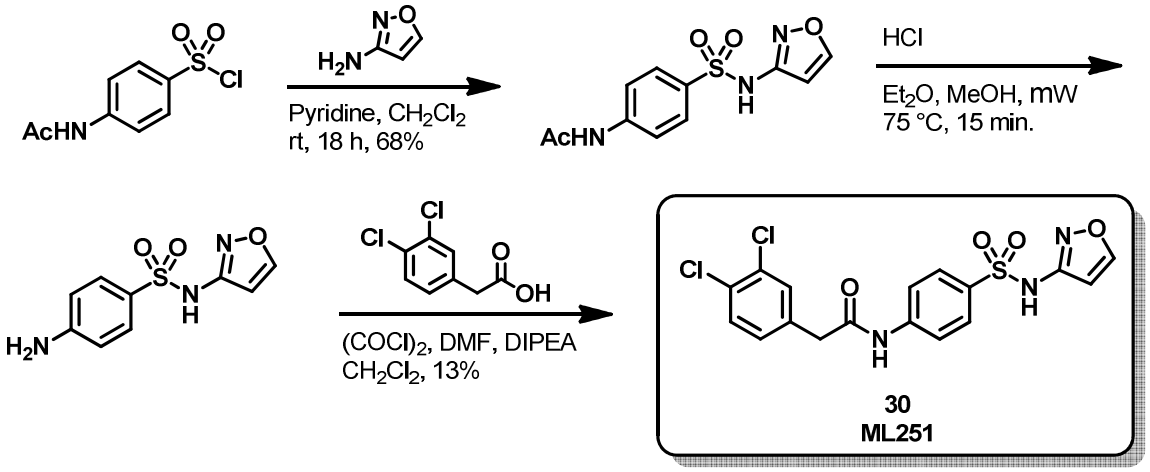
Supplmentary Figure 1. Secondary plots to derive inhibition constants for **42** are shown for a) F6P (K_i) and b) saturating ATP ($K_{i'}$).



Supplemental Figure 2. Rabbit muscle PFK and *T. brucei* PFK inhibition assay performed at 1 μM of **42**.

General Methods for Chemistry. All air or moisture sensitive reactions were performed under positive pressure of nitrogen with oven-dried glassware. Anhydrous solvents such as dichloromethane, *N,N*-dimethylformamide (DMF), acetonitrile, methanol and triethylamine were purchased from Sigma-Aldrich. Preparative purification was performed on a Waters semi-preparative HPLC system. The column used was a Phenomenex Luna C18 (5 micron, 30 x 75 mm) at a flow rate of 45 mL/min. The mobile phase consisted of acetonitrile and water (each containing 0.1% trifluoroacetic acid). A gradient of 10% to 50% acetonitrile over 8 minutes was used during the purification. Fraction collection was triggered by UV detection (220 nM). Analytical analysis was performed on an Agilent LC/MS (Agilent Technologies, Santa Clara, CA). Method 1: A 7 minute gradient of 4% to 100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) was used with an 8 minute run time at a flow rate of 1 mL/min. A Phenomenex Luna C18 column (3 micron, 3 x 75 mm) was used at a temperature of 50 °C. Method 2: A 3 minute gradient of 4% to 100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) was used with a 4.5 minute run time at a flow rate of 1 mL/min. A Phenomenex Gemini Phenyl column (3 micron, 3 x 100 mm) was used at a temperature of 50 °C. Purity determination was performed using an Agilent Diode Array Detector for all Methods. Mass determination was performed using an Agilent 6130 mass spectrometer with electrospray ionization in the positive mode. ^{1}H NMR spectra were recorded on Varian 400 MHz spectrometers. Chemical Shifts are reported in ppm with undeuterated solvent (DMSO-h6 at 2.50 ppm) as internal standard for DMSO-d6 solutions. All of the analogs tested in the biological assays have a purity greater than 95% based on both analytical methods. High-resolution mass spectrometry was recorded on an Agilent 6210 Time-of-Flight LC/MS system. Confirmation of molecular formulae was accomplished using electrospray ionization in the positive mode with the Agilent Masshunter software (version B.02).

Analogs **1-6, 8 - 27** and **30** were synthesize by the procedure exemplified in Figure 1b in the manuscript which entailed sulfonamide formation, deprotection of the nitrogen, then amide, sulfonamide coupling. **7** utilized a reductive amination. Analogs **28, 29** and **31 – 42** utilized the scheme shown in Figure 1c in the manuscript. Preparation of **30** is given below.



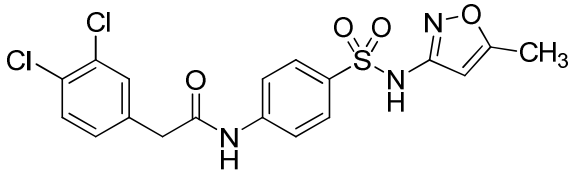
Step 1: Pyridine (0.208 mL, 2.57 mmol) and 4-acetamidobenzene-1-sulfonyl chloride (0.500 g, 2.14 mmol) were added sequentially to a solution of isoxazol-3-amine (0.198 g, 2.35 mmol) in CH_2Cl_2 (21.4 mL) were added. The reaction was capped and allowed to stir at ambient temperature overnight. Following concentration *in vacuo*, the crude residue was purified via silica gel chromatography using a 95:5 – 0:100, hexane/EtOAc (v/v) gradient to give N-(4-(N-isoxazol-3-ylsulfamoyl)phenyl)acetamide as a viscous oil (413 mg, 68%).

Step 2: A 2.0 M solution of HCl in Et_2O (0.800 mL, 1.60 mmol) was added to a solution of N-(4-(N-isoxazol-3-ylsulfamoyl)phenyl)acetamide (0.225 g, 0.80 mmol) in MeOH (8.0 mL). The reaction vial was capped and heated to 75 °C for 15 min in a microwave reactor. After cooling, the reaction was concentrated *in vacuo* to give crude 4-amino-N-(isoxazol-3-yl)benzenesulfonamide, HCl as a white solid that was judged to be >95% pure by LC/MS analysis. This material was therefore used in subsequent reactions without further purification.

Step 3: Oxalyl chloride (0.092 mL, 1.05 mmol) and DMF (5.8 μL, 0.08 mmol) were added to a solution of 2-(3,4-dichlorophenyl)acetic acid (0.154 g, 0.75 mmol) in CH_2Cl_2

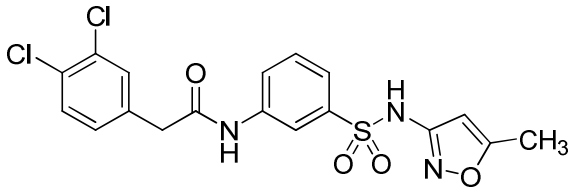
(1.25 mL), which resulted in vigorous evolution of gas. The reaction was left to stir for 2.5 hr and was then concentrated *in vacuo*. The residue was then taken up in CH_2Cl_2 (1.25 mL) and added to a solution of 4-amino-N-(isoxazol-3-yl)benzenesulfonamide (0.090 g, 0.38 mmol) and DIPEA (0.197 mL, 1.13 mmol) in CH_2Cl_2 (1.25 mL) at ambient temperature. After 30 min at the same temperature, the reaction was concentrated *in vacuo* and the residue was taken up in EtOAc (15 mL), washed with water (2 x 15 mL) and brine (2 x 15 mL), and dried over anhydrous Na_2SO_4. The mixture was filtered and concentrated *in vacuo* to give an orange oil that was taken up in 1.5 mL DMSO and purified via reverse phase chromatography to give 2-(3,4-dichlorophenyl)-N-(4-(N-isoxazol-3-ylsulfamoyl)phenyl)acetamide, TFA (ML251) as a white solid (21 mg, 13%).

