Supporting Information

Identification and optimisation of a series of N-(4-anilino-2-pyridyl)acetamide activin-receptor like kinase 1 (ALK1) inhibitors

Procedures and characterisation for compounds 3, 11-20, 22 (21 is in experimental of main manuscript)

N-(4-chloropyridin-2-yl)acetamide. To a solution of 4-chloropyridin-2-amine (1 g, 7.78 mmol) in pyridine (10 mL) at 0°C, acetyl chloride (0.553 mL, 7.78 mmol) was added dropwise. The reaction mixture was stirred for 1 h at 0°C then was allowed to warm to rt overnight. The reaction mixture was concentrated *in vacuo* and the residue was redissolved in DCM, washed with water and dried ove $MgSO_4$. The crude oil was triturated with 10% diethyl ether in isohexane to afford the title compound (1.10 g, 83%) as a cream solid; ^{1}H NMR (300 MHz, DMSO) 2.11 (3H, s), 7.21 (1H, dd), 8.15 (1H, d), 8.29 (1H, d), 10.80 (1H, s); m/z MH^+ 171.

N-(4-(5-chlorobenzo[d][1,3]dioxol-4-ylamino)pyridin-2-yl)acetamide **3**.
5-Chlorobenzo[d][1,3]dioxol-4-amine (1 g, 5.83 mmol), N-(4-chloropyridin-2-yl)acetamide (0.994 g, 5.83 mmol), 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene (0.405 g, 0.70 mmol), palladium acetate (0.065 g, 0.29 mmol) and cesium carbonate (3.80 g, 11.7 mmol) were suspended in DMA (12 mL) and sealed into a microwave tube. The reaction was at 150°C for 30 min in a microwave and cooled to rt. The reaction mixture was concentrated and diluted with EtOAc, then washed with water and sat. brine. The organic layer was dried over $MgSO_4$, filtered and evaporated. The crude oil was triturated with EtOAc, Et_2O and isohexane to give a solid which was impure product (680 mg). The filtrate was concentrated and triturated again to give the desired pure title compound **3** (800 mg, 45%) as a pale orange solid; ^{1}H NMR (500 MHz, DMSO) 2.01 (3H, s), 6.05 (2H, s), 6.30 (1H, dd, *J* 2.2, 5.7), 6.85 (1H, d, *J* 8.4), 7.03 (1H, d, *J* 8.4), 7.43 (1H, s), 7.79 - 7.93 (1H, m), 8.53 (1H, s), 10.11 (1H,

s). ^{13}C NMR (126 MHz, DMSO) 24.4, 98.1, 102.5, 105.8, 106.7, 120.6, 122.6, 123.3, 143.7, 147.7, 148.1, 152.5, 153.1, 169.4; m/z MH^+ = 306; HRMS (ESI) calcd for $C_{14}H_{13}N_3O_3Cl$ (MH^+) 306.0645, found 306.0653.

N4-(5-chlorobenzo[d][1,3]dioxol-4-yl)pyridine-2,4-diamine. A solution of NaOH (419 mg, 10.5 mmol) in water (4 mL) was added to a stirred suspension of N-(4-(5-chlorobenzo[d][1,3]dioxol-4-ylamino)pyridin-2-yl)acetamide **3** (800 mg, 2.62 mmol). The resulting suspension was stirred at 80°C for 16 h. The reaction was monitored by TLC (EtOAc) as the retention times on HPLC were very similar. The reaction mixture was concentrated *in vacuo*, and the residue was acidified with 10% aq. HCl then adjusted to pH 8 with aq. $NaHCO_3$. The resulting aqueous layer was extracted with EtOAc, and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting crude oil was triturated with DCM / Et_2O / isohexane to afford the title compound (690 mg, 100%) as a beige solid; ^{1}H NMR (300 MHz, DMSO) 5.46 (2H, s), 5.55 - 5.58 (1H, m), 5.92 - 5.95 (1H, m), 6.08 (2H, s), 6.84 (1H, d), 7.01 (1H, d), 7.54 (1H, d), 7.99 (1H, s); m/z MH^+ 264.

