Supporting Information

The identification of a novel and selective series of Itk inhibitors via a template-hopping strategy

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General Methods

Unless stated otherwise, starting materials were commercially available. All solvents and commercial reagents were of laboratory grade and were used as received. 1H NMR spectra were recorded on a Bruker DRX 400 (400MHz) instrument or a Bruker AVII+ 600 (600MHz). The following abbreviations have been used: s, singlet; d, doublet; t, triplet; Hz, Hertz. Flash chromatography was carried out using pre-packed Biotage "Isolute" flash silica cartridges on a Biotage "Flashmaster 2" system. The following method was used for LCMS (liquid chromatography - mass spectral) analysis:

LCMS Method:

The analysis was conducted on an Acquity UPLC BEH C18 column (50mm x 2.1mm internal diameter 1.71µm packing diameter) at 40°C.

The solvents employed were:
A = 0.1% v/v solution of formic acid in water.
B = 0.1% v/v solution of formic acid in acetonitrile.

The gradient employed was as follows:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0	1	97	3
1.5	1	0	100
1.9	1	0	100
2.0	1	97	3

The UV detection was an averaged signal from wavelength of 210nm to 350nm and mass spectra were recorded on a mass spectrometer using alternate-scan positive and negative mode electrospray ionization.

The following illustrates the mobile phases and gradients used when compounds underwent purification by mass-directed autopreparative HPLC.

Mass-Directed Autopreparative HPLC (Formic Acid Modifier)

The HPLC analysis was conducted on a Sunfire C18 column (150mm x 30mm internal diameter, 5Iµm packing diameter) at ambient temperature.

The solvents employed were:

A = 0.1% v/v solution of formic acid in water.
B = 0.1% v/v solution of formic acid in acetonitrile.

Mass-Directed Autopreparative HPLC (Trifluoroacetic Acid Modifier)

The HPLC analysis was conducted on a Sunfire C18 column (150mm x 30mm internal diameter, 5Iµm packing diameter) at ambient temperature.

The solvents employed were:

A = 0.1% v/v solution of trifluoroacetic acid in water.
B = 0.1% v/v solution of trifluoroacetic acid in acetonitrile.

Mass-Directed Autopreparative HPLC (Ammonium Bicarbonate Modifier)

The HPLC analysis was conducted on an XBridge C18 column (150mm x 30mm internal diameter, 5Iμm packing diameter) at ambient temperature.

The solvents employed were:

A = 10 mM ammonium bicarbonate in water adjusted to pH 10 with ammonia solution.
B = acetonitrile.

For each of the mass-directed autopreparative purifications, irrespective of the modifier used, the gradient employed was dependent upon the retention time of the particular compound undergoing purification as recorded in the analytical LCMS, and was as follows:

For compounds with an analytical LCMS retention time below 0.6 minutes the following gradient was used:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0	40	99	1
1	40	99	1
10	40	70	30
11	40	1	99
15	40	1	99

For compounds with an analytical LCMS retention time between 0.6 and 0.9 minutes the following gradient was used:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0	40	85	15
1	40	85	15
10	40	45	55
11	40	1	99
15	40	1	99

For compounds with an analytical LCMS retention time between 0.9 and 1.2 minutes the following gradient was used:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0	40	70	30
1	40	70	30
10	40	15	85

11	40	1	99
15	40	1	99

For compounds with an analytical LCMS retention time between 1.2 and 1.4 minutes the following gradient was used:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0	40	50	50
1	40	50	50
10	40	1	99
11	40	1	99
15	40	1	99

For compounds with an analytical LCMS retention time greater than 1.4 minutes the following gradient was used:

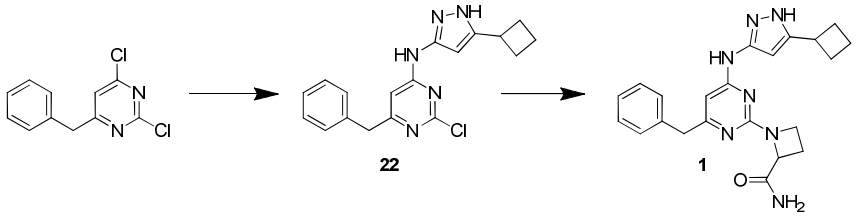
Time (minutes)	Flow Rate (mL/min)	%A	%B
0	40	20	80
1	40	20	80
10	40	1	99
11	40	1	99
15	40	1	99

The UV detection was an averaged signal from wavelength of 210nm to 350nm and mass spectra were recorded on a mass spectrometer using alternate-scan positive and negative mode electrospray ionization.

Section 1: Synthesis and characterization of compounds and intermediates

Experimental procedures for intermediates **19**, **20**, **21f, 26** and compounds **3e-f**, **5**, **9-17** can be found in reference 15.