Compound Characterization



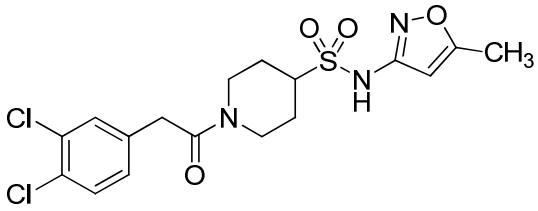
2-(3,4-dichlorophenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (1)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, br. s.), 10.59 (1 H, s), 7.70 - 7.83 (4 H, m), 7.53 - 7.63 (2 H, m), 7.31 (1 H, dd, *J*=8.2, 2.0 Hz), 6.10 (1 H, s), 3.73 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.761 min and t_2 (Method 2) = 3.530 min; HRMS calculated for $C_{18}H_{16}Cl_2N_3O_4S$ $(M+H)^+$ 440.0233, found 440.0225.



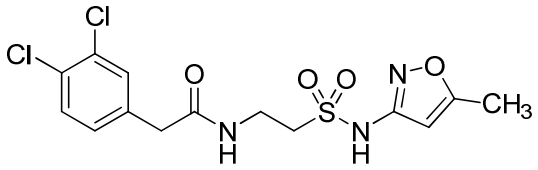
2-(3,4-dichlorophenyl)-N-(3-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (2)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.47 (1 H, s), 10.55 (1 H, s), 8.19 - 8.25 (1 H, m), 7.74 - 7.80 (1 H, m), 7.57 - 7.63 (2 H, m), 7.47 - 7.56 (2 H, m), 7.32 (1 H, dd, *J*=8.4, 1.8 Hz), 6.09 (1 H, s), 3.72 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.675 min and t_2 (Method 2) = 3.557 min. HRMS calculated for $C_{18}H_{16}Cl_2N_3O_4S$ $(M+H)^+$ 440.0233, found 440.0222.



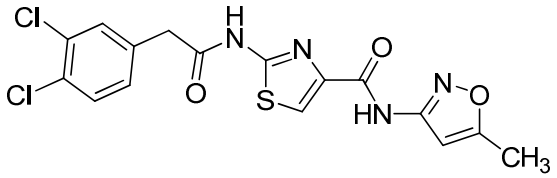
1-(2-(3,4-dichlorophenyl)acetyl)-N-(5-methylisoxazol-3-yl)piperidine-4-sulfonamide (3)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.97 (1 H, br. s.), 7.55 (1 H, d, *J*=8.2 Hz), 7.49 (1 H, d, *J*=2.0 Hz), 7.20 (1 H, dd, *J*=8.2, 2.0 Hz), 6.12 (1 H, s), 4.37 - 4.52 (1 H, m), 4.00 - 4.13 (1 H, m), 3.69 - 3.83 (2 H, m), 3.46 - 3.59 (1 H, m), 3.02 - 3.14 (1 H, m), 2.57 - 2.69 (1 H, m), 2.34 (3 H, s), 1.90 - 2.04 (2 H, m), 1.35 - 1.61 (2 H, m); LC-MS Retention Time: t_1 (Method 1) = 5.395 min and t_2 (Method 2) = 3.352 min; HRMS calculated for $C_{17}H_{20}Cl_2N_3O_4S$ $(M+H)^+$ 432.0546; found 432.0549.



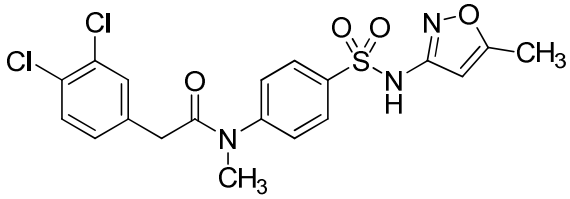
2-(3,4-dichlorophenyl)-N-(2-(N-(5-methylisoxazol-3-yl)sulfamoyl)ethyl)acetamide (NCGC00244110-01) (4)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.92 (1 H, s), 8.13 - 8.27 (1 H, m), 7.55 (1 H, d, *J*=8.2 Hz), 7.49 (1 H, d, *J*=2.0 Hz), 7.21 (1 H, dd, *J*=8.2, 2.0 Hz), 6.09 (1 H, s), 3.36 - 3.48 (6 H, m), 2.35 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.174 min and t_2 (Method 2) = 3.240 min. HRMS calculated for $C_{14}H_{16}Cl_2N_3O_4S$ $(M+H)^+$ 392.0233, found 392.0217.



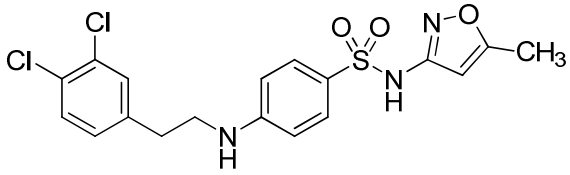
2-(2-(3,4-dichlorophenyl)acetamido)-N-(5-methylisoxazol-3-yl)thiazole-4-carboxamide (5).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.66 (s, 1H), 10.46 (s, 1H), 8.10 (s, 1H), 7.67 – 7.54 (m, 2H), 7.33 (dd, *J* = 8.3, 2.1 Hz, 1H), 6.73 (q, *J* = 0.8 Hz, 1H), 3.85 (s, 2H), 2.41 (d, *J* = 0.9 Hz, 3H). LC-MS Retention Time: t_1 (Method 1) = 6.024 min and t_2 (Method 2) = 3.696 min; HRMS calculated for $C_{16}H_{13}Cl_2N_4O_3S$ $(M+H)^+$ 411.008, found 411.0066.



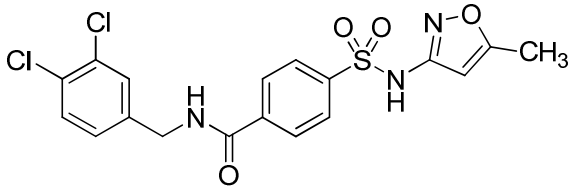
2-(3,4-dichlorophenyl)-N-methyl-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (6)

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.51 (s, 1H), 7.93 – 7.86 (m, 2H), 7.64 – 7.57 (m, 2H), 7.49 (d, J = 8.3 Hz, 1H), 7.38 (s, 1H), 7.07 (d, J = 8.3 Hz, 1H), 6.17 (q, J = 0.8 Hz, 1H), 3.62 (d, J = 7.6 Hz, 2H), 3.25 (s, 3H), 2.30 (d, J = 0.9 Hz, 3H). LC-MS Retention Time: t_1 (Method 1) = 5.435 min and t_2 (Method 2) = 3.422 min; HRMS calculated for $C_{19}H_{18}Cl_2N_3O_4S$ $(M+H)^+$ 454.039, found 454.0392.



