Supporting Information

Rapid Generation of a High Quality Lead for Transforming Growth Factor-β (TGF-β) Type I Receptor (ALK5)

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Contents

1. Experimental Section

General

All reagents and solvents were used without further purification or drying. All reactions were performed under nitrogen, unless otherwise specified. All mass spectral data have been obtained using electrospray; data were obtained using a Waters ZMD or Waters ZQ LC/mass spectrometer acquiring both positive and negative ion data. ^{1}H NMR spectra were collected on either 300MHz or 400MHz Bruker spectrometer; unless otherwise stated NMR spectra were obtained at 300MHz in d^{6}-DMSO. Unless otherwise stated, column chromatography (by the flash procedure, FCC) and medium pressure liquid chromatography (MPLC) were performed on Merck Kieselgel silica (Art. 9385) or on Silicycle cartridges (40-63 microns silica, 12 to 120 g weight) using an Isco Combi Flash Companion system. Preparative HPLC was performed on C18 reversed-phase silica on a Waters 'Xterra' or 'XBridge' preparative reversed-phase column (5 microns silica, 19 mm diameter, 100 mm length) or on a Phenomenex "Gemini" or 'AXIA' preparative reversed-phase column (5 microns silica, 110A, 21.1 mm diameter, 100 mm length) using decreasingly polar mixtures of water (containing 1% formic acid or 1% aqueous ammonium hydroxide) and acetonitrile. Other abbreviations used: DMF, dimethylformamide; THF, tetrahydrofuran; DMA, dimethylacetamide; FCC, flash column chromatography; DCM, dichloromethane; TFA, trifluoroacetic acid; SCX, strong cation exchange.

The purity of all test compounds was >95% except for **11** where the measured purity was 85% as indicated in the experimental for that compound. Purity was ascertained from AUC (UV detection) by analytical HPLC using a Waters ZQ2000 single quad MS with a waters 2795 LC and a Waters 2996 PDA controlled by MassLynx 4.0, performed on C18 reversed-phase silica, on a Phenomenex "Gemini" reversed-phase column (5 microns silica, 110 A, 2 mm diameter, 50 mm length). Analytical HPLC method used a ramp from 95:5 A:C to 95:5 B:C over 4 minutes, then held at 95:5 B:C for 0.5 minutes before returning to 95:5 A:C for 0.5 minutes, where eluents A, B and C are: A = 100% water, B = 100% Acetonitrile, C = 50:50 actonitrile:1% aq. NH_4OH. Detection is by DAD from 220-300nm and the MS is a Waters ZQ operating in pos/neg EScI mode from 100-1400da.

4-(6-Methyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(3,4,5-trimethoxyphenyl)pyridin-2-amine (5). A mixture of 3-(2-chloropyridin-4-yl)oxy-6-methyl-2-pyridin-2-yl-pyridine (0.125 g), 3,4,5-trimethoxyaniline (0.092 g), Xantphos (0.029 g), $Pd_2(dba)_3$ (0.015 g), Cs_2CO_3 (0.41 g) and DMA (5 ml) was heated to 130°C for 30 minutes by microwave in a sealed tube. After cooling, the mixture was filtered. The filtrate was diluted with ethyl acetate (20 ml) and washed with saturated brine (15 ml). The organic portion was dried ($MgSO_4$) and concentrated *in vacuo*. Purification by FCC using a gradient of 0-5% methanolic ammonia (7M) in DCM afforded the title compound (26 mg, 14%); ^{1}H NMR: 2.61 (3H, s), 3.72 (6H, s), 3.74 (3H s), 6.15 (1H, d), 6.19 (1H, q), 6.27 (1H, s),

6.41 (2H, s), 7.13-7.18 (2H, m), 7.35 (1H, d), 7.60-7.64 (1H, m), 7.70 (1H, d), 7.90 (1H, d), 8.59 (1H, d); *m/z*: MH^+ 445.5.

