Supporting Information

Rapid Development of Piperidine Carboxamides as Potent and Selective Anaplastic Lymphoma Kinase Inhibitors.

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Contents

Supporting information is provided for compound purification, spectroscopic characterisation, high resolution accurate mass measurement for compounds of series **7** and **11**, and HPLC conditions for determination of chemical purity and enantiomeric excess. Synthetic protocols for the preparations of compounds **13,15,16,17,19,20,22,23** are included. Details for performing the ALK enzymatic and cellular assays as well as the IGF1R enzymatic assay are included. Supporting information for crystallographic data collection and refinement statistics is included for compound **1** in Table S1**.** Selected Ambit kinome data is included for compounds **1** and **11w** in Table S2.

General. All reagents and solvents were obtained from commercial suppliers and used without further purification. All reactions were carried out under an inert atmosphere of nitrogen unless otherwise notes. Silica gel chromatography was performed using prepacked silica gel cartridges (Biotage). ^{1}H spectra were obtained on either a Bruker UltraShield 300 MHz or Bruker DRX 400 (400 MHz) spectrometer and reported as ppm downfield from the deuterated solvent. All tested compounds were purified to >95% purity as determined by HPLC. HPLC analysis was obtained on an Agilent 1100, using one of the following two methods: [A] Agilent SB-C18 column (50 × 3.0 mm, 2.5 µm) at 40 °C with a 1.5 mL/min flow rate using a

gradient of 5% to 95% [0.1% TFA in acetonitrile] in [0.1% TFA in water] over 3.5 min; [B] Agilent Zorbax SB-C18 (50 × 3.0 mm, 3.5 µm) at 40 °C with a 1.5 mL/min flow rate using a gradient of 5% to 95% [0.1% TFA in acetonitrile] in [0.1% TFA in water] over 3.5 min. Enantiomeric excess was obtained by SFC using a Chiralpak AD-H column (4.6 mm x 15 cm, 5 µm particle size) with carbon dioxide (Gradient A) and methanol with 0.2% diethylamine (Gradient B) as the mobile phase. A gradient of 5% B to 60% for a run time of 7 min (40 ºC, 4.0 ml/min, back pressure at 100 bars). Analysis software were MassWare™ v. 4.01, MassLynx™ v. 4.0 SP1 and Agilent LC/MSD Chemstation Rev.B.03.01. Exact mass measurements were performed on a Fourier-Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer operating at 7 tesla (Bruker Daltonics, Billerica MA). Ions were generated by electrospray ionization (positive mode). The instrument was externally calibrated with a PEG300/600 solution using the standard Francel equation. The calculated mass error for each calibrant ion was less than 1.0 ppm from the measured value. For each spectra 512 k data points were collected using a 1.25 MHz sweep width of detection. The time domain data were not processed prior to performing a magnitude mode Fourier transform.

(*S*)-1-(2-(Methylamino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7a). HRMS: Calc'd for $(C_{19}H_{25}N_5O_1)H+$: 340.2127; Found: 340.2132.

(*S*)-N-(4-Methylbenzyl)-1-(2-(phenylamino)-4-pyrimidinyl)-3-piperidinecarboxamide (7b). HRMS: Calc'd for $(C_{24}H_{27}N_5O_1)H+$: 402.22828; Found: 402.22850.

(*S*)-1-(2-(Benzylamino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7c). HRMS: Calc'd for $(C_{25}H_{29}N_5O_1)H+$: 416.2439; Found: 416.2445.

(*S*)-1-(2-((3-Chlorophenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7d). HRMS: Calc'd for ($C_{24}Cl_1H_{26}N_5O_1$)H+: 436.18938; Found: 436.18952.

(*S*)-1-(2-((4-Chlorophenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7e). HRMS: Calc'd for ($C_{24}Cl_1H_{26}N_5O_1$)H+: 436.1894; Found: 436.1905.

(*S*)-1-(2-((3-Methoxyphenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7f). HRMS: Calc'd for ($C_{25}H_{29}N_5O_2$)H+: 432.23878; Found:. 432.23922.