*General procedure for acylation of N4-(5-chlorobenzo[d][1,3]dioxol-4-yl)pyridine-2,4-diamine with a carboxylic acid, for synthesis of compounds **11**, **12**, **14**, **15***. A solution of N4-(5-chlorobenzo[d][1,3]dioxol-4-yl)pyridine-2,4-diamine (70 mg, 0.27 mmol) in DMA (1 mL) was added to the appropriate carboxylic acid (0.5 mmol). DIPEA (0.14 mL, 0.81 mmol) was added followed by a solution of HATU (190 mg, 0.50 mmol) in DMA (1 mL). The reaction mixture was heated at 60°C overnight. The reaction mixture was allowed to cool to rt, and purified by reverse phase HPLC without further work-up to afford the following products: *N-[4-[(5-chloro-1,3-benzodioxol-4-yl)amino]-2-pyridyl]propanamide* **11** (30 mg, 35%); ^{1}H NMR (500 MHz, DMSO) 1.01 (3H, t, *J* 7.6), 2.31 (2H, q, *J* 7.5), 6.05 (2H, s), 6.31 (1H, dd, *J* 2.2, 5.7), 6.86 (1H, d, *J* 8.4), 7.03 (1H, d, *J* 8.4), 7.43 (1H, d, *J* 1.6), 7.85 (1H, d, *J* 5.7), 8.51 (1H, s), 10.04 (1H, s); ^{13}C NMR (126 MHz, DMSO) 10.0, 29.8, 98.0, 102.5, 105.8, 106.8, 120.6, 122.6, 123.3, 143.8, 147.7, 148.1, 152.5, 153.2, 173.1; HRMS (ESI) calcd for $C_{15}H_{15}N_3O_3Cl$ (MH^+) 320.0802, found 320.0806. *N-[4-[(5-chloro-1,3-benzodioxol-4-yl)amino]-*

2-pyridyl]-2-methyl-propanamide **12** (28 mg, 31%); ^{1}H NMR (500 MHz, DMSO) 1.03 (6H, d, *J* 6.8), 2.68 (1H, sep, *J* 6.8), 6.05 (2H, s), 6.32 (1H, dd, *J* 2.1, 5.7), 6.86 (1H, d, *J* 8.4), 7.03 (1H, d, *J* 8.4), 7.43 (1H, d, *J* 2.1), 7.86 (1H, d, *J* 5.7), 8.50 (1H, s, 1H), 10.03 (1H, s); ^{13}C NMR (126 MHz, DMSO) 19.9 (2C), 34.9, 98.1, 102.5, 105.9, 106.8, 120.6, 122.6, 123.4, 143.8, 147.7, 148.1, 152.5, 153.3, 176.3. HRMS **(**ESI) calcd for $C_{16}H_{17}ClN_3O_3$ (MH^+) 334.0958, found 334.0952. *N-[4-[(5-chloro-1,3-benzodioxol-4-yl)amino]-2-pyridyl]cyclobutanecarboxamide* **14** (36 mg, 39%); ^{1}H NMR (500 MHz, DMSO) 1.7 – 1.8 (1H, m), 1.83 – 1.93 (1H, m), 1.99 – 2.08 (2H, m), 2.1 – 2.2 (2H, m), 3.27-3.34 (1H, m), 6.06 (2H, s), 6.32 (1H, dd, *J* 2.2, 5.7), 6.87 (1H, d, *J* 8.4), 7.04 (1H, d, *J* 8.4), 7.45 (1H, s), 7.85 (1H, d, *J* 5.7), 8.52 (1H, s), 9.91 (1H, s); ^{13}C NMR (126 MHz, DMSO) 18.1, 24.9 (2C), 39.7, 98.1, 102.5, 105.8, 106.8, 120.6, 122.6, 123.3, 143.8, 147.7, 148.1, 152.5, 153.2, 173.9. HRMS (ESI) calcd for $C_{17}H_{17}ClN_3O_3$ (MH^+) 346.0958, found 346.0980. *N-[4-[(5-chloro-1,3-benzodioxol-4-yl)amino]-2-pyridyl]cyclopentanecarboxamide* **15** (30 mg, 31%); ^{1}H NMR (500 MHz, DMSO) 1.43 – 1.56 (2H, m), 1.58 – 1.69 (4H, m), 1.73 – 1.84 (2H, m), 2.86 (1H, p, *J* 7.9), 6.05 (2H, s), 6.32 (1H, dd, *J* 2.1, 5.7), 6.86 (1H, d, *J* 8.4), 7.03 (1H, d, *J* 8.4), 7.44 (1H, d, *J* 2.1), 7.86 (1H, d, *J* 5.7), 8.50 (1H, s), 10.04 (1H, s); ^{13}C NMR (126 MHz, DMSO) 26.2 (2C), 30.5 (2C), 45.3, 98.1, 102.4, 105.8, 106.8, 120.6, 122.6, 123.3, 143.8, 147.7, 148.0, 152.5, 153.2, 175.5; HRMS **(**ESI) calcd for $C_{18}H_{19}ClN_3O_3$ (MH^+) 360.1115, found 360.1132.