4-Pyridin-3-yloxy-*N*-(3,4,5-trimethoxyphenyl)pyridin-2-amine (7). 3,4,5-Trimethoxyaniline (66 mg), Xantphos (25 mg), $Pd(OAc)_2$ (15 mg), Cs_2CO_3 (234 mg) and 2-chloro-4-pyridin-3-yloxy-pyridine (75 mg) were combined in 1,4-dioxane (2.5 ml). The mixture was heated to 150°C for 40 minutes in a microwave. After cooling the reaction mixture was taken up in ethyl acetate and water and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by acidic reverse phase HPLC afforded the title compound (20 mg, 16%) as a solid; ^{1}H NMR: 3.60 (3H, s), 3.72 (6H, s), 6.20 (1H, d), 6.46 (1H, dd), 6.96 (2H, s), 7.54 (1H, dd), 7.68-7.72 (1H, m), 8.09 (1H, d), 8.51-8.53 (2H, m), 8.90 (1H, s); *m/z*: MH^+ 354.0.

The following examples were made by an analogous procedure to **(7)** by using the appropriate 4-(2-chloropyridin-4-yl)oxyheterocycle and the appropriate aniline:

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(3,4,5-trimethoxyphenyl)pyridin-2-amine (1). ^{1}H NMR: ($CDCl_3$): 2.34 (3H, s), 2.61 (3H, s), 3.78 (6H, s), 3.81 (3H, s), 6.21-6.26 (2H, m), 6.32 (1H, s), 6.49 (2H, s), 7.17-7.22 (1H, m), 7.64-7.70 (1H, m), 7.76-7.79 (1H, m), 7.97 (1H, d), 8.63-8.66 (1H, m); *m/z*: MH^+ 459.4.

4-(2,6-Dimethylpyridin-3-yl)oxy-*N*-(3,4,5-trimethoxyphenyl)-pyridin-2-amine (6). ^{1}H NMR: 2.29 (3H, s), 2.48 (3H, s), 3.60 (3H, s), 3.72 (6H, s), 6.02 (1H, dd), 6.80 (1H, d), 6.96-6.96 (2H, m), 7.21 (1H, d), 7.48 (1H, d), 8.06 (1H, d), 8.85 (1H, s); *m/z*: MH^+ 382.7.

4-(6-Methylpyridin-3-yl)oxy-*N*-(3,4,5-trimethoxyphenyl)-pyridin-2-amine (8). ^{1}H NMR: 2.49 (3H, s), 3.60 (3H, s), 3.72 (6H, s), 6.15 (1H, d), 6.44 (1H, dd), 6.97 (2H, s), 7.38 (1H, d), 7.58 (1H, dd), 8.07 (1H, d), 8.37 (1H, d), 8.87 (1H, s); *m/z*: MH^+ 367.8.

4-(2-Methylpyridin-3-yl)oxy-*N*-(3,4,5-trimethoxyphenyl)-pyridin-2-amine (9). ^{1}H NMR: 2.35 (3H, s), 3.60 (3H, s), 3.72 (6H, s), 6.06 (1H, d), 6.43 (1H, dd), 6.95 (2H, s), 7.35-7.40 (1H, m), 7.61 (1H, dd), 8.08 (1H, d), 8.42 (1H, dd), 8.86 (1H, s); *m/z*: MH^+ 368.1.

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(2-methoxyphenyl)pyridin-2-amine (10). ^{1}H NMR: 2.23 (3H, s), 2.5 (3H, under DMSO), 3.67 (3H, s), 6.18 (1H, dd), 6.29 (1H, d), 6.70-6.85 (3H, m), 7.18-7.22 (1H, m), 7.40 (1H, s), 7.64-7.74 (2H, m), 7.81 (1H, d), 7.92 (1H, s), 8.01-8.05 (1H, m), 8.39 (1H, d); *m/z*: MH^+ 399.3.

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(2-morpholin-4-ylphenyl)pyridin-2-amine (11). ^{1}H NMR: 2.34 (3H, s), 2.5 (3H, under DMSO), 2.77 (4H, t), 3.71 (4H, t), 6.25 (1H, dd), 6.41 (1H, d), 6.92-7.03 (2H, m), 7.08 (1H, d), 7.32 (1H, t), 7.53 (1H, s), 7.76 (1H, d), 7.81-7.85 (3H, m), 7.92 (1H, d), 8.50 (1H, d); *m/z*: MH^+ 454.4. Product was only 85% pure by LCMS but material was tested without further purification.