(*S*)-1-(2-((4-Methoxyphenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7g). HRMS: Calc'd for ($C_{25}H_{29}N_5O_2$)H+: 432.23878; Found: 432.23946.

(*S*)-1-(2-((3-Ethylphenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7h). HRMS: Calc'd for ($C_{26}H_{31}N_5O_1$)H+: 430.25948; Found: 430.25994.

(*S*)-1-(2-((4-Ethylphenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7i). HRMS: Calc'd for ($C_{26}H_{31}N_5O_1$)H+: 430.25948; Found: 430.25979.

(*S*)-1-(2-((3,4-Dimethoxyphenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7j). HRMS: Calc'd for ($C_{26}H_{31}N_5O_3$)H+: 462.24928; Found: 462.24982.

(*S*)-1-(2-(2,3-Dihydro-1,4-benzodioxin-6-ylamino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7k). HRMS: Calc'd for ($C_{26}H_{29}N_5O_3$)H+: 460.23368; Found: 460.23367.

(*S*)-1-(2-((3,5-Dimethoxyphenyl)amino)-4-pyrimidinyl)-N-(4-methylbenzyl)-3-piperidinecarboxamide (7l). HRMS: Calc'd for ($C_{26}H_{31}N_5O_3$)H+: 462.24928; Found: 462.25005.

(*S*)-N-Phenyl-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11a). HRMS: Calc'd for ($C_{25}H_{29}N_5O_4$)H+: 464.22858; Found: 464.22892.

(*S*)-N-Benzyl-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11b). HRMS: Calc'd for ($C_{26}H_{31}N_5O_4$)H+: 478.24418; Found: 478.24470.

(*S*)-N-(2-Phenylethyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11c). HRMS: Calc'd for ($C_{27}H_{33}N_5O_4$)H+: 492.2598; Found: 492.2607.

(*S*)-N-(3-Phenylpropyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11d). HRMS: Calc'd for ($C_{28}H_{35}N_5O_4$)H+: 506.27618; Found:506.27559.

(*S*)-N-(2-Oxo-2-phenylethyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11e). HRMS: Calc'd for ($C_{27}H_{31}N_5O_5$)H+: 506.23908; Found: 506.2397.

(*S*)-N-((*S*)-1-Phenylethyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11f). HRMS: Calc'd for ($C_{27}H_{33}N_5O_4$)H+: 492.25978; Found: 492.25997.

(*S*)-N-((*R*)-1-Phenylethyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11g). HRMS: Calc'd for ($C_{27}H_{33}N_5O_4$)H+: 492.25978; Found: 492.26028.

(*S*)-N-(2-Methylbenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11h). HRMS: Calc'd for ($C_{27}H_{33}N_5O_4$)H+: 492.25978; Found: 492.26019.

(*S*)-N-(3-Methylbenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11i). HRMS: Calc'd for ($C_{27}H_{33}N_5O_4$)H+: 492.25978; Found: 492.26017.

(*S*)-N-(3-(Trifluoromethoxy)benzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11j). HRMS: Calc'd for ($C_{27}F_3H_{30}N_5O_5$)H+: 562.22648; Found: 562.2267.

(*S*)-N-(4-(Trifluoromethoxy)benzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11k). HRMS: Calc'd for ($C_{27}F_3H_{30}N_5O_5$)H+: 562.22648; Found: 562.2261.

(*S*)-N-(2-Chlorobenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11l). HRMS: Calc'd for ($C_{26}C_{l1}H_{30}N_5O_4$)H+: 512.20528; Found: 512.20556.

(*S*)-N-(3-Chlorobenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11m). HRMS: Calc'd for ($C_{26}C_{l1}H_{30}N_5O_4$)H+: 512.20528; Found: 512.20556.

(*S*)-N-(4-Chlorobenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11n). HRMS: Calc'd for ($C_{26}C_{l1}H_{30}N_5O_4$)H+: 512.20528; Found: 512.20560.

(*S*)-N-(3-Nitrobenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11o). HRMS: Calc'd for ($C_{26}H_{30}N_6O_6$)H+: 523.22928; Found: 523.22929.

(*S*)-N-(4-Nitrobenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11p). HRMS: Calc'd for ($C_{26}H_{30}N_6O_6$)H+: 523.22928; Found: 523.22945.