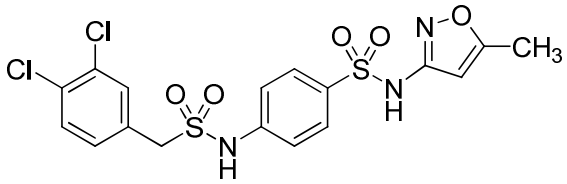
4-(3,4-dichlorophenethylamino)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (7)

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.94 (s, 1H), 7.59 – 7.49 (m, 4H), 7.26 (dd, J = 8.3, 2.1 Hz, 1H), 6.70 (t, J = 5.6 Hz, 1H), 6.68 – 6.62 (m, 2H), 6.09 (t, J = 0.8 Hz, 1H), 3.36 – 3.27 (m, 2H), 2.83 (t, J = 7.2 Hz, 2H), 2.28 (d, J = 0.9 Hz, 3H). LC-MS Retention Time: t_1 (Method 1) = 6.302 min and t_2 (Method 2) = 3.779 min. HRMS calculated for $(M+H)^+$ 426.0440, found= 426.0423.

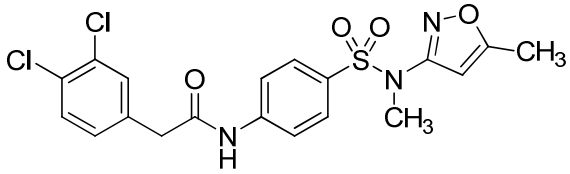


N-(3,4-dichlorobenzyl)-4-(N-(5-methylisoxazol-3-yl)sulfamoyl)benzamide (8)

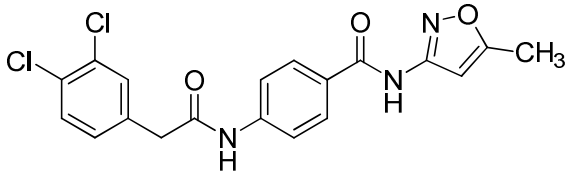
^{1}H NMR (400 MHz, DMSO-d_6) δ 11.58 (s, 1H), 9.28 (t, J = 5.9 Hz, 1H), 8.07 – 8.01 (m, 2H), 7.97 – 7.92 (m, 2H), 7.61 – 7.56 (m, 2H), 7.34 – 7.29 (m, 1H), 6.14 (q, J = 0.8 Hz, 1H), 4.47 (d, J = 5.9 Hz, 2H), 2.29 (d, J = 0.9 Hz, 3H). LC-MS Retention Time: t_1 (Method 1) = 5.631 min and t_2 (Method 2) = 3.600 min. HRMS calculated for $(M+H)^+$ 440.0233, found= 440.0229.



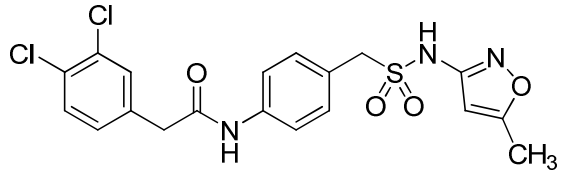
4-((3,4-dichlorophenyl)methylsulfonamido)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (9): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.35 (1 H, br. s.), 10.50 (1 H, br. s.), 7.78 (2 H, d, *J*=8.6 Hz), 7.62 (1 H, d, *J*=8.2 Hz), 7.51 (1 H, d, *J*=2.0 Hz), 7.30 (2 H, d, *J*=9.0 Hz), 7.23 (1 H, dd, *J*=8.6, 2.0 Hz), 6.13 (1 H, s), 4.69 (2 H, s), 2.30 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.652 min and t_2 (Method 2) = 3.562 min; HRMS calculated for $C_{17}H_{16}Cl_2N_3O_5S_2$ $(M+H)^+$ 475.9903, found 475.9895.



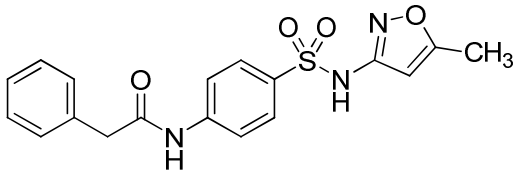
2-(3,4-dichlorophenyl)-N-(4-(N-methyl-N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (10): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.65 (1 H, s), 7.77 - 7.85 (2 H, m), 7.70 - 7.77 (2 H, m), 7.55 - 7.64 (2 H, m), 7.31 (1 H, dd, *J*=8.2, 2.0 Hz), 6.49 (1 H, s), 3.74 (2 H, s), 3.18 (3 H, s), 2.36 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 6.480 min and t_2 (Method 2) = 3.885 min; HRMS calculated for $C_{19}H_{18}Cl_2N_3O_4S$ $(M+H)^+$ 454.039, found 454.0398.



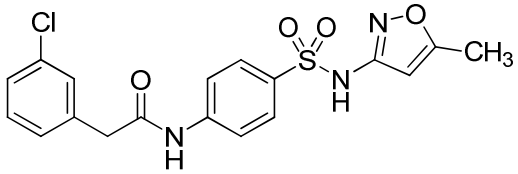
4-(2-(3,4-dichlorophenyl)acetamido)-N-(5-methylisoxazol-3-yl)benzamide (11) ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.14 (s, 1H), 10.48 (s, 1H), 8.04 – 7.95 (m, 2H), 7.70 (d, *J* = 8.8 Hz, 1H), 7.65 – 7.53 (m, 2H), 7.33 (dd, *J* = 8.3, 2.1 Hz, 1H), 6.73 (d, *J* = 1.0 Hz, 1H), 3.74 (s, 2H), 2.40 (d, *J* = 0.9 Hz, 3H). LC-MS Retention Time: t_1 (Method 1) = 5.871 min and t_2 (Method 2) = 3.659 min. HRMS calculated for $C_{19}H_{16}Cl_2N_3O_3$ $(M+H)^+$ 404.0563, found 404.0567.



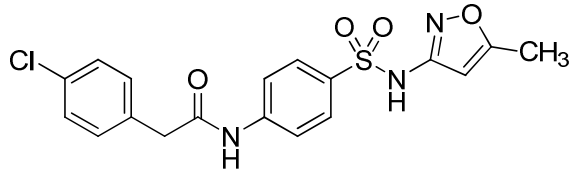
2-(3,4-dichlorophenyl)-N-(4-((N-(5-methylisoxazol-3-yl)sulfamoyl)methyl)phenyl)acetamide (12): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.80 (1 H, s), 10.25 (1 H, s), 7.57 - 7.62 (2 H, m), 7.54 (2 H, d, *J*=8.6 Hz), 7.31 (1 H, dd, *J*=8.2, 2.0 Hz), 7.22 (2 H, d, *J*=8.6 Hz), 5.95 (1 H, s), 4.53 (2 H, s), 3.69 (2 H, s), 2.30 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.784 min and t_2 (Method 2) = 3.605 min; HRMS calculated for $C_{19}H_{18}Cl_2N_3O_4S$ $(M+H)^+$ 454.0390, found 454.0390.