N-[4-[(5-Chloro-1,3-benzodioxol-4-yl)amino]-2-pyridyl]cyclopropanecarboxamide **13**. To a mixture of compound **8** (200 mg, 1.02 mmol), 5-chloro-1,3-benzodioxol-4-amine (227 mg, 1.33 mmol), Pd_2dba_3 (53 mg, 0.092 mmol) and xantphos (58 mg, 0.10 mmol) in toluene (2 mL) in a microwave tube was added DBU (381 µl, 2.55 mmol). The reaction mixture was degassed with nitrogen and heated in a microwave reactor at 150°C for 7 h. The solvent was removed by evaporation. The residue was diluted with DCM, washed with water and brine, then purified by flash silica chromatography, eluting with 2% MeOH in DCM, then triturated with ether and pentane, then further purified by preparative HPLC to afford the title compound (150 mg, 44%) as a beige solid; ^{1}H NMR (500 MHz, DMSO) 0.63 - 0.81 (4H, m), 1.85 - 2.06 (1H, m), 6.04 (2H, s), 6.33 (1H, dd, *J* 2.2, 5.7), 6.85 (1H, d,

J 8.4), 7.02 (1H, d, *J* 8.4), 7.40 (1H, d, *J* 2.0 Hz), 7.87 (1H, d, *J* 5.7), 8.50 (1H, s), 10.41 (1H, s); ^{13}C NMR (126 MHz, DMSO) 7.9 (2C), 14.6, 98.0, 102.4, 105.9, 106.8, 120.6, 122.6, 123.3, 143.7, 147.7, 148.1, 152.5, 153.1, 172.7. HRMS (ESI) calc. for $C_{16}H_{15}ClN_3O_3$ (MH^+) 332.0802, found 332.0812.

N-[4-[(5-chloro-1,3-benzodioxol-4-yl)amino]-2-pyridyl]benzamide **16**. HATU (131 mg, 0.35 mmol) was added to N4-(5-chlorobenzo[d][1,3]dioxol-4-yl)pyridine-2,4-diamine (70 mg, 0.27 mmol), benzoic acid (38.9 mg, 0.32 mmol) and DIPEA (0.092 mL, 0.53 mmol) in DMA (2 mL), and the reaction mixture was stirred at rt for 16 h. As the reaction was incomplete by LCMS, additional benzoic acid (19 mg, 0.16 mmol), 0.7eq HATU (72 mg, 0.19 mmol) and DIPEA (0.092 mL, 0.53 mmol) were added and the reaction mixture was stirred for 2 days at rt. The crude reaction mixture was purified by reverse phase HPLC to afford the title compound (39 mg, 40%) as a beige solid; ^{1}H NMR (300 MHz, DMSO) 6.10 (2H, s), 6.40 - 6.43 (1H, m), 6.90 (1H, d), 7.06 (1H, d), 7.45 - 7.51 (2H, m), 7.54 - 7.60 (2H, m), 7.95 - 8.00 (3H, m), 8.67 (1H, s), 10.43 (1H, s); m/z MH^+ 368.

N-[4-(1,3-benzodioxol-4-ylamino)-2-pyridyl]cyclopropanecarboxamide **18**. Benzo[d][1,3]dioxol-4-amine (100 mg, 0.73 mmol), N-(4-chloropyridin-2-yl)cyclopropanecarboxamide (143 mg, 0.73 mmol), xantphos (51 mg, 0.09 mmol), palladium acetate (8 mg, 0.04 mmol) and cesium carbonate (475 mg, 1.46 mmol) were suspended in DMA (3 mL), and the reaction mixture was heated at 150°C in a microwave reactor, and then allowed to cool to rt. The reaction mixture was directly purified by ion exchange chromatography on a SCX column, eluting with 0.35 M NH_3/MeOH, then further purified by reverse phase HPLC to afford the title compound (110 mg, 51%) as a cream solid; ^{1}H NMR (300 MHz, DMSO) 0.72 - 0.78 (4H, m), 1.92 - 2.00 (1H, m), 5.99 (2H, s), 6.42 - 6.44 (1H, m), 6.73 - 6.73 (2H, m), 6.81 - 6.86 (1H, m), 7.57 (1H, d), 7.87 (1H, d), 8.61 (1H, s), 10.39 (1H, s); m/z MH^+ 298.