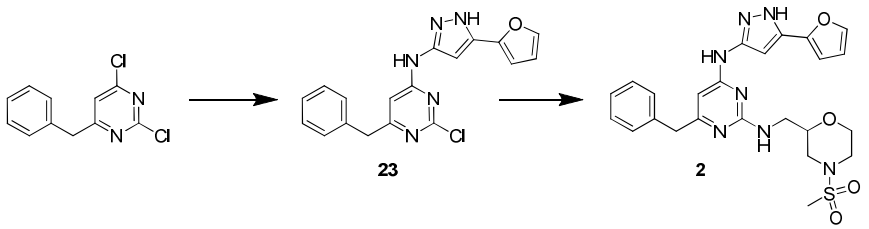
Synthesis of compound 1



A mixture of 2,4-dichloro-6-(phenylmethyl)pyrimidine (233 mg, 0.975 mmol), 5-cyclobutyl-1*H*-pyrazol-3-amine (134 mg, 0.975 mmol) and triethylamine (0.136 mL, 0.975 mmol) in isopropanol (1 mL) was stirred for 96 hours at room temperature. The reaction mixture was concentrated and purified by automated column chromatography (silica column, 0-25 % dichloromethane: methanol over 30 minutes). The desired fractions were combined and evaporated to dryness to afford 2-chloro-*N*-(3-cyclobutyl-1*H*-pyrazol-5-yl)-6-(phenylmethyl)-4-pyrimidinamine **22** (175 mg, 53% yield) which was approx 74% pure. Material was used directly in the next reaction. LCMS: Rt 1.15 minutes; m/z 339.96, 341.91 (MH+).

A mixture of 2-chloro-*N*-(5-cyclobutyl-1*H*-pyrazol-3-yl)-6-(phenylmethyl)-4-pyrimidinamine **22** (80.0 mg, 0.235 mmol) and azetidine-2-carboxamide (87.0 mg, 0.798 mmol) in isopropanol (1 mL) was heated at 150 °C for 75 minutes in a Biotage "Initiator" microwave. The crude reaction mixture was concentrated *in vacuo* and purified by mass-directed autopreparative HPLC (triflouroacetic acid modifier) to afford 1-[4-[(5-cyclobutyl-1*H*-pyrazol-3-yl)amino]-6-(phenylmethyl)-2-pyrimidinyl]-2-azetidinecarboxamide **1** (33.5 mg, 35% yield). LCMS: Rt 0.85 minutes; m/z 404.1 (MH+). ^{1}H NMR (DMSO-d_6, 400MHz): δ (ppm) 11.01 (br. s., 1H), 7.70 (br. s., 1H), 7.25-7.46 (m, 6H), 6.49 (br. s., 1H), 6.02 (s, 1H), 4.76-4.95 (m, 1H), 4.10-4.33 (m, 2H), 3.97 (br. s., 2H), 3.36-3.50 (m, 1H), 2.64-2.80 (m, 1H), 2.04-2.36 (m, 5H), 1.88-2.02 (m, 1H), 1.73-1.87 (m, 1H).

Synthesis of compound 2

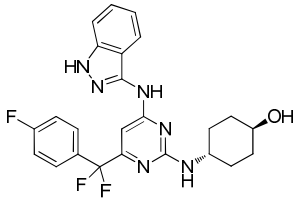


A solution of 2,4-dichloro-6-(phenylmethyl)pyrimidine (4.33 g, 18.11 mmol), 5-(2-furanyl)-1H-pyrazol-3-amine (2.70 g, 18.11 mmol) and triethylamine (5.1 mL, 36.2 mmol) in isopropanol (25 mL) was heated to 80 ºC for 96 hours. The reaction mixture was concentrated and purified by automated column chromatography (silica column, 0-50% cyclohexane: ethyl acetate (+1% triethylamine) over 30 minutes). The desired fractions were

combined and evaporated to dryness to afford 6-benzyl-2-chloro-*N*-(5-(furan-2-yl)-1*H*-pyrazol-3-yl)pyrimidin-4-amine **23** (1.64 g, 26% yield). LCMS: Rt 1.11 minutes; m/z 351.84, 353.81 (MH+). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) 12.97 (br. s., 1H) 10.44 (br. s., 1H) 7.78 (s, 1H) 7.19 - 7.42 (m, 5H) 6.83 (d, *J*=3.0 Hz, 1H) 6.61 (br. s., 1H) 3.91 (s, 2H) 3.34 (s, 4H).

To a mixture of 6-benzyl-2-chloro-N-(5-(furan-2-yl)-1H-pyrazol-3-yl)pyrimidin-4-amine **23** (26.7 mg, 0.076 mmol) and (4-(methylsulfonyl)morpholin-2-yl)methanamine (29.1 mg, 0.15 mmol) in isopropanol (0.4 mL) was added acetic acid (1 drop) and the mixture heated at 220°C for 2 hours in a CEM "Discover" microwave. The crude reaction mixture was concentrated *in vacuo* and purified by mass-directed autopreparative HPLC (ammonium bicarbonate modifier) to afford *N*4-[5-(2-furanyl)-1*H*-pyrazol-3-yl]-*N*2-{[4-(methylsulfonyl)-2-morpholinyl]methyl}-6-(phenylmethyl)-2,4-pyrimidinediamine **2** (3.9 mg, 10% yield). LCMS: Rt 0.73 minutes; m/z 509.8 (MH+).