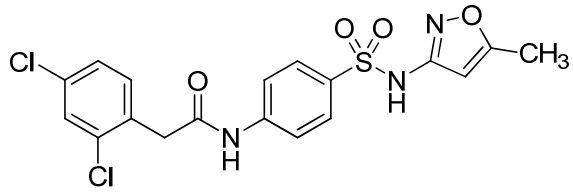
N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)-2-phenylacetamide (13): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.30 (1 H, s), 10.57 (1 H, s), 7.72 - 7.85 (4 H, m), 7.28 - 7.38 (4 H, m), 7.21 - 7.28 (1 H, m,), 6.10 (1 H, s), 3.67 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.159 min and t_2 (Method 2) = 3.408 min; HRMS calculated for $C_{18}H_{18}N_3O_4S$ $(M+H)^+$, 372.1013; found 372.1020.



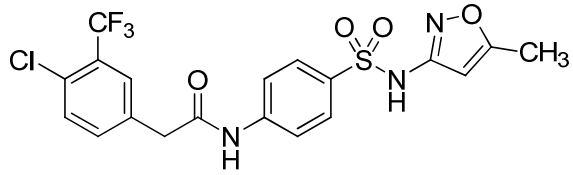
2-(3-chlorophenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (14): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, s), 10.58 (1 H, s), 7.73 - 7.82 (4 H, m), 7.37 - 7.42 (1 H, m), 7.30 - 7.37 (2 H, m), 7.25 - 7.30 (1 H, m), 6.10 (1 H, s), 3.71 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.541 min and t_2 (Method 2) = 3.445 min. HRMS calculated for $C_{18}H_{17}ClN_3O_4S$ $(M+H)^+$, 406.0623; found 406.0633.



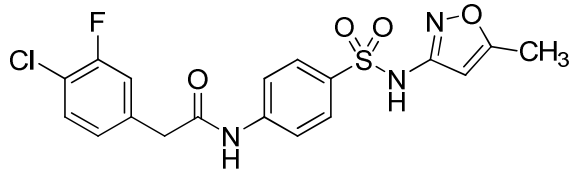
2-(4-chlorophenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (15): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, s), 10.58 (1 H, s), 7.73 - 7.83 (4 H, m), 7.36 - 7.42 (2 H, m), 7.30 - 7.36 (2 H, m), 6.10 (1 H, s), 3.69 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.543 min and t_2 (Method 2) = 3.451 min; HRMS calculated for $C_{18}H_{17}ClN_3O_4S$ $(M+H)^+$ 406.0623, found 406.0640.



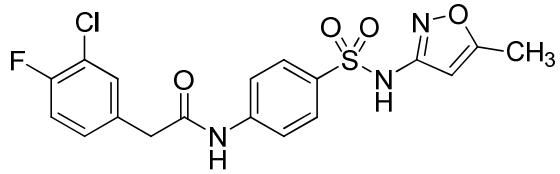
2-(2,4-dichlorophenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (16): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, s), 10.65 (1 H, s), 7.70 - 7.85 (4 H, m), 7.61 (1 H, s), 7.37 - 7.49 (2 H, m), 6.11 (1 H, s), 3.88 (2 H, s), 2.29 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.803 min and t_2 (Method 2) = 3.629 min. HRMS calculated for $C_{18}H_{16}Cl_2N_3O_4S$ $(M+H)^+$ 440.0233, found 440.0231.



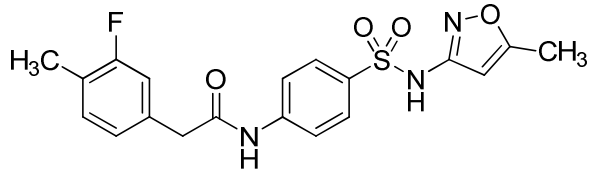
2-(4-chloro-3-(trifluoromethyl)phenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (17): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, s), 10.63 (1 H, s), 7.81 - 7.85 (1 H, m), 7.73 - 7.81 (4 H, m), 7.66 - 7.71 (1 H, m), 7.59 - 7.65 (1 H, m), 6.11 (1 H, s), 3.84 (2 H, s), 2.28 (3 H, s); ^{19}F NMR (376 MHz, DMSO-d_6) δ ppm -66.25; LC-MS Retention Time: t_1 (Method 1) = 5.984 min and t_2 (Method 2) = 3.675 min. HRMS calculated for $C_{19}H_{16}ClF_3N_3O_4S$ $(M+H)^+$ 474.0497, found 474.0488.



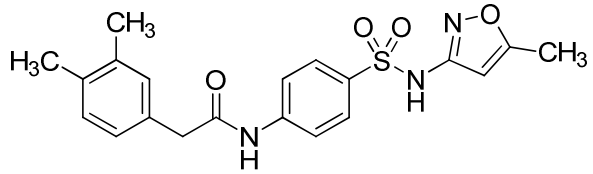
2-(4-chloro-3-fluorophenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (18): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, br. s.), 10.59 (1 H, s), 7.72 - 7.82 (4 H, m), 7.53 (1 H, dd, *J*=8.2, 7.8 Hz), 7.37 (1 H, dd, *J*=10.4, 1.8 Hz), 7.18 (1 H, dd, *J*=8.2, 1.2 Hz), 6.10 (1 H, s), 3.74 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.636 min and t_2 (Method 2) = 3.563 min; HRMS calculated for $C_{18}H_{16}ClFN_3O_4S$ $(M+H)^+$ 424.0529; found 424.0534.



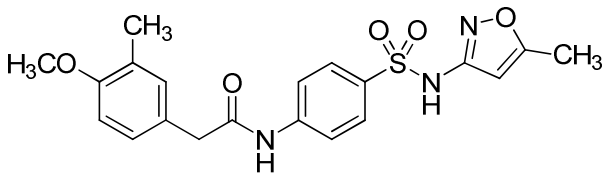
2-(3-chloro-4-fluorophenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (19): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, s), 10.57 (1 H, s), 7.72 - 7.82 (4 H, m), 7.54 (1 H, dd, *J*=7.0, 2.0 Hz), 7.35 - 7.40 (1 H, m), 7.28 - 7.35 (1 H, m), 6.10 (1 H, s), 3.71 (2 H, s), 2.28 (3 H, s); ^{19}F NMR (376 MHz, DMSO-d_6) δ ppm -124.61; LC-MS Retention Time: t_1 (Method 1) = 5.612 min and t_2 (Method 2) = 3.550 min; HRMS calculated for $C_{18}H_{16}ClFN_3O_4S$ $(M+H)^+$ 424.0529, found 424.0528.



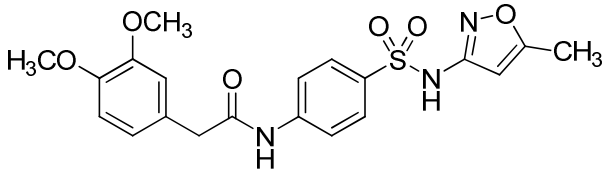
2-(3-fluoro-4-methylphenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (20): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (1 H, s), 10.55 (1 H, s), 7.72 - 7.83 (4 H, m), 7.22 (1 H, t, *J*=8.0 Hz), 7.09 (1 H, d, *J*=11.3 Hz), 7.04 (1 H, d, *J*=7.8 Hz), 6.10 (1 H, s), 3.66 (2 H, s), 2.28 (3 H, s), 2.20 (3 H, s); ^{19}F NMR (376 MHz, DMSO-d_6) δ ppm -123.23; LC-MS Retention Time: t_1 (Method 1) = 5.546 min and t_2 (Method 2) = 3.527 min; HRMS calculated for $C_{19}H_{19}FN_3O_4S(M+H)^+$ 404.1075, found 404.1081.