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(3-methoxyphenyl)pyridin-2-amine (12). ^{1}H NMR: 2.36 (3H, s), 2.53 (3H, s), 3.70 (3H, s), 6.10 (1H, d), 6.36 (1H, dd), 6.42-6.45 (1H, m), 7.10 (2H, d), 7.30-7.35 (2H, m), 7.57 (1H, s), 7.78-7.88 (2H, m), 7.98 (1H, d), 8.50 (1H, d), 8.86 (1H, s); *m/z*: MH^+ 399.3.

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(3-morpholin-4-ylphenyl)pyridin-2-amine (13). ^{1}H NMR: 2.35 (3H, s), 2.5 (3H, under DMSO), 3.02 (4H, t), 3.72 (4H, t), 6.09 (1H, d), 6.33 (1H, dd), 6.45-6.49 (1H, m), 7.01-7.09 (2H, m), 7.17 (1H, s), 7.30-7.35 (1H, m), 7.56 (1H, s), 7.76-7.88 (2H, m), 7.96 (1H, d), 8.50 (1H, d), 8.74 (1H, s); *m/z*: MH^+ 454.3.

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(4-methoxyphenyl)pyridin-2-amine (14). ^{1}H NMR: 2.35 (3H, s), 2.5 (3H, under DMSO), 3.70 (3H, s), 6.01 (1H, d), 6.27 (1H, dd), 6.81 (2H, d), 7.33 (1H, t), 7.45 (2H, d), 7.55 (1H, s), 7.76-7.88 (2H, m), 7.91 (1H, d), 8.51 (1H, d), 8.65 (1H, s); *m/z*: MH^+ 399.7.

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(4-morpholin-4-ylphenyl)pyridin-2-amine (15). ^{1}H NMR: 2.35 (3H, s), 2.5 (3H, under DMSO), 3.00 (4H, t), 3.72 (4H, t), 6.00 (1H, d), 6.26 (1H, dd), 6.84 (2H, d), 7.33 (1H, ddd), 7.41 (2H, d), 7.55 (1H, s), 7.77 (1H, d), 7.85 (1H, td), 7.90 (1H, d), 8.51 (1H, d), 8.60 (1H, s); *m/z*: MH^+ 454.3.

4-{[4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxypyridin-2-yl]amino}benzenesulfonamide (16). ^{1}H NMR: 2.36 (3H, s), 2.54 (3H, s), 6.18 (1H, d),

6.46 (1H, dd), 7.10 (2H, s), 7.32 (1H, ddd), 7.59 (1H, s), 7.66 (2H, d), 7.75-7.88 (4H, m), 8.05 (1H, d), 8.49 (1H, d), 9.34 (1H, s); *m/z*: MH^+ 448.7.

4-{[4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxypyridin-2-yl]amino}-*N*,*N*-dimethyl-benzamide (17). ^{1}H NMR: 2.36 (3H, s), 2.54 (3H, s), 2.95 (6H, s), 6.14 (1H, d), 6.41 (1H, dd), 7.28-7.34 (3H, m), 7.58 (1H, s), 7.66 (2H, d), 7.79-7.87 (2H, m), 8.01 (1H, d), 8.50 (1H, d), 9.10 (1H, s); *m/z*: MH^+ 440.6.

4-(5,6-Dimethyl-2-pyridin-2-yl-pyridin-3-yl)oxy-*N*-(4-fluorophenyl)pyridin-2-amine (18). ^{1}H NMR: 2.36 (3H, s), 2.53 (3H, s), 6.05 (1H, d), 6.35 (1H, dd), 7.05 (2H, dd), 7.32 (1H, ddd), 7.57 (1H, s), 7.60 (2H, ddd), 7.78-7.87 (2H, m), 7.95 (1H, d), 8.50 (1H, d), 8.88 (1H, s); *m/z*: MH^+ 387.2.