Methyl 4-(((((3*S*)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinyl)carbonyl)amino)methyl)benzoate (11r). HRMS: Calc'd for ($C_{28}H_{33}N_5O_6$)H+: 536.24958; Found:536.25000.

(*S*)-N-(4-biphenylylmethyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11t). HRMS: Calc'd for ($C_{32}H_{35}N_5O_4$)H+: 554.2754; Found: 554.2768

(*S*)-N-(3-Phenoxybenzyl)-1-(2-((3,4,5-trimethoxyphenyl)amino)-4-pyrimidinyl)-3-piperidinecarboxamide (11u). HRMS: Calc'd for ($C_{32}H_{35}N_5O_5$)H+: 570.27028; Found: 570.27077.

(*S*)-4-Methylbenzyl 1-(2-(3,4,5-trimethoxyphenylamino)pyrimidin-4-yl)piperidine-3-carboxylate (13). A solution of **3** (50 mg), DMAP (4 mg) and DCC (27 mg) in DCM (3218 µL) was stirred at 23 °C for 30 min. *p*-Tolylmethanol (24 mg) was then added and the reaction was stirred at 23 °C over 24 h. The reaction was then filtered, the filtrate was concentrated and the reaction mixture was purified by mass-triggered preparative HPLC (Phenomenex Gemini-NX C18 110A column (100 × 21 mm, 5µm), 44 mL/min flow rate, 5% to 95% [0.1% TFA in acetonitrile] in [0.1% TFA in water] over 10 min, mass spectral data were acquired from 100-850 amu in electrospray positive mode using MS - Waters SQ, UV - Waters 2487 or Waters PD) to provide the desired products as the salt (72% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.88 (s, 1H), 7.93 (d, *J* = 6.16 Hz, 1H), 7.18 - 7.24 (m, 2H), 7.09 - 7.16 (m, 4H), 6.24 (d, *J* = 6.16 Hz, 1H), 4.98 - 5.11 (m, 2H), 4.31 (br. s., 1H), 3.94 (br. s., 0H), 3.17 - 3.29 (m, 0H), 2.53 - 2.71 (m, 2H), 2.27 (s, 3H), 1.91 - 2.03 (m, 1H), 1.61 - 1.79 (m, 2H), 1.50 (t, *J* = 10.56 Hz, 1H); HRMS: Calc'd for ($C_{27}H_{32}N_4O_5$)H+: 493.2438; Found: 493.2452.

(*S*)-*tert*-Butyl 1-(2-chloropyrimidin-4-yl)piperidin-3-ylcarbamate (15). To a solution of (*S*)-3-(boc-amino)piperidine (1g) and TEA (694 µL) in EtOH (50 mL) at 4 °C was added 2,4-dichloropyrimidine (744 mg) and the resulting solution was allowed to warm to 23 °C while stirring. After 18 h, the reaction was concentrated and the crude material was absorbed onto a plug of silica gel and purified by chromatography through two 25 g silica gel columns, eluting with 10% to 100% EtOAc in hexanes, to provide (*S*)-*tert*-butyl 1-(2-chloropyrimidin-4-

yl)piperidin-3-ylcarbamate (1.26 g, 4.03 mmol, 81 % yield) as white solid. MS (ESI, pos. ion) *m/z*: 313.0 [M+H].

(*S*)-4-(3-Aminopiperidin-1-yl)-N-(3,4,5-trimethoxyphenyl)pyrimidin-2-amine (16). Compound **15** (1260 mg) and 3,4,5-trimethoxyaniline (738 mg) were combined in DMSO (16 mL) and heated to 90 °C while stirring. After 18 h, additional 3,4,5-trimethoxyaniline (50 mg) was introduced and the reaction was stirred at 90 °C. After an additional 18 h, the reaction was cooled to 23 °C and concentrated to give (*S*)-*tert*-butyl 1-(2-(3,4,5-trimethoxyphenylamino)pyrimidin-4-yl)piperidin-3-ylcarbamate as a purple solid. The crude product was brought up in DCM (10 ml) and treated with TFA (1496 µL) over 18 h. Additional TFA (1496 µL) was added. After 18 h, the reaction was concentrated to give (*S*)-4-(3-aminopiperidin-1-yl)-N-(3,4,5-trimethoxyphenyl)pyrimidin-2-amine as the TFA salt and taken on as is. MS (ESI, pos. ion) *m/z*: 360.2 [M+H].