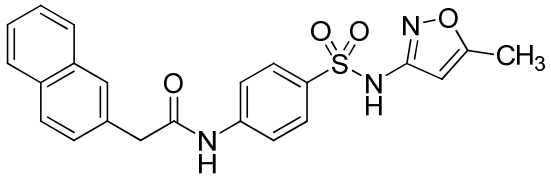
2-(3,4-dimethylphenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (21): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.30 (1 H, s), 10.51 (1 H, s), 7.73 - 7.81 (4 H, m), 7.04 - 7.10 (2 H, m), 6.99 - 7.04 (1 H, m), 6.10 (1 H, s), 3.58 (2 H, s), 2.28 (3 H, s), 2.19 (3 H, s), 2.18 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.658 min and t_2 (Method 2) = 3.454 min; HRMS calculated for $C_{20}H_{22}N_3O_4S$ $(M+H)^+$ 400.1326, found 400.1326.



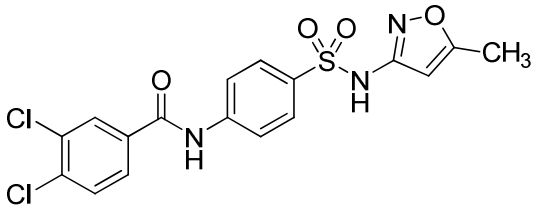
2-(4-methoxy-3-methylphenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (22): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.30 (1 H, s), 10.49 (1 H, s), 7.72 - 7.82 (4 H, m), 7.04 - 7.14 (2 H, m), 6.86 (1 H, d, *J*=8.2 Hz), 6.10 (1 H, s), 3.75 (3 H, s), 3.55 (2 H, s), 2.28 (3 H, s), 2.12 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.462 min and t_2 (Method 2) = 3.364 min; HRMS calculated for $C_{20}H_{22}N_3O_5S$ $(M+H)^+$ 416.1275, found 416.1277.



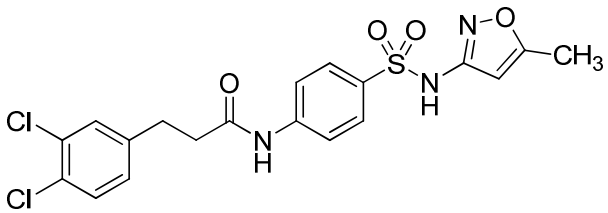
2-(3,4-dimethoxyphenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (23): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.30 (1 H, br. s.), 10.50 (1 H, s), 7.73 - 7.81 (4 H, m), 6.93 (1 H, d, *J*=1.6 Hz), 6.86 - 6.91 (1 H, m), 6.80 - 6.85 (1 H, m), 6.10 (1 H, s), 3.73 (3 H, s), 3.72 (3 H, s), 3.58 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 4.855 min and t_2 (Method 2) = 3.108 min; HRMS calculated for $C_{20}H_{22}N_3O_6S$ $(M+H)^+$ 432.1244; found 432.1229.



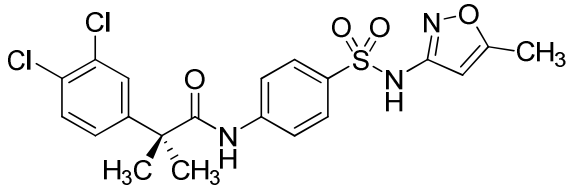
N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)-2-(naphthalen-2-yl)acetamide (24): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.30 (1 H, s), 10.65 (1 H, s), 7.85 - 7.92 (3 H, m), 7.82 (1 H, s), 7.74 - 7.81 (4 H, m), 7.44 - 7.56 (3 H, m), 6.10 (1 H, s), 3.86 (2 H, s), 2.28 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.705 min and t_2 (Method 2) = 3.517 min; HRMS calculated for $C_{22}H_{20}N_3O_4S$ $(M+H)^+$ 422.1169, found 422.1175.



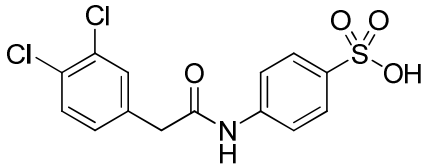
3,4-dichloro-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)benzamide (25): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.36 (1 H, s), 10.74 (1 H, s), 8.22 (1 H, d, *J*=2.0 Hz), 7.90 - 7.99 (3 H, m), 7.81 - 7.88 (3 H, m), 6.14 (1 H, s), 2.30 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.958 min and t_2 (Method 2) = 3.676 min. HRMS calculated for $C_{17}H_{14}Cl_2N_3O_4S$ $(M+H)^+$ 426.0077, found 426.0075.



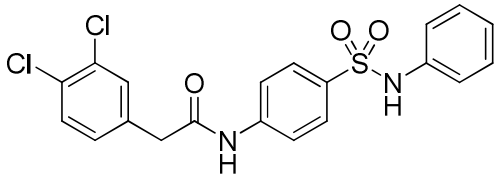
3-(3,4-dichlorophenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)propanamide (26): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.30 (1 H, s), 10.33 (1 H, s), 7.70 - 7.82 (4 H, m), 7.50 - 7.58 (2 H, m), 7.25 (1 H, dd, *J*=8.4, 1.8 Hz), 6.11 (1 H, s), 2.91 (2 H, t, *J*=7.4 Hz), 2.68 (2 H, t, *J*=7.4 Hz), 2.29 (3 H, s); LC-MS Retention Time: t_1 (Method 1) = 6.011 min and t_2 (Method 2) = 3.618 min. HRMS calculated for $C_{19}H_{18}Cl_2N_3O_4S$ $(M+H)^+$ 454.0390, found 454.0399.



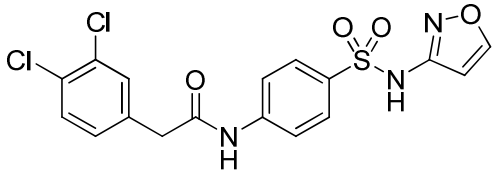
2-(3,4-dichlorophenyl)-2-methyl-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)propanamide (27): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.30 (1 H, s), 9.54 (1 H, s), 7.72 - 7.84 (4 H, m), 7.55 - 7.63 (2 H, m), 7.26 (1 H, dd, *J*=8.6, 2.3 Hz), 6.10 (1 H, s), 2.28 (3 H, s), 1.56 (6 H, s); LC-MS Retention Time: t_1 (Method 1) = 6.355 min and t_2 (Method 2) = 3.802 min. HRMS calculated for $C_{20}H_{20}Cl_2N_3O_4S$ $(M+H)^+$ 468.0546, found 468.0541.