4-({4-[(2,6-Dimethylpyridin-3-yl)oxy]pyridin-2-yl}amino)benzenesulfonamide (19). A mixture of 3-(2-chloropyridin-4-yl)oxy-2,6-dimethyl-pyridine (0.2 g, 0.85 mmol), sulfanilamide (0.191 g), Cs_2CO_3 (0.417 g), $Pd(OAc)_2$ (0.013 g), Xantphos (49 mg) and DMA (2 ml) was heated at 150ºC for 10 minutes in a microwave. After cooling the crude product was semi-purified by ion exchange chromatography, using an SCX column, eluting with 7M NH_3/MeOH. Further purification by preparative HPLC using decreasingly polar mixtures of water (containing 0.1% TFA) and acetonitrile as eluent afforded the title compound (0.17 g, 54%) as a solid; ^{1}H NMR: 2.30 (3H, s), 2.49 (3H, s), 6.13 (1H, d), 6.52-6.56 (1H, m), 7.10 (2H, s), 7.23 (1H, d), 7.50 (1H, d), 7.68 (2H, d), 7.79 (2H, d), 8.14 (1H, d), 9.38 (1H, s); *m/z*: MH^+ 371.0.

2-Chloro-4-pyridin-3-yloxy-pyridine. A mixture of CuI (0.114 g), 2-chloro-4-iodo pyridine (0.718 g), 3-hydroxy pyridine (0.289 g), Cs_2CO_3 and DMF (12 ml) was heated in a microwave at 150ºC for 40 mins. After cooling the mixture was taken up in ethyl acetate and water and extracted with ethyl acetate. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Purification by FCC, eluting with 0-100% ethyl acetate in *iso*-hexane, afforded the title compound (0.478 g, 77%) as a solid; ^{1}H NMR: 7.01 (1H, dd), 7.11 (1H, d), 7.55 (1H, dd), 7.74 (1H, d), 8.33 (1H, d), 8.55 (2H, d); *m/z*: MH^+ 207.3.

3-(2-Chloropyridin-4-yl)oxy-2,6-dimethyl-pyridine. To a suspension of NaH (60% dispersion in mineral oil, 0.136 g) in DMF (5 ml) was added sequentially a solution of 2,6-dimethylpyridin-3-ol (0.209 g) in DMF (2 ml) and 2,4-dichloropyridine (0.3 g) in DMF (3 ml). After stirring for 20 minutes at room temperature, the mixture was heated at 100°C for 2h. The reaction mixture was then quenched with water, taken up in ethyl acetate and water and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by FCC, eluting with 0-70% ethyl acetate in *iso*-hexane, afforded the title compound (0.212 g, 53%) as a yellow solid; ^{1}H NMR: 2.28 (3H, s), 2.48 (3H, s), 6.90 (1H, dd), 6.99 (1H, d), 7.22 (1H, d), 7.52 (1H, d), 8.29 (1H, d); *m/z*: MH^+ 235.7.

2-Chloro-4-(6-methylpyridin-3-yl)oxy-pyridine. This compound was prepared according to the method described for 3-(2-chloropyridin-4-yl)oxy-2,6-dimethyl-pyridine using 2,4-dichloropyridine and 6-methylpyridin-3-ol, to afford the title compound (0.441 g, 67%) as a colourless oil; ^{1}H NMR: (300MHz, $CDCl_3$) 2.61 (3H, s), 6.79 (1H, dd), 6.82 (1H, d), 7.25 (1H, d), 7.34 (1H, dd), 8.26 (1H, d), 8.35 (1H, d); *m/z*: MH^+ 221.4.

2-Chloro-4-(2-methylpyridin-3-yl)oxy-pyridine. This compound was prepared according to the method described for 3-(2-chloropyridin-4-yl)oxy-2,6-dimethyl-pyridine using 2,4-dichloropyridine and 2-methylpyridin-3-ol, to afford the title compound (0.54 g, 82%) as a cream solid; ^{1}H NMR: 2.34 (3H, s), 6.92 (1H, dd), 7.03 (1H, d), 7.36-7.40 (1H, m), 7.64 (1H, dd), 8.31 (1H, d), 8.44 (1H, dd); *m/z*: MH^+ 221.4.