N-[4-(2-Chloroanilino)-2-pyridyl]cyclopropanecarboxamide **17** was prepared in a similar manner to **18**, starting from 2-chloroaniline; ^{1}H NMR (500 MHz, DMSO) 0.71 - 0.77 (4H, m), 1.94 (1H, dt, *J*

12.4, 6.1), 6.47 (1H, dd, *J* 5.8, 2.2), 7.18 (1H, td, *J* 8.3, 1.7), 7.34 (1H, td, *J* 7.6, 1.5), 7.39 (1H, dd, *J* 8.0, 1.7), 7.53 (1H, dd, *J* 8.0, 1.4), 7.56 (1H, s), 7.90 (1H, d, *J* 5.7), 8.52 (1H, s), 10.45 (1H, s); HRMS (ESI) calc. for $C_{15}H_{15}ClN_3O$ (MH^+) 288.0904, found 288.0910.

N-(4-anilino-2-pyridyl)cyclopropanecarboxamide **19**. Prepared in a similar manner to **18**, from aniline, in 35% yield; ^{1}H NMR (700 MHz, DMSO) 0.75 - 0.80 (4H, m), 1.98 (1H, m), 6.62 (1H, dd), 7.02 (1H, dd), 7.18 (2H, d), 7.34 (2H, dd), 7.81 (1H, d), 7.93 (1H, d), 8.79 (1H, s), 10.44 (1H, s); m/z MH^+ 254.

N-[4-(4-pyridylamino)-2-pyridyl]cyclopropanecarboxamide **20**. Prepared in a similar manner to **18**, from 4-aminopyridine, in 3% yield; ^{1}H NMR (700 MHz, DMSO) 0.62 – 0.98 (4H, m), 1.85 – 2.1 (1H, m), 6.84 (1H, dd, *J* 2.2, 5.7), 7.01 – 7.19 (2H, m), 7.98 (1H, d, *J* 2.0), 8.09 (1H, d, *J* 5.6), 8.26 – 8.44 (2H, m), 9.33 (1H, s), 10.64 (1H, s); m/z MH^+ 255.

N-[4-[(6-chloro-[1,3]dioxolo[4,5-b]pyridin-7-yl)-methyl-amino]-2-pyridyl]cyclopropanecarboxamide **22**. Iodomethane (0.004 mL, 0.06 mmol) was added to N-(4-(6-chloro-[1,3]dioxolo[4,5-b]pyridin-7-ylamino)pyridin-2-yl)cyclopropanecarboxamide **21** (20 mg, 0.06 mmol) and cesium carbonate (23 mg, 0.07 mmol) in DMF (2 mL). The reaction mixture was stirred at rt for 4 h. The crude mixture was filtered and purified by reverse phase HPLC to afford the title compound **22** (13 mg, 61%) as a white solid; ^{1}H NMR (400 MHz, DMSO) 0.77 (4H, m), 1.98 (1H, m), 3.24 (3H, s), 6.23 (2H, s), 6.38 (1H, dd, *J* 5.8, 2.2), 7.47 (1H, d, *J* 1.9), 7.83 (1H, s), 7.98 (1H, d, *J* 5.9), 10.55 (1H, s). m/z MH^+ 347.

ALK5 expression and purification for crystallography.

The construct 6-His-TEV-GGG-ALK5(200-503) was produced in SF9 cell using a baculoviral expression system. Cells were lysed by acceleration disruption in a buffer containing 20mM Tris pH8, 300mM NaCl, 1mM TCEP, 20mM imidazole, 10%v/v glycerol, 4 Roche EDTA free protease inhibitor tablets, 50µl Novagen Benzonase. The lysate was then applied to a Ni-NTA column which was in turn washed with 20mM Tris pH8, 300mM NaCl, 1mM TCEP, 20mM imidazole, 10%v/v glycerol and then eluted with 20mM Tris pH8, 300mM NaCl, 1mM TCEP, 250mM imidazole, 10%v/v glycerol. After overnight TEV cleavage of the 6-His tag, the sample was re-purified on a Ni-NTA column as above, followed by gel filtration on a Superdex 75 16/60 size exclusion column, then concentrated to 10mg/ml.