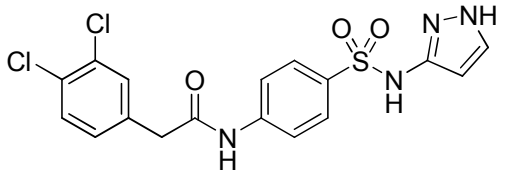
4-(2-(3,4-dichlorophenyl)acetamido)benzenesulfonic acid (28): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.25 (1 H, s), 7.56 - 7.62 (2 H, m), 7.52 (4 H, s), 7.32 (1 H, dd, *J*=8.4, 1.8 Hz), 3.70 (2 H, s), sulfonic acid proton not observed.; LC-MS Retention Time: t_1 (Method 1) = 4.539 min and t_2 (Method 2) = 3.351 min. HRMS calculated for $C_{14}H_{12}Cl_2NO_4S$ $(M+H)^+$ 359.9859, found 359.9867.



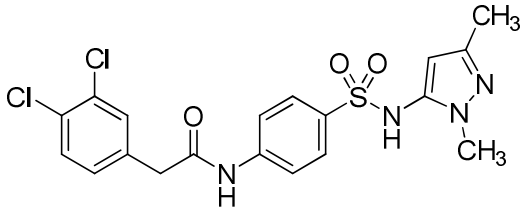
2-(3,4-dichlorophenyl)-N-(4-(N-phenylsulfamoyl)phenyl)acetamide (29): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.69 (1 H, br. s.), 10.55 (1 H, s), 7.68 - 7.82 (6 H, m), 7.54 - 7.61 (2 H, m), 7.29 - 7.16 (4 H), 3.71 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = 6.261 min and t_2 (Method 2) = 3.656 min. HRMS calculated for $C_{20}H_{17}Cl_2N_2O_3S$ $(M+H)^+$ 435.0331, found 435.0335.



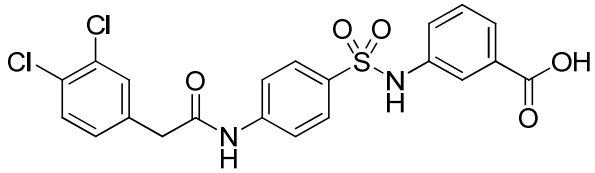
2-(3,4-dichlorophenyl)-N-(4-(N-isoxazol-3-ylsulfamoyl)phenyl)acetamide (30): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.45 (1 H, br. s.), 10.60 (1 H, s), 8.70 (1 H, d, *J*=1.4 Hz), 7.78 - 7.83 (2 H, m), 7.72 - 7.78 (2 H, m), 7.55 - 7.62 (2 H, m), 7.30 (1 H, dd, *J*=8.3, 1.7 Hz), 6.40 (1 H, d, *J*=1.6 Hz), 3.73 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.734 min and t_2 (Method 2) = 3.624 min; HRMS calculated for $C_{17}H_{14}Cl_2N_3O_4S$ $(M+H)^+$ 426.0077; found 426.0077.



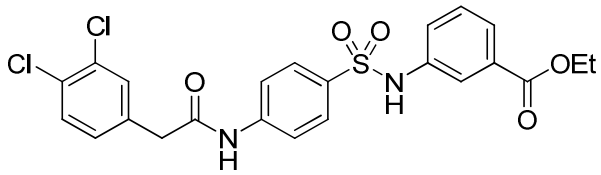
N-(4-(N-1H-pyrazol-3-ylsulfamoyl)phenyl)-2-(3,4-dichlorophenyl)acetamide (31): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.65 (1 H, s), 7.94 (1 H, d, *J*=2.7 Hz), 7.72 - 7.81 (4 H, m), 7.55 - 7.62 (2 H, m), 7.30 (1 H, dd, *J*=8.2, 2.0 Hz), 5.83 (1 H, d, *J*=2.7 Hz), 5.57 (2 H, s), 3.74 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.611 min and t_2 (Method 2) = 3.508 min; HRMS calculated for $C_{17}H_{15}Cl_2N_4O_3S$ $(M+H)^+$ 425.0236, found 425.0237.



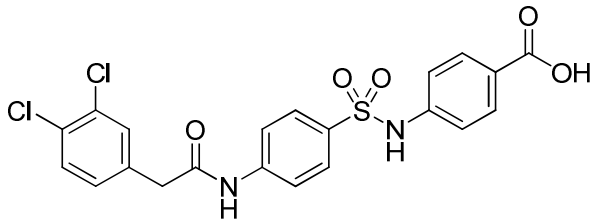
2-(3,4-dichlorophenyl)-N-(4-(N-(1,3-dimethyl-1H-pyrazol-5-yl)sulfamoyl)phenyl)acetamide (32); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 10.12 (s, 1H), 7.80 – 7.76 (m, 2H), 7.69 – 7.64 (m, 2H), 7.62 – 7.58 (m, 2H), 7.32 (dd, *J* = 8.3, 2.1 Hz, 1H), 5.43 (d, *J* = 0.6 Hz, 1H), 3.75 (s, 2H), 3.46 (s, 3H), 2.00 (s, 3H). LC-MS Retention Time: t_1 (Method 1) = 5.526 min and t_2 (Method 2) = 3.465 min. HRMS calculated for $C_{19}H_{19}Cl_2N_4O_3S$ $(M+H)^+$ 453.0549, found 453.0552.



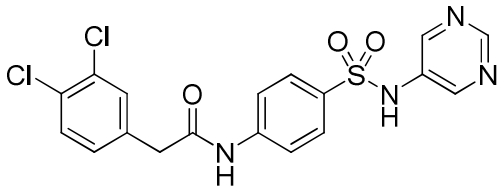
3-(4-(2-(3,4-dichlorophenyl)acetamido)phenylsulfonamido)benzoic acid (33): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.01 (1 H, br. s.), 10.54 (1 H, s), 10.39 (1 H, br. s.), 7.65 - 7.74 (5 H, m), 7.54 - 7.60 (3 H, m), 7.26 - 7.38 (3 H, m), 3.71 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.630 min and t_2 (Method 2) = 3.466 min. HRMS calculated for $C_{21}H_{17}Cl_2N_2O_5S$ $(M+H)^+$ 479.0230, found 479.0241.



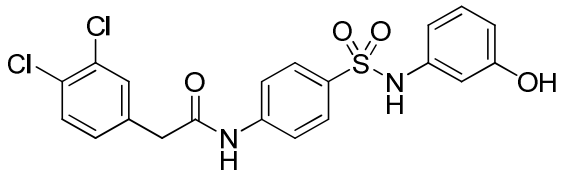
Ethyl 3-(4-(2-(3,4-dichlorophenyl)acetamido)phenylsulfonamido)benzoate (34): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.54 (1 H, s), 10.43 (1 H, s), 7.65 - 7.74 (5 H, m), 7.54 - 7.61 (3 H, m), 7.31 - 7.40 (2 H, m), 7.29 (1 H, dd, *J*=8.2, 2.0 Hz), 4.26 (2 H, q, *J*=7.0 Hz), 3.71 (2 H, s), 1.28 (3 H, t, *J*=7.0 Hz); LC-MS Retention Time: t_1 (Method 1) = 6.453 min and t_2 (Method 2) = 3.828 min; HRMS calculated for $C_{23}H_{21}Cl_2N_2O_5S$ $(M+H)^+$ 507.0543, found 507.0537.