5-(2-Chloropyridin-4-yl)oxy-2,3-dimethyl-6-pyridin-2-yl-pyridine. NaH (69 mg, 1.72 mmol, 60% dispersion in mineral oil) and 2,4-dichloropyridine were sequentially added to a solution of 5,6-dimethyl-2-pyridin-2-yl-pyridin-3-ol (prepared according to WO2005080377, 173 mg, 0.86 mmol) in DMF (2 ml), and the resulting mixture was heated at reflux for 40 minutes and was then allowed to cool to room temperature The mixture was quenched with water, taken up in ethyl acetate and water and extracted with ethyl acetate. The combined organic layers were washed with water then with

brine, dried over $MgSO_4$ and concentrated *in vacuo*. The resulting residue was partially purified by FCC, eluting with 0-4% MeOH in DCM, then further purified by FCC, eluting with 3% MeOH in DCM to obtain impure title compound (136 mg, 51% if pure) as a white solid. This material was used in subsequent steps without further purification; ^{1}H NMR: (300MHz, $CDCl_3$) 2.38 (3H, s), 2.63 (3H, s), 6.68-6.71 (1H, m), 6.73 (1H, d), 7.17-7.22 (1H, m), 7.28 (1H, s), 7.67-7.73 (1H, m), 7.82-7.86 (1H, m), 8.13 (1H, d), 8.55-8.58 (1H, m); *m/z*: MH^+ 312.4.

3-(2-Chloropyridin-4-yl)oxy-6-methyl-2-pyridin-2-yl-pyridine. A mixture of 3-(2-chloropyridin-4-yl)oxy-2-iodo-6-methylpyridine (0.3 g), 2-pyridylzinc bromide (0.232 g), $Pd(PPh_3)_4$ (0.1 g), THF (2 ml) and DMA (1 ml) was heated at 150°C for 3h in a microwave. After cooling, the mixture was diluted with ethyl acetate (20 ml) and washed with saturated brine (15 ml). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Purification by FCC, eluting with 10-50% ethyl acetate in *iso*-hexane afforded the title compound (0.145 g, 56%) as a beige solid; *m/z*: MH^+ 298.2.

3-(2-Chloropyridin-4-yl)oxy-2-iodo-6-methyl-pyridine. NaH (0.286 g) was added portionwise to a solution of 2-iodo-6-methylpyridin-3-ol (2.80 g) in DMF (20 ml). The resulting suspension was stirred for 10 minutes at room temperature. 2,4-Dichloropyridine (1.47 g) was then added portionwise and the mixture was heated at 140ºC for 20 minutes. The resulting solution was stirred at 140°C for 2h. The mixture

was then concentrated *in vacuo*, and the residue was dissolved in DCM (150 ml) and washed with water (50 ml). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Purification by FCC, eluting with 0-5% ethyl acetate in DCM afforded the title compound (3.43 g, 100%) as a beige solid; ^{1}H NMR: 2.61 (3H, s), 6.74-6.79 (2H, m), 7.18 (1H, d), 7.24 (1H, d), 8.28 (1H, d); *m/z*: MH^+ 347.1.

2. Enzyme Compound activity

A fluorescence polarisation assay was used to assess the ALK5 binding capacity and hence biochemical activity of compounds. 6His tagged human ALK5 (residues162-503) was grown and purified from insect cells and stored at –80°C in aliquots. Assay measurements were performed in 50 mM HEPES pH7.4, 10 mM MgCl2, 1mM DTT, 0.01% CHAPS in a final assay volume of 12 µl. Briefly 6 µl of ALK5 protein, final concentration of 3 µg/ml, was added to each well of a 384 well assay plate containing 12 nl of each concentration of test compound dissolved in DMSO. 6 µl of a solution containing an ALK5 FP probe ((2Z)-3-(6-{4-[2-({4-[(6-chloro[1,3]dioxolo[4,5-b]pyridin-7-yl)amino]-5-(1-methylethoxy)quinazolin-7-yl}oxy)ethyl]piperazin-1-yl}-6-oxohexyl)-2-[(2E,4E)-5-(3-ethyl-1,1-dimethyl-6,8-disulfo-1H-benzo[e]indolium-2-yl)penta-2,4-dien-1-ylidene]-1,1-dimethyl-6-sulfo-2,3-dihydro-1H-benzo[e]indole-8-sulfonate), final concentration 50 nM, and ATP, final concentration 1 mM. Following incubation for 30 minutes at room temperature, fluorescence polarisation was measured

on a BMG Labtech PHERAstar plate reader using a 650/690 nm optic module. mP values were then used to calculate IC_{50} values for each compound tested.