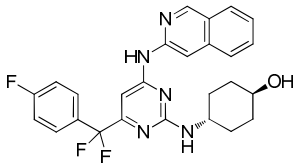
Synthesis of compound 3a



1*H*-Indazol-3-amine (109 mg, 0.819 mmol) was added portionwise to a solution of 2,4-dichloro-6-[difluoro(4-fluorophenyl)methyl]pyrimidine **20** (200 mg, 0.682 mmol) in acetonitrile (5 mL). The reaction mixture was stirred at 50 °C for 17 hours. It was then concentrated and purified by automated column chromatography (silica column, 0-50 % ethyl acetate: cyclohexane over 1 hour). The desired fractions were combined and evaporated to dryness to afford *N*-{2-chloro-6-[difluoro(4-fluorophenyl)methyl]-4-pyrimidinyl}-1*H*-indazol-3-amine **21a** (178mg, 67 % yield). LCMS: Rt 1.18 minutes; m/z 389.9, 391.9 (MH+).

To a mixture of *N*-{2-chloro-6-[difluoro(4-fluorophenyl)methyl]-4-pyrimidinyl}-1*H*-indazol-3-amine **21a** (33.5 mg, 0.086 mmol) and *N,N*-diisopropylethylamine (0.030 mL, 0.172 mmol) in isopropanol (1mL) was added *trans*-4-aminocyclohexanol (30 mg, 0.260 mmol) and the reaction was heated at 160 °C for 3 hours in a Biotage "Initiator" microwave. The crude reaction mixture was concentrated *in vacuo* and purified by mass-directed autopreparative HPLC (formic acid modifier) to afford *trans*-4-{[4-[difluoro(4-fluorophenyl)methyl]-6-(1*H*-indazol-3-ylamino)-2-pyrimidinyl]amino}cyclohexanol **3a** (22 mg, 55% yield). LCMS: Rt 1.03 minutes; m/z 469.0 (MH+). ^{1}H NMR ($CDCl_3$, 400MHz): δ (ppm) 7.70 (d, *J* = 8.0 Hz, 1H), 7.62 (dd, *J* = 8.5, 5.5 Hz, 2H), 7.42-7.49 (m, 2H), 7.15-7.23 (m, 2H), 7.09 (t, *J* = 8.5 Hz, 2H), 3.58-3.81 (m, 2H), 1.92-2.14 (m, 4H), 1.14-1.43 (m, 4H).

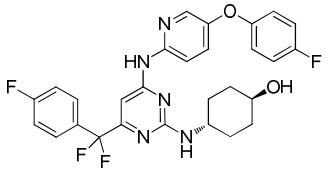
Synthesis of compound 3b



A mixture of 2,4-dichloro-6-[difluoro(4-fluorophenyl)methyl]pyrimidine **20** (300 mg, 1.0 mmol), isoquinolin-3-amine (177 mg, 1.2 mmol) and sodium hydride (60% dispersion in mineral oil) (82 mg, 2.0 mmol) in tetrahydrofuran (10 mL) was stirred at 50 °C overnight. The reaction was quenched by the addition of methanol and concentrated *in vacuo* to afford crude *N*-(2-chloro-6-(difluoro(4-fluorophenyl)methyl)pyrimidin-4-yl)isoquinolin-3-amine **21b** (410 mg) which was used directly in the next reaction. LCMS: Rt 1.38 minutes; m/z 400.9, 402.8 (MH+).

A mixture of *N*-{2-chloro-6-[difluoro(4-fluorophenyl)methyl]-4-pyrimidinyl}-3-isoquinolinamine **21b** (205 mg, 0.511 mmol), *trans*-4-aminocyclohexanol (118 mg, 1.02 mmol) and *N,N*-diisopropylethylamine (0.18 mL, 1.02 mmol) in isopropanol (5 mL) was heated at 170 °C for 2.5 hours in a Biotage "Initiator" microwave. The crude reaction mixture was concentrated *in vacuo* and purified by mass-directed autopreparative HPLC (formic acid modifier) to afford *trans*-4-{[4-[difluoro(4-fluorophenyl)methyl]-6-(3-isoquinolinylamino)-2-pyrimidinyl]amino}cyclohexanol **3b** (32 mg, 11% yield). LCMS: Rt 1.08 minutes; m/z 480.28 (MH+). ^{1}H NMR (DMSO-d_6, 400MHz): δ (ppm) 10.03-10.40 (m, 1H), 9.13 (br. s., 1H), 8.54-8.95 (m, 1H), 6.98-8.12 (m, 9H), 6.73 (br. s., 1H), 3.08-3.90 (m, 2H), 1.70-2.16 (m, 4H), 1.05-1.53 (m, 4H).