(*S*)-2-p-Tolyl-N-(1-(2-(3,4,5-trimethoxyphenylamino)pyrimidin-4-yl)piperidin-3-yl)acetamide (**17**). To a solution of **16** (1448 mg) and TEA (1685 µL) in DCM (20 mL) was added HATU (1686 mg) followed by *p*-tolylacetic acid (701 mg) and the reaction was stirred at 23 °C. After 24 h, additonal HATU (1686 mg) and p-tolylacetic acid (701 mg) was added and the reaction was stirred at 23 °C for an additional 4 h. The reaction was then concentrated and the crude material was absorbed onto a plug of silica gel and purified by chromatography through a 100 g silica gel column, eluting with 1.5% MeOH in DCM, to provide the crude product. The crude material was purified by reverse-phase preparative HPLC (150*30mm C18 column, 10% to 90% [0.1% TFA in acetonitrile] in [0.1% TFA in water] over 12 min to provide (*S*)-2-p-tolyl-N-(1-(2-(3,4,5-trimethoxyphenylamino)pyrimidin-4-yl)piperidin-3-yl)acetamide (9% yield) as the TFA salt. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ 11.66 (br. s., 1H), 7.50 - 7.68 (m, 1H), 6.91 - 7.18 (m, 4H),

6.85 (s, 2H), 5.97 - 6.21 (m, 1H), 5.30 - 5.55 (m, 1H), 4.05 - 4.34 (m, 1H), 3.72 - 4.00 (m, 11H), 3.32 - 3.70 (m, 4H), 2.32 (s, 3H), 1.96 (br. s., 1H), 1.62 (br. s., 3H); HRMS: Calc'd for $(C_{27}H_{33}N_5O_4)H+$: 492.2598; Found: 492.2614.

N-(4-Methylbenzyl)-1-(piperidin-3-yl)methanamine hydrochloride (19). To a stirred solution of 3-formyl-piperidine-1-carboxylic acid *tert*-butyl ester (623 µL) and 4-methylbenzylamine (361 µL) in DCM (28 mL) was added sodium triacetoxyborohydride (781 mg) and the resultant mixture was stirred at 23 °C. After 36 h, the reaction was diluted with DCM and extracted with water, 1N NaOH and saturated aqueous sodium bicarbonate. The organic layer was dried over sodium sulfate, filtered and concentrated to give the crude product. The crude material was absorbed onto a plug of silica gel and purified by chromatography through a 25 g silica gel column, eluting with 10% to 80% EtOAc in hexanes to provide *tert*-butyl 3-((4-methylbenzylamino)methyl)piperidine-1-carboxylate as a colorless film. To this was added dioxane (28 ml) followed by HCl, 1 M in dioxane (5680 µl) and the resulting mixture was stirred at 60 °C. After 3 d, the reaction was cooled to 23 °C and concentrated to give the desired product (35.5% yield over two steps) as a yellow oil. MS (ESI, pos. ion) *m/z*: 219.2 [M+H].

4-(3-((4-Methylbenzylamino)methyl)piperidin-1-yl)-N-(3,4,5 trimethoxyphenyl) pyrimidin-2-amine (20). To a solution of N-(4-methylbenzyl)-1-(piperidin-3-yl)methanamine hydrochloride (255 mg) and TEA (278 µL) in EtOH (9996 µL) at 4 °C was added 2,4-dichloropyrimidine (149 mg) and the resulting solution was allowed to warm to 23 °C over 2 h. The reaction was concentrated and the crude material was absorbed onto a plug of silica gel and purified by chromatography through a 25g silica gel columns, eluting with 0.5% to 5% MeOH in DCM, to provide 1-(1-(2-chloropyrimidin-4-yl)piperidin-3-yl)-N-(4-methylbenzyl)methanamine (28 %