3. Cell Compound Activity

Cellular activity of compounds was assessed by measuring the ability of compounds to inhibit nuclear translocation of a GFP-2/Smad2 fusion reporter in MDA-MB-468 cells (Smad2 Redistribution® Assay, BioImage). Briefly cells were seeded at 8000 cells per well in an appropriate 96 well assay plate in 100 µl RPMI1640, 10% FCS, 1% L-glutamine and grown for 48 hrs at 37°C, 5% CO_2. Compounds in 5 nl DMSO were dosed using sonic dispensing and TGFβ1 (Calbiochem) added to a final concentration of 3 ng/ml and plates incubated at 37°C, 5% CO_2 for 90 minutes. Cells were fixed in 4% formaldehyde, washed in PBS and stained with 2 µg/ml Hoechst33342 (Molecular Probes). Nuclear translocation was measured using Cellomics ArrayScan imaging.

4. ALK5 expression and purification for crystallography

ALK5(200-503) N-terminaly tagged with a 6-his tag, followed by a TEV (Tobacco Etch Virus) protease cleavage site was produced in SF21 cells using a baculoviral expression system, in *Sf-900 II* media (Invitrogen) harvesting after 48 hours. Cells were lysed by acceleration disruption in a buffer containing 20 mM Tris pH8, 300 mM NaCl, 1 mM TCEP, 20 mM imidazole, 10% v/v glycerol, Complete EDTA free

protease inhibitor tablets (Roche), and 6u/ml Benzonase (Novagen). The lysate was then applied to a Ni-NTA (Qiagen) column which was in turn washed with 20 mM Tris pH8, 300 mM NaCl, 1 mM TCEP, 20 mM imidazole, 10% v/v glycerol and then eluted with 20 mM Tris pH8, 300 mM NaCl, 1 mM TCEP, 250 mM imidazole, 10% v/v glycerol. After overnight TEV cleavage of the 6-His tag, and dialysis into 20mM Tris pH8, 300mM NaCl, 1mM TCEP, 10%v/v glycerol, the sample was re-purified on a Ni-NTA column, collecting the unbound fraction, followed by concentration and gel filtration on a Superdex 75 16/60 size exclusion column (GE Healthcare) in 50mM Tris pH8, 200mM NaCl, 8%v/v glycerol, 1mM TCEP, then concentrated to 10mg/ml.

5. ALK5 Crystallisation, data collection and structure solution

Diffraction quality crystals of apo-ALK5 were obtained at room temperature using the sitting drop method with a 1:1 ratio of protein (10 mg/ml ALK5 in 20 mM Tris pH8, 200 mM NaCl, 1 mM TCEP, 1 mM DTT, 1 mM TCEP, 8% v/v glycerol 10 mM MgATP) and precipitant (20-30% v/w PEG8000, 200 mM Na-acetate, 100 mM Tris pH8-9.2). Apo crystals were soaked in a solution containing 10mM compound in the mother liquor for several days and then flash-cooled at 100K for data collection using 15% v/v ethylene-glycol as cryoprotectant. Datasets were collected on a Rigaku FRE rotating anode equipped with VariMax multilayer mirrors and a Saturn994 CCD detector. Data were processed using MOSFLM and SCALA. Ligand parameters were calculated and the ligands were fitted using AFITT. Structures were solved with

molecular replacement using AMORE and refined with REFMAC with intermediate rounds of model building in COOT.