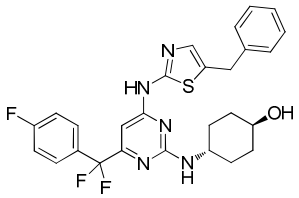
Synthesis of compound 3c



2-Chloro-6-(difluoro(4-fluorophenyl)methyl)-*N*-(5-(4-fluorophenoxy)pyridin-2-yl)pyrimidin-4-amine **21c** was prepared similarly to intermediate **21b** using 5-(4-fluorophenoxy)pyridin-2-amine and was used directly in the next reaction. LCMS: Rt 1.45 minutes; m/z 461.0, 463.0 (MH+).

trans-4-((4-(Difluoro(4-fluorophenyl)methyl)-6-((5-(4-fluorophenoxy)pyridin-2-yl)amino)pyrimidin-2-yl)amino)cyclohexanol **3c** was prepared similarly to **3b** from **21c**. LCMS: Rt 1.18 minutes; m/z 540.4 (MH+). ^{1}H NMR (CD_3OD, 400MHz): δ (ppm) 8.15 (d, J=9.0 Hz, 1H), 8.03 (d, J=2.5 Hz, 1H), 7.62 (dd, J=8.5, 5.5 Hz, 2H), 7.38 (dd, J=9.0, 3.0 Hz, 1H), 7.17 (t, J=8.8 Hz, 2H), 7.06-7.13 (m, 2H), 6.98-7.06 (m, 2H), 6.78 (s, 1H), 3.60-3.79 (m, 1H), 3.47-3.60 (m, 1H), 1.89-2.13 (m, 4H), 1.20-1.42 (m, 4H).

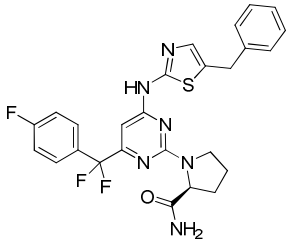
Synthesis of compound 3d



Sodium hydride (60% dispersion in mineral oil) (82 mg, 2.05 mmol) was added to a solution of 5-(phenylmethyl)-1,3-thiazol-2-amine (234 mg, 1.23 mmol) in tetrahydrofuran (5 mL) and the mixture was stirred at room temperature for 10 minutes. This mixture was added to a solution of 2,4-dichloro-6-[difluoro(4-fluorophenyl)methyl]pyrimidine **20** (300 mg, 1.02 mmol) in tetrahydrofuran (5 mL). The mixture was stirred at 50 °C overnight. The reaction was quenched by the addition of methanol and concentrated *in vacuo* to afford crude 2-chloro-6-[difluoro(4-fluorophenyl)methyl]-*N*-[5-(phenylmethyl)-1,3-thiazol-2-yl]-4-pyrimidinamine **21d** (457 mg) which was used directly in the next reaction. LCMS: Rt 1.26 minutes; m/z 447.0, 449.0 (MH+).

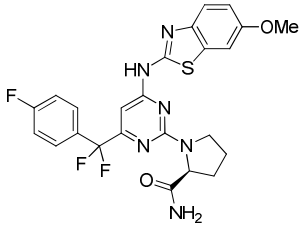
trans-4-((4-((5-Benzylthiazol-2-yl)amino)-6-(difluoro(4-fluorophenyl)methyl)pyrimidin-2-yl)amino)cyclohexanol **3d** was prepared similarly to **3b** from **21d**. LCMS: Rt 1.14 minutes; m/z 526.1 (MH+). ^{1}H NMR (CD_3OD, 400MHz): δ (ppm) 7.54-7.68 (m, 2H), 6.99-7.37 (m, 8H), 6.46 (s, 1H), 4.09 (s, 2H), 3.81-3.96 (m, 1H), 3.48-3.63 (m, 1H), 1.85-2.13 (m, 4H), 1.17-1.49 (m, 4H).

Synthesis of compound 4d



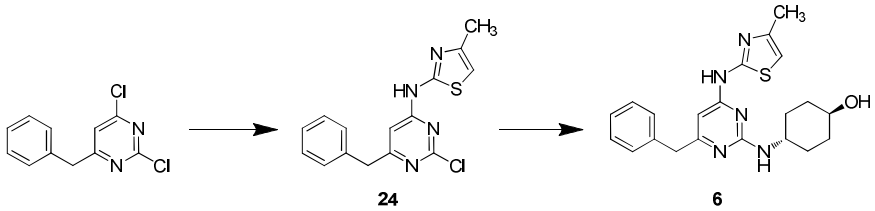
A mixture of 2-chloro-6-[difluoro(4-fluorophenyl)methyl]-*N*-[5-(phenylmethyl)-1,3-thiazol-2-yl]-4-pyrimidinamine **21d** (228 mg, 0.510 mmol), L-prolinamide (116 mg, 1.02 mmol) and *N,N*-diisopropylethylamine (0.18 mL, 1.02 mmol) was heated at 160 °C for 1 hour in a Biotage "Initiator" microwave. The crude reaction mixture was concentrated *in vacuo* and purified by mass-directed autopreparative HPLC (formic acid modifier) to afford 1-(4-[difluoro(4-fluorophenyl)methyl]-6-{[5-(phenylmethyl)-1,3-thiazol-2-yl]amino}-2-pyrimidinyl)-L-prolinamide **4d** (8.6 mg, 3% yield). LCMS: Rt 1.24 minutes; m/z 525.0 (MH+).