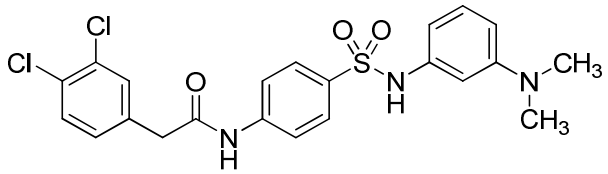
4-(4-(2-(3,4-dichlorophenyl)acetamido)phenylsulfonamido)benzoic acid (35): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.69 (1 H, br. s.), 10.55 (1 H, s), 7.68 - 7.82 (6 H, m), 7.54 - 7.61 (2 H, m), 7.29 (1 H, dd, *J*=8.4, 1.8 Hz), 7.16 (2 H, d, *J*=8.6 Hz), 3.71 (2 H, s), carboxylic acid proton not observed; LC-MS Retention Time: t_1 (Method 1) = 5.552 min and t_2 (Method 2) = 3.474 min; HRMS calculated for $C_{21}H_{17}Cl_2N_2O_5S$ $(M+H)^+$ 479.0230, found 479.0233.



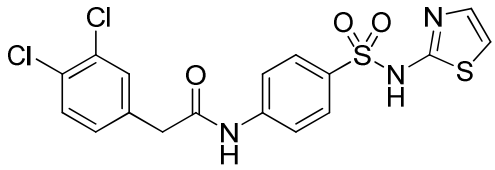
2-(3,4-dichlorophenyl)-N-(4-(N-pyrimidin-5-ylsulfamoyl)phenyl)acetamide (36): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.72 (1 H, s), 10.59 (1 H, s), 8.88 (1 H, s), 8.49 (2 H, s), 7.70 - 7.79 (4 H, m), 7.55 - 7.62 (2 H, m), 7.30 (1 H, dd, *J*=8.2, 2.0 Hz), 3.72 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = t_1 (Method 1) = 4.355 min and t_2 (Method 2) = 3.410 min; HRMS calculated for $C_{18}H_{15}Cl_2N_4O_3S$ $(M+H)^+$ 437.0236, found 437.0236.



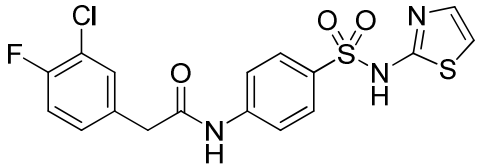
2-(3,4-dichlorophenyl)-N-(4-(N-(3-hydroxyphenyl)sulfamoyl)phenyl)acetamide (37): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.53 (1 H, s), 10.02 (1 H, s), 9.40 (1 H, s), 7.65 - 7.74 (4 H, m), 7.55 - 7.61 (2 H, m), 7.30 (1 H, dd, *J*=8.2, 2.0 Hz), 6.96 (1 H, t, *J*=8.0 Hz), 6.52 - 6.57 (1 H, m), 6.46 - 6.52 (1 H, m), 6.38 (1 H, dd, *J*=8.2, 1.6 Hz), 3.71 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.684 min and t_2 (Method 2) = 3.520 min; HRMS calculated for $C_{20}H_{17}Cl_2N_2O_4S$ $(M+H)^+$ 451.0281, found 451.0282.



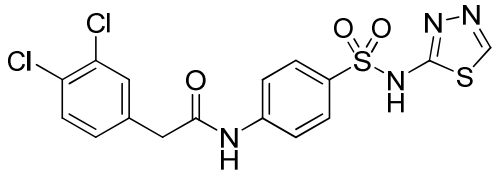
2-(3,4-dichlorophenyl)-N-(4-(N-(3-(dimethylamino)phenyl)sulfamoyl)phenyl)acetamide (38): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.52 (1 H, s), 9.94 (1 H, s), 7.67 - 7.74 (4 H, m), 7.55 - 7.61 (2 H, m), 7.29 (1 H, dd, *J*=8.2, 2.0 Hz), 6.97 (1 H, t, *J*=8.2 Hz), 6.41 - 6.46 (1 H, m), 6.33 - 6.40 (2 H, m), 3.71 (2 H, s), 2.79 (6 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.393 min and t_2 (Method 2) = 3.394 min; HRMS calculated for $C_{22}H_{22}Cl_2N_3O_3S$ $(M+H)^+$ 478.0753, found 478.0758.



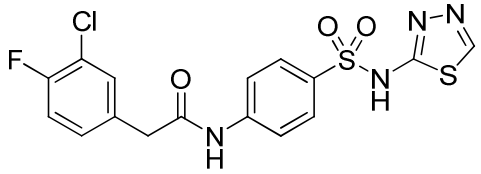
2-(3,4-dichlorophenyl)-N-(4-(N-thiazol-2-ylsulfamoyl)phenyl)acetamide (39): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.66 (1 H, br. s.), 10.50 (1 H, s), 7.67 - 7.76 (4 H, m), 7.55 - 7.62 (2 H, m), 7.31 (1 H, dd, *J*=8.2, 2.0 Hz), 7.23 (1 H, d, *J*=4.7 Hz), 6.80 (1 H, d, *J*=4.7 Hz), 3.72 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.353 min and t_2 (Method 2) = 3.441 min; HRMS calculated for $C_{17}H_{14}Cl_2N_3O_3S_2$ $(M+H)^+$ 441.9848, found 441.983.



2-(3-chloro-4-fluorophenyl)-N-(4-(N-thiazol-2-ylsulfamoyl)phenyl)acetamide (40): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.66 (s, 1H), 10.49 (s, 1H), 7.77 – 7.68 (m, 4H), 7.54 (dd, J = 7.3, 2.1 Hz, 1H), 7.40 – 7.28 (m, 2H), 7.23 (d, J = 4.6 Hz, 1H), 6.80 (d, J = 4.6 Hz, 1H), 3.70 (d, J = 0.8 Hz, 2H). LC-MS Retention Time: t_1 (Method 1) = 5.075 min and t_2 (Method 2) = 3.296 min; HRMS calculated for $C_{17}H_{14}ClFN_3O_3S_2$ $(M+H)^+$ 426.0144, found 426.0145.



N-(4-(N-1,3,4-thiadiazol-2-ylsulfamoyl)phenyl)-2-(3,4-dichlorophenyl)acetamide (41): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.54 (1 H, s), 8.73 (1 H, s), 7.69 - 7.77 (4 H, m), 7.56 - 7.63 (2 H, m), 7.31 (1 H, dd, J=8.2, 2.0 Hz), 3.73 (2 H, s); LC-MS Retention Time: t_1 (Method 1) = 5.350 min and t_2 (Method 2) = 3.302 min. HRMS calculated for $C_{16}H_{13}Cl_2N_4O_3S_2$ $(M+H)^+$ 442.9801, found 442.9800.



N-(4-(N-1,3,4-thiadiazol-2-ylsulfamoyl)phenyl)-2-(3-chloro-4-fluorophenyl)acetamide (42): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.52 (1 H, s), 8.74 (1 H, s), 7.73 (4 H, s), 7.54 (1 H, dd, J=7.0, 2.0 Hz), 7.36 - 7.41 (1 H, m), 7.28 - 7.35 (2 H, m), 3.71 (2 H, s); ^{19}F NMR (376 MHz, DMSO-d_6) δ ppm -124.65; LC-MS Retention Time: t_1 (Method 1) = 5.148 min and t_2 (Method 2) = 3.205 min. HRMS calculated for $C_{16}H_{12}ClFN_4NaO_3S_2$ $(M+Na)^+$ 448.9916, found 448.9936.