Data processing and refinement statistics:

	cmpd 1	cmpd 19
PDB accession code	2WOT	2WOU
Space group:	P212121	P212121
Cell constants a; b; c (Å)	41.933 78.218	41.8813 77.0418
	89.074 90.000	90.3875 90.0000
	90.000 90.000	90.0000 90.0000
Resolution limit (Å)	1.85	2.20
Resolution range (Å)	22.50 1.85	20 2.2
Completeness overall (%)	*97.77*	*99.1*
Reflections, unique	25080	13415
Multiplicity	*3.53*	*3.74*
*R*merge $_{overall}$ [1]	*0.090*	*0.168*
*R*value $_{overall}$ (%) [2]	17.88	22.59
*R*value $_{free}$ (%)	22.13	30.21
Non hydrogen protein atoms	2399	2357
Non hydrogen ligand atoms	34	26
Solvent molecules	294	136
R.m.s. deviations from ideal values		
Bond lengths (Å)	0.007	0.009
Bond angles (°)	1.100	1.135

Average *B* values (Å^2)		
Protein main chain atoms	18.782	21.462
Protein all atoms	19.186	21.549
Ligand	16.024	23.393
Solvent	30.212	25.000
Φ, Ψ angle distribution for residues [3]		
In most favoured regions (%)	91.9	92.2
In additional allowed regions (%)	8.1	7.8
In generously regions (%)	0.0	0.0
In disallowed regions (%)	0.0	0.0

1 $R_{merge} = \Sigma_{hkl} [(\Sigma_i |I_i - \langle I \rangle|)/ \Sigma_i I_i]$

2 $R_{value} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$

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

3 Ramachandran statistics as defined by PROCHECK

MOSFLM: Leslie, A.G.W., (1992), Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, No. 26.

SCALA: P.R.Evans, "Data reduction", Proceedings of CCP4 Study Weekend, 1993, on Data Collection & Processing, pages 114-122

AMORE: J.Navaza, Acta Cryst. A50, 157-163 (1994)

REFMAC: Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Acta Crystallogr. 1997, D53, 240-255.

AFITT: Wlodek, S., Skillman, A. G. and Nicholls, A., Acta Cryst. D, **2006**, *62*, 741.

COOT: Emsley, P.; Cowtan, K. Acta Crystallogr. 2004, D60, 2126.

PYMOL: DeLano, W. L. (2003) The PyMOL molecular graphics system. DeLano Scientific, San Carlos, CA, **http://www.pymol.org**

6. Computational chemistry

The 1RW8 crystal structure was used as a template structure for the docking calculations. The protein was prepared in MacroModel (Schrödinger: San Diego, CA 92122-1003) by relaxing the hydrogen atoms on the protein-ligand complex using the OPLS (2005) forcefield. All heavy atoms were fixed during this optimisation. The protein-ligand docking software Glide (Schrödinger: San Diego, CA 92122-1003) was used for this study. The bound inhibitor in 1RW8 was used to define the active site for the docking. All water molecules were deleted apart from the described water molecule in the selectivity pocket which was maintained in a number of the docking runs. Leatherface was used to enumerate all enantiomeric forms of ligand structures along with likely protonation states and tautomers. CORINA (Gasteiger Research; Erlangen, Germany) was run on the 2D ligand structures to generate a 3D start point. Based on these modelled binding modes the published inhibitors were manually fragmented into their respective hinge, selectivity pocket and solvent channel binding regions. Additional hinge region fragments were added from in-house kinase inhibitor structures. The fragments were then hybridised together in different combinations, with

all generated compounds containing a hinge region and selectivity pocket group and in some cases a solvent channel group. Designed ligands were optimised using the OPLS (2005) forcefield before docking. Glide was run in SP and XP modes and the output was compared and a judgement made for each ligand as to which provided the most reliable docked pose. In some cases SP gave a more satisfactory docked pose and in some cases XP provided a more acceptable pose. For some compounds positional and hydrogen bonding constraints were also applied to the hinge and selectivity pocket interactions to guide the docking. Often further relaxing was required of the protein-ligand complex (using MacroModel) to provide a satisfactory binding mode as an element of protein movement was required. Note the 'Docking Score' itself was not specifically used to rank the ideas but the ability of the docking algorithm to produce the required binding mode was essential. The energetics of the modelled binding modes was further analysed in ConQuest through comparisons against relevant bond angle and torsions in the Cambridge Crystallographic Structural Database (CSD).