Synthesis of compound 4f



1-(4-[Difluoro(4-fluorophenyl)methyl]-6-{[6-(methyloxy)-1,3-benzothiazol-2-yl]amino}-2-pyrimidinyl)-L-prolinamide **4f** was prepared similarly to **4d** from **21f**. LCMS: Rt 1.15 minutes; m/z 514.9 (MH+). ^{1}H NMR (DMSO-d_6, 600MHz): δ (ppm) 11.79 (br. s., 1H), 7.67-7.77 (m, 2H), 7.40-7.61 (m, 3H), 7.26-7.38 (m, 2H), 7.00 (dd, *J*=8.8, 2.6 Hz, 1H), 6.91-7.02 (m, 1H), 6.56-6.64 (m, 1H), 4.33-4.85 (m, 1H), 3.81 (s, 3H), 3.40-4.01 (m, 2H), 2.18-2.32 (m, 1H), 1.79-2.12 (m, 3H).

Synthesis of compound 6



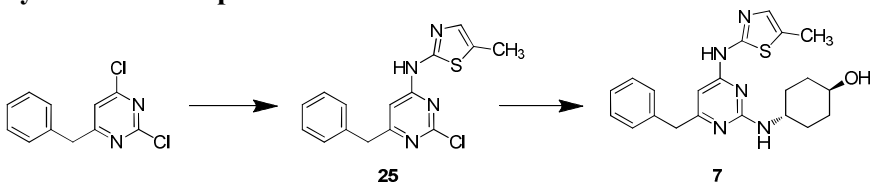
24 **6**

4-methyl-1,3-thiazol-2-amine (0.72 g, 6.27 mmol) was dissolved in tetrahydrofuran (25 mL) and cooled to 0 °C under nitrogen. Sodium hydride (60% dispersion in mineral oil) (0.51 g, 12.4 mmol) was added in three portions and the mixture stirred for 20 minutes at this temperature. A solution of 2,4-dichloro-6-(phenylmethyl)pyrimidine (1.5 g, 6.3 mmol) in tetrahydrofuran (25 mL) was added slowly and the mixture allowed to warm to room temperature overnight. The reaction was quenched by the addition of methanol and concentrated *in vacuo*. Water was added and the mixture extracted with dichloromethane. It was then concentrated and purified by automated column chromatography (silica column, 0-50 % ethyl acetate: cyclohexane over 1 hour). Desired fractions combined and concentrated *in vacuo* to afford 2-chloro-*N*-(4-methyl-1,3-thiazol-2-yl)-6-(phenylmethyl)-4-pyrimidinamine **24** (1.04 g, 52% yield) as an off-white solid. LCMS: Rt 1.18 minutes; m/z 317.1, 319.2 (MH+). ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.28-7.36 (m, 3H), 7.22-7.26 (m, 2H), 6.51 (s, 1H), 6.33 (s, 1H), 4.02 (s, 2H), 2.17 (s, 3H).

2-Chloro-*N*-(4-methyl-1,3-thiazol-2-yl)-6-(phenylmethyl)-4-pyrimidinamine **24** (50 mg, 0.16mmol), *trans*-4-aminocyclohexanol (36 mg, 0.32 mmol) and *N,N*-diisopropylethylamine (0.083 mL, 0.473 mmol) in isopropanol (2.5 mL) heated to 160 °C for 4 hours in a Biotage "Initiator" microwave. The crude reaction mixture was concentrated *in vacuo* and purified by mass-directed autopreparative HPLC (formic acid modifier) to afford *trans*-4-{[4-[(4-methyl-1,3-thiazol-2-yl)amino]-6-(phenylmethyl)-2-pyrimidinyl]amino}cyclohexanol **6** (27.8 mg, 45% yield) as an off-white solid. LCMS: Rt 0.75 minutes; m/z 395.92 (MH+). ^{1}H NMR (DMSO-d_6, 400MHz): δ (ppm) 11.08 (br. s., 1H), 7.14-7.35 (m, 5H), 6.83-6.98 (m, 1H), 6.66

(s, 1H), 5.95 (s, 1H), 3.75-3.89 (m, 1H), 3.71 (s, 2H), 3.30-3.49 (m, 1H), 2.22 (s, 3H), 1.77-2.01 (m, 4H), 1.13-1.40 (m, 4H).