yield) as a yellow film. The intermediate (92 mg) and 3,4,5-trimethoxyaniline (51 mg) were combined in DMSO (2772 µL) and heated to 90 °C while stirring. After 18 h, the reaction was cooled to 23 °C and concentrated. The crude material was absorbed onto a plug of silica gel and purified by chromatography through a 25g silica gel column, eluting with 0.5% to 10% MeOH in DCM, to provide 4-(3-((4-methylbenzylamino)methyl)piperidin-1-yl)-N-(3,4,5-trimethoxyphenyl)pyrimidin-2-amine (43.5 mg, 32.9 % yield) as a purple solid. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ 7.91 (br. s., 1H), 7.70 (br. s., 1H), 7.17 - 7.24 (m, 2H), 7.11 (d, *J* = 7.60 Hz, 2H), 6.93 (s, 2H), 6.06 (d, *J* = 4.24 Hz, 1H), 4.08 (d, *J* = 11.55 Hz, 2H), 3.72 - 3.89 (m, 11H), 3.03 - 3.19 (m, 2H), 2.49 - 2.62 (m, 2H), 2.31 (s, 3H), 1.86 (br. s., 2H), 1.65 (br. s., 1H), 1.42 - 1.57 (m, 1H), 1.23 - 1.39 (m, 1H); HRMS: Calc'd for ($C_{27}H_{35}N_5O_3$)H+: 478.2805; Found: 478.2819.

(*R*)-1-(2-(3,4,5-Trimethoxyphenylamino)-pyrimidin-4-yl)piperidine-3-carboxylic acid (22). **22** was prepared in a method analogous to **3** using (*R*)-(-)-nipecotic acid ethyl ester in place of (*S*)-(+)-nipecotic acid ethyl ester to provide the desired product as a light purple solid. ^{1}H NMR (300 MHz, CD_3OD) δ ppm 1.67 (m, 1 H); 1.80 - 1.96 (m, 2 H); 2.10 (m, 1 H); 2.66 (m, 1 H); 3.77 – 3.84 (m, 12 H); 4.00 - 4.09 (m, 1 H); 6.61 (d, *J*=7.45 Hz, 1 H); 6.79 (s, 2 H); 7.69 (d, *J*=7.16 Hz, 1 H); MS (ESI, pos. ion) *m/z*: 389.1 [M+H].

(*R*)-N-(4-Methylbenzyl)-1-(2-(3,4,5-trimethoxy-phenylamino) pyrimidin-4-yl)piperidine-3-carboxamide (23). **23** was prepared using General Procedure B using **22** in place of **3** to provide the desired product (79% yield) as a light purple solid. ^{1}H NMR (400 MHz, CHLOROFORM-d) δ 7.95 (d, *J* = 6.06 Hz, 1H), 7.35 (br. s., 1H), 7.08 (s, 4H), 6.83 (s, 2H), 6.44 (br. s., 1H), 6.01 (d, *J* = 6.06 Hz, 1H), 4.25 - 4.41 (m, 2H), 4.22 (d, *J* = 13.30 Hz, 1H), 3.94 - 4.08 (m, 1H), 3.77 - 3.85 (m, 9H), 3.52 (dd, *J* = 9.39, 13.50 Hz, 1H), 3.06 - 3.17 (m, 1H), 2.30 - 2.39 (m, 1H), 2.29 (s, 3H), 1.90 -

2.02 (m, 2H), 1.71 (td, *J* = 4.01, 13.30 Hz, 1H), 1.52 (dtd, *J* = 4.89, 9.45, 13.96 Hz, 1H); HRMS: Calc'd for $(C_{27}H_{33}N_5O_4)H+$: 492.25978; Found: 492.26048.

ALK inhibition in enzyme assay. The cytoplasmic domain (amino acids 1058-1620) of wild-type human ALK (NP_004295.2) was expressed in SF9 cells as an N-terminal GST fusion protein. Kinase activity of the purified protein was assessed using a Lance® TR-FRET assay. The kinase reaction was performed in a 384-well microtiter plate using 2 nM enzyme in 20 mM HEPES (pH 7.5), 0.05% BSA, 2 mM DTT, 10 mM $MgCl_2$, 1 μM peptide substrate (Biotin-