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 (10mg/ml ALK5 in 20mM Tris pH8, 200mM NaCl, 1mM TCEP, 1mM DTT, 1mM TCEP, 8%v/v glycerol 10mM MgATP) and precipitant (20-30 v/w% PEG8000, 200mM Na-acetate, 100mM 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 15v/v% ethylene-glycol as cryoprotectant. Diffraction data were collected at the ID29 beamline at the ESRF. Data were processed using MOSFLM and SCALA. The structure was solved with molecular replacement using AMORE and refined with REFMAC with rounds of manual model building in COOT. Data processing and refinement statistics are summarised below:

PDB accession code	5FRI
Space group:	P212121
Cell constants a; b; c (Å)	41.960 76.700 89.069 90.000 90.000 90.000
Resolution limit (Å)	1.80
Resolution range (Å)	58.12 - 1.80
Completeness overall (%)	94.2
Reflections, unique	25748
Multiplicity	6.4
*R*merge $_{overall}$ [1]	0.115

*R*value $_{overall}$ (%) [2]	16.35
*R*value $_{free}$ (%)	19.34
Non hydrogen protein atoms	2412
Non hydrogen ligand atoms	23
Solvent molecules	249
R.m.s. deviations from ideal values	
Bond lengths (Å)	0.009
Bond angles (º)	1.179
Average *B* values (Å^2)	
Protein main chain atoms	12.68
Protein all atoms	15.44
Ligand	11.87
Solvent	22.26
Φ, Ψ angle distribution for residues [3]	
In most favoured regions (%)	96.97
In allowed regions (%)	3.03
In disallowed regions (%)	0.0

1 $R_{merge} = \Box\Box_{hkl} [(\Sigma_i |I_i - \langle I \rangle|)/ \Sigma_i I_i]$
2 $R_{value} = \Box\Box_{hkl} ||F_{obs}| - |F_{calc}|| / \Box_{hkl} |F_{obs}|$
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

References for crystallography

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.

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

Kinase panel data for compound 21, from Dundee university:

Kinase	ATP conc µM (at or below the calculated K_m)	Compound 21 activity (%inhibition at 1 µM)
EPHA2	50	78.4
YES1	20	75.4
src	50	47.2
MNK1	50	46.1
BTK	50	25.8
JNK1	20	25
CDK2	20	23.3
CSK	20	23.1
GSK3b	5	21.6
FGFR1	20	21.3
p38b	20	21.1
CAMKKbeta	20	20.9
TBK1	50	20
FLT1	20	17.2
MAPK13	5	14.9
IKKb	5	14.2
MELK	50	12.6
PBK	20	12.6
IKKe	50	11.8
PAK4	5	10.8
DYRK1A	50	9
MARK3	5	8.8
PKD1	50	7.6
CAMK1d	50	6.2
MAPK9	20	6
AurKB	20	5.6
MAPKAPK1B	50	4.6
MSK1	20	3.8
P70S6K	20	3.7
PKCz	5	2.4
PIM3	20	2.1
HIPK2	5	2
CHK2	20	1
PLK1	5	1
Src	50	0.7
SRPK1	50	0.5
IGF1R	5	0.1
NEK2a	50	0.1
DYRK3	5	-0.5
INSR	20	-0.5
MAPKAPK5	20	-0.7
CK2	5	-1.1
p38a	50	-1.2
PKCa	20	-2.8
PKA	5	-4
MST4	20	-4.9
MAPKAPK2	20	-5
AKT2	50	-5.3

SYK	20	-5.9
PRK2	5	-6.6
smMLCK	50	-10.7
EF2K	5	-13.5
EPHB3	5	-17.8
NEK6	50	-20.6
PDPK1	20	-29.9

ALK family enzyme data for compound 21, in FRET assays at Thermofisher:

Kinase	**Compound 21 IC_{50} (nM), geometric mean**	**n**
ALK1	1.1	3
ALK2	1.2	2
ALK3	2.4	2
ALK4	31	2
ALK5	1.9	3
ALK6	1300	2