Synthesis of compound 7

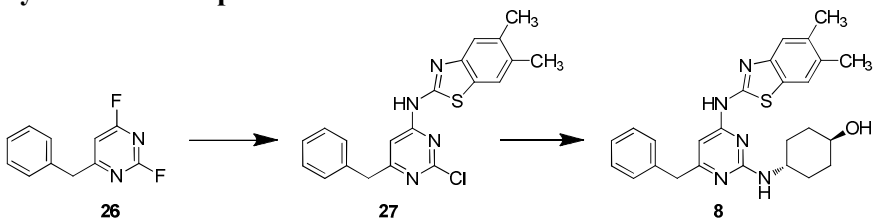


25 **7**

5-Methyl-1,3-thiazol-2-amine (172 mg, 1.51 mmol) was dissolved in tetrahydrofuran (10 mL) and cooled to 0 °C. Sodium hydride (60% dispersion in mineral oil) (50 mg, 1.26 mmol) was added portion-wise and the mixture was left to stir at 0 °C for 30 minutes. A solution of 2,4-dichloro-6-(phenylmethyl)pyrimidine (300 mg, 1.26 mmol) in tetrahydrofuran (10 mL) was added slowly and the mixture allowed to warm to room temperature over 2 hours. Water was added and the mixture extracted with dichloromethane. The organic layer was then concentrated and purified by automated column chromatography (silica column, 0-50% ethyl acetate : cyclohexane over 1 hour). Desired fractions were combined and concentrated *in vacuo* to afford 2-chloro-*N*-(5-methyl-1,3-thiazol-2-yl)-6-(phenylmethyl)-4-pyrimidinamine **25** (103 mg) which was approx 63% pure. Material was used directly in the next reaction. LCMS: Rt 1.16 minutes; m/z 316.9, 318.8 (MH+).

A mixture of 2-chloro-*N*-(5-methyl-1,3-thiazol-2-yl)-6-(phenylmethyl)-4-pyrimidinamine **25** (103 mg, 0.21 mmol), *trans*-4-aminocyclohexanol (49 mg, 0.42 mmol) and *N,N*-diisopropylethylamine (0.07 mL, 0.42 mmol) in isopropanol (5 mL) was heated at 170 °C for 2.5 hours in a Biotage "Initiator" microwave. Starting material remained after this time so additional portions of *trans*-4-aminocyclohexanol (49 mg, 0.42 mmol) and *N,N*-diisopropylethylamine (0.07 mL, 0.42 mmol) were added and the mixture returned to the microwave for a further hour at 170 °C. The crude reaction mixture was concentrated *in vacuo* and purified by mass-directed autopreparative HPLC (formic acid modifier) to afford *trans*-4-{[4-[(5-methyl-1,3-thiazol-2-yl)amino]-6-(phenylmethyl)-2-pyrimidinyl]amino}cyclohexanol **7** (18.8mg, 20% yield). LCMS: Rt 0.68 minutes; m/z 396.26 (MH+). ^{1}H NMR (DMSO-d_6, 400MHz): δ (ppm) 10.97 (br. s., 1H), 7.15-7.39 (m, 5H), 7.03 (s, 1H), 6.85-6.98 (m, 1H), 5.93 (s, 1H), 4.55 (br. s., 1H), 3.75-3.91 (m, 1H), 3.70 (s, 2H), 3.35-3.48 (m, 1H), 2.32 (s, 3H), 1.76-2.06 (m, 4H), 1.14-1.38 (m, 4H).

Synthesis of compound 8



26 **27** **8**

To an ice-cooled solution of 5,6-dimethyl-1,3-benzothiazol-2-amine (130 mg, 0.727 mmol) in tetrahydrofuran (10 mL) under nitrogen was added sodium hydride (60% dispersion in mineral oil) (58.2 mg, 1.46 mmol) and the mixture was stirred for 15 minutes. A solution of 2,4-difluoro-6- (phenylmethyl)pyrimidine **26** (150 mg, 0.727 mmol) in tetrahydrofuran (1

mL) was added and the mixture allowed to stir and slowly warm to ambient temperature. After 3 hours the mixture was treated with saturated ammonium chloride (20 mL) and ethyl acetate (20 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated. The crude material was purified by automated column chromatography (silica column, 0-50% ethyl acetate: cyclohexane over 1 hour) to afford *N*-[2-fluoro-6-(phenylmethyl)-4-pyrimidinyl]-5,6-dimethyl-1,3-benzothiazol-2-amine **27** (39 mg, 15% yield). LCMS: Rt 1.36 minutes; m/z 364.91 (MH+).

A mixture of *trans*-4-aminocyclohexanol (28.4 mg, 0.247 mmol) and *N*-[2-fluoro-6-(phenylmethyl)-4-pyrimidinyl]-5,6-dimethyl-1,3-benzothiazol-2-amine **27** (30 mg, 0.082 mmol) in isopropanol (2 mL) was heated in a Biotage "Initiator" microwave.at 160 °C for 30 minutes. The mixture was evaporated to dryness and the product purified by mass-directed autopreparative HPLC (ammonium bicarbonate modifier) to afford *trans*-4-{[4-[(5,6-dimethyl-1,3-benzothiazol-2-yl)amino]-6-(phenylmethyl)-2pyrimidinyl]amino}cyclohexanol **8** (25 mg, 66% yield) as a white solid. LCMS: Rt 0.93 minutes; m/z 460.0 (MH+). ^{1}H NMR (DMSO-d_6) δ: 11.33 (br. s., 1H), 7.55 (s, 1H), 7.44 (s, 1H), 7.19-7.34 (m, 5H), 6.98-7.09 (m, 1H), 6.03 (s, 1H), 4.50-4.61 (m, 1H), 3.82-3.98 (m, 1H), 3.74 (s, 2H), 3.37-3.49 (m, 1H), 2.31 (s, 6H), 1.83-2.06 (m, 4H), 1.21-1.48 (m, 4H).