General methods for Biology

T. brucei PFK qHTS assay

Inhibition of *T. brucei* PFK was screened by coupling PFK-mediated ADP production to a modified luciferase-based detection. For the primary PFK reaction, both substrates (ATP and F6P) were included at K_m concentrations to allow for robust measurement of inhibition, and the enzyme concentration and incubation time were optimized to provide linear turnover with clean Michaelis-Menten kinetics. The PFK-mediated production of ADP in the primary reaction was then coupled to the commercially available ADP-Glo kit (ADP-Glo, Promega, Madison, WI) as an endpoint reaction, allowing for an ADP-dependent luminescent signal. The ADP-Glo utilizes several coupled enzyme reactions to 1) deplete all unconverted ATP remaining from the initial PFK reaction, 2) reconvert all PFK-hydrolyzed ADP back to ATP and 3) produce a luminescent signal using the classic ATP-dependent luciferase reaction. Thus, a decrease in luminescence is indicative of PFK inhibition. As no PFK-specific inhibitors have been previously identified, an enzyme-free control was included on each plate for 100% inhibition data normalization. Both substrate and enzyme reagents were prepared in 0.1M triethanolamine (TEA) buffer, pH 8.0, containing final concentrations of 5 mM MgCl2 and 0.01% Tween20 (to prevent aggregation artifacts). Bovine serum albumin (BSA) was additionally added to the PFK buffer to a final concentration of 0.1% to limit nonspecific binding of PFK to plastic tubing and wells. A stepwise description of the 1536-well assay is shown in Supplementary Table 1.

Tb PFK ATP-depletion secondary assay

Confirmed qHTS active compounds were tested in a modified qHTS assay using identical enzyme and substrate conditions to the primary assay, yet with an alternative measure of PFK activity. The primary qHTS assay provides a luminescence-based detection of ADP production, and thus a direct measurement of *Tb* PFK product formation. An alternative detection kit, Kinase-Glo Plus (Promega, Madison, WI), utilizes a comparable luminescence-based

technology to measure ATP concentration. When used in conjunction with optimized *Tb* PFK conditions, this orthogonal assay provides a measurement of PFK activity as a function of ATP depletion. Upon inhibition of PFK, luminescent signal remains high relative to uninhibited enzyme, providing a change in signal opposite to that seen in the primary assay. This allows for both confirmation of inhibitor activity, as well as a controlled counter-screen to those compounds that act on luciferase or otherwise inhibit the detection reagent. Both kits detect final nucleotide levels with similar coupling enzymes, and therefore compounds that directly inhibit the detection step should lead to a reduction in luminescent signal in both ADP and ATP assays, whereas true inhibitors should lead to opposing changes in luminescence, generally with comparable potency. The *Tb* PFK reaction was run using identical conditions to the qHTS PFK assay, with Kinase-Glo Plus reagent used in place of ADP-Glo, and with identical assay buffers. A stepwise description of the 1536-well assay is shown in Supplementary Table 2.

Human cell line toxicity counterscreen

Confirmed qHTS active compounds were tested for toxicity against the MRC-5 human lung fibroblast cell line. This line has historically been used as surrogate host in *in vitro Trypanosoma* assays, and so provides a relevant surrogate for human cells in testing *T. brucei* PFK inhibitor toxicity. Briefly, MRC-5 cultured cells were plated in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 1,000 cells per well (5 µL total per well) in 1536-well plates, dosed with titrated compounds in DMSO and incubated for 72 hours at 37 °C. Toxicity was assessed using the CellTiter Glo kit (Promega, Madison, WI), which uses intracellular ATP concentration as a viability marker; 2.5 µL of CellTiter Glo reagent was added to each well and incubated at room temperature for 10 minutes, after which the resulting luminescence was measured in a ViewLux detector using a one second exposure. As inhibitors of glycolysis could have potentially affected total cellular ATP levels, an ATP-independent viable dye protocol was prepared (MTT, Invitrogen, Carlsbad, CA),

though it was not required, as none of the active compounds demonstrated appreciable toxicity in the CellTiter Glo assay.

Tb PFK substrate competition assay

The mechanism of *Tb* PFK inhibition was additionally tested for select compounds, using substrate competition to examine whether compounds competed with ATP for F6P for binding. Competition was assayed by coupling production of ADP by *Tb* PFK to rabbit pyruvate kinase (PyK), which converts ADP and phosphoenol-pyruvate (PEP) to ATP and pyruvate. The reaction is further coupled to lactate dehydrogenase (LDH), which converts pyruvate and NADH to lactate and NAD^+, respectively. NADH conversion was monitored kinetically using an NADH fluorescence at ex340/em450. Briefly, a master substrate mix consisting of 2 nM *Tb* PFK, saturating concentrations of one substrate (0.5 mM ATP or 2mM F6P), excess rabbit PyK, PEP and LDH (500 nM, 0.5 mM and 500 nM, respectively), and 0.18 mM NADH was prepared. Titrations of the competing substrate (either ATP or F6P) and small molecule inhibitor spanning their respective K_ms and IC_{50}s were also prepared. All reagents were prepared in triethanolamine (TEA) buffer, pH 7.6, containing 10 mM MgCl2 and 100 mM KCl. Individual aliquots of substrate mix, competing substrate and inhibitor, were then mixed to begin the reaction, which was immediately transferred to a black multiwell plate and measured on a PerkinElmer ViewLux; kinetic change in fluorescence was measured every 15 seconds. Changes in fluorescence were plotted for each pair of substrate and inhibitor concentrations to determine initial velocities, which were then transformed to Lineweaver-Burk plots using Prism graphical software (GraphPad Software, Inc.; La Jolla, CA) for demonstration of binding site competition and Ki / Ki' determinations.

T. brucei in vitro parasite growth assay

Selected active compounds were additionally tested in a well-validated *in vitro* parasite assay to determine the relative potencies of lead compounds against live *T. brucei* cultures. Briefly, dilutions of *T. brucei brucei* (strain Lister 427)

were incubated in modified Iscove's cell culture medium (supplemented with 10% FBS, 0.05 mM bathocuproine sulfonate, 1.5 mM L-cysteine, 1 mM hypoxanthine, 0.2 mM 2-mercaptoethanol, 1 mM sodium pyruvate and 0.16 mM thymidine) with titrations of compound for 72 hours. The initial cell density was adapted so that cells were at the end of the logarithmic growth phase (~$5x10^4$ tryps/mL) after 72 hours of incubation. General morphology and motility were recorded after incubation, and Alamar Blue dye was added as a fluorescent marker of viability (ex530/em590) and read on a fluorescent plate reader following 4 hrs of incubation at 37 °C.

Rabbit PFK counterassay

Recombinant rabbit PFK (*Rm* PFK) was generated by transforming the pET5a/RmPFK plasmid into the PFK-deficient E. coli host strain, DF1020DE3. Expressed *Rm* PFK was then isolated and purified as described previously using ATP-N6-agarose (Banaszak, K., et al., *Journal of Molecular Biology*, **2011**. *407*(2): p. 284-97). *Rm* PFK activity was assayed using a coupled reaction measuring oxidation of NADH (coupled to PyK and LDH, similar to the substrate competition assays) in the presence of ATP and F6P at pH 7.0.