Section 2: Assay protocols

Generation of reagents

Generation of Flag tagged full length human Itk

Recombinant human Itk (full length) is expressed in insect cells (in pFastBac-1 vector Invitrogen) fused to a Flag tag at its N terminus. The sequence of the Itk part is identical to Genbank entry L10717. The FLAG-Itk fusion protein is extracted from insect cells and purified by immunoaffinity chromatography on anti-FLAG (M2) agarose affinity resin. Further purification is by size exclusion chromatography. Purified protein is stored at - 80 ^{0}C in Tris-HCI (50 mM), NaCI (200 mM), sorbitol (500 mM), DTT (2 mM), pH 8.0.

Generation of biotinylated GST SAM68

Truncated human SAM-68 (R331-Y443) is expressed in E. coli (using a pGex-4T vector Pharmacia) as a GST-thrombin cleavage site-Avi-tag-Sam68:331-443 fusion. The Sam68 part of the fusion (R331-Y443) is identical to the sequence of Genbank database entry NM_006559. GST-SAM68 is purified by affinity chromatography on glutathione- sepharose. Specific biotinylation of the Avitag sequence of GST-SAM68 is performed at room temperature in the presence of: ATP, (5 mM), D-biotin, (1 mM), DTT, (1 mM) and biotin ligase, (1 μM), and is complete in 2 hours. The biotinylated protein is further purified by size exclusion chromatography and stored at -80 °C in Tris-HCI (50 mM), NaCI (250 mM), glycerol (10%), DTT (2 mM), pH 8.0.

Itk enzyme assay methodology

ATP and Streptavidin-APC concentrations shown in brackets describe conditions for the compounds tested at higher ATP concentrations.

For each test sample, 3 μL Substrate Reagent (100 nM biotinylated GST SAM68, 20 mM $MgCl_2$, 40 μM (or 2mM) ATP in assay buffer (50 mM Hepes, 1 mM DTT, 0.0025% Tween-20, pH 7.4) was added to 100 nl test solution containing compound in 100% DMSO in a black low volume 384 well plate (Greiner). Itk stock solutions were pre-incubated in 100 μM (or 1mM) ATP, 10 mM $MgCl_2$ for 30mins at 20 ^{o}C, before dilution in assay buffer to a working concentration which ensures linearity proportional to time and enzyme concentration (typically 5-10 nM to give a 2.5-5 nM final assay concentration). 3 μL diluted enzyme was added to each sample before incubating at 20 ^{o}C for the duration of the assays linear initial rate phase (typically 30 minutes). After this time 3 μL of Stop/read solution, typically 150 nM (or 45 nM) Streptavidin-APC (Perkin-Elmer), 1.5 nM Europium labelled Antiphosphotyrosine (Perkin-Elmer), 40 mM HEPES, 150 mM NaCl, 0.03% w/v BSA, 60 mM EDTA) was added to each sample and incubated for 45 minutes at 20 ^{o}C. The plate was then read on an Envision using wavelengths of λ_{Ex}: 337 nm, λ_{Em}: 615 nm (Europium), 665 nm (APC).

Lck IMAP assay methodology

Lck was preactivated before use in the assay by overnight incubation at 4 °C in the presence of 1 mM ATP, 1 mM $MgCl_2$. After incubation preactivated enzyme was snap frozen on dry ice and stored at -80 °C until needed.

For each test sample, 3μL Substrate Reagent (10 μM ATP, 100 nM Tamra-p34cdc-derived peptide) and 3 μL Enzyme Reagent (20 nM Lck) in assay buffer (IMAP Reaction buffer) was added to 100 nL test solution containing compound in 100% DMSO in a black low volume 384 well plate (Greiner) and incubated at room temperature for 30 minutes. After this time 3μL of Detection Reagent (IMAP Progressive Binding buffer A, IMAP Progressive Binding buffer B (50:50 v/v), IMAP Progressive Binding Reagent (1:900 dilution)) was added and samples incubated for a further 30 minutes at room temperature then read on the Envision at wavelengths of λ_{Ex}: 530 nm, λ_{Em}: 580 nm (Cy3 TAMRA).
IMAP reagents / Tamra-p34cdc-derived from Molecular Devices, Lck in house.

AuroraB assay methodology

3 μL AuroraB (typically at 2 nM) enzyme solution (25 mM HEPES, 25 mM NaCl, 0.0025% Tween-20, 0.15 mg/ml BSA, 1 mM DTT) was added to 100 nL test solution containing compound in 100% DMSO in a black low volume 384 well plate (Greiner). This was preincubated for 30 minutes. 3 μL substrate solution (200 μM 5FAM-PKAtide, 25 mM HEPES, 25 mM NaCl, 0.0025% Tween-20, 0.15 mg/ml BSA, 1mM DTT, 4 mM $MgCl_2$, 4 mM ATP) was then added to the plate and incubated for 120 minutes at room temperature. Reaction was terminated with the addition of 6 μL stop buffer (18.9% Progressive Binding Buffer A (Molecular Devices), 1% Progressive Binding Buffer B (Molecular Devices), 0.2% IMAP Binding Reagent (beads) in 79.9% water. Samples incubated for a further 30-90 minutes at room temperature then read on Envision at wavelengths of λ_{Ex}: 485 nM, λ_{Em}: 530 nM.

CytoStim induced IFNγ release peripheral blood mononuclear cells (PBMC)

Blood was obtained by venepuncture from human volunteers into heparin (10 U/mL). All donors provided written informed consent for use of their samples, and the collection and use of the samples received Institutional Ethics Committee approval. PBMC were isolated using histopaque-1077, and re-suspended in media (RPMI, 10% heat inactivated foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). PBMC were plated out into flat-well, 96-well plates (100,000 cells per well, 150 μl/well) containing compound (1 μl, in DMSO). The PBMCs were incubated with drug for 1 hour (37 °C, 5% CO_2) before activation with a sub-maximal concentration of cytostim (50 μl, to give a final dilution of 1:3200 in the assay). Following a further incubation (37 °C, 5% CO_2, for approximately 18 hours) the supernatant was removed and assayed for IFNγ using an MSD assay.

Section 3: Crystallographic Methods

Cloning and expression

The ORF for 6xHis-thrombin cleavage site-human Itk(357-620, Y512E) was cloned into Baculovirus expression vector pFastBac1 and expressed in insect Sf9 cells using the Bac-to-Bac Baculovirus expression system (Invitrogen/ Life technologies). The protein was purified from the Sf9 cells by immobilized metal-ion affinity chromatography on a HisTrap Crude column followed by thrombin digestion to remove the 6xHis-tag. Subsequent purification was by ion-exchange chromatography on a Source 15Q column, hydrophobic interaction chromatography on Source ISO resin and size exclusion chromatography on Superdex S75. The purified protein was buffer exchanged into storage buffer (25 mM Tris-HCl pH 7.6, 50 mM NaCl, 2 mM Dithiothreitol, pH 7.6) and concentrated to 12.6 mg/ml.

Crystallisation, Data Collection and Refinement

Complex formation was facilitated by concentrating the protein from 1.2 mg/ml to ~10 mg/ml in storage buffer in presence of 100 μM inhibitor (**3e**). Cocrystallisation was carried out by hanging drop vapour diffusion with micro-seeding. Each drop comprised an equal volume of protein/inhibitor solution and seeding solution. The seeding solution was obtained using the seed bead method (Hampton Research Corp., California, USA) with reservoir solution (0.85M Ammonium Sulphate. 0.2M Magnesium Acetate, 10 mM Dithiothreitol, 0.1 M Sodium Citrate pH 5.7) and an ITK(357-620, Y512E)/inhibitor (**1**) cocrystal that had been initially generated under comparable conditions without seeding. The resultant ITK(357-620, Y512E)/inhibitor (**3e**) cocrystal was cryoprotected by brief immersion into cryobuffer (1.2 M Ammonium Sulphate, 0.2 M MgAc, 0.1 M NaCitrate pH 5.7, 10 mM DTT, 20% Glycerol). X-ray diffraction data were collected at 100 K at the ESRF (station ID23.1) using an ADSC Q315R CCD detector. Data were processed using MOSFLM (*Leslie, A. G. W. (1992). Jnt CCP4/ESF-EACMB Newslett. Protein Crystallogr.* ***26****.*) and SCALA (*P.R.Evans, "Data reduction", Proceedings of CCP4 Study Weekend, 1993, on Data Collection & Processing, pages 114-122*) (within the CCP4 programming suite (*Collaborative Computational Project, Number 4.* ***"****The CCP4 Suite: Programs for Protein Crystallography." (1994) Acta Cryst****.,*** ***D50****, 760-763.*)). The complex was solved by Fourier Synthesis using ITK protein coordinates determined in-house (data not shown). Refinement was carried out using REFMAC (*Murshudov, G.N., Vagin, A.A. and Dodson, E.J. (1997) Acta Cryst.* ***D53****, 240-255*) and model building using COOT (*P. Emsley and K. Cowtan (2004) Coot: model-building tools for molecular graphics. Acta Cryst.,* ***D60****, 2126-2132*). The final R_{factor} and R_{free} achieved for the structure were 17.2% and 22.1% respectively. The structure was deposited into the PDB as entry 4L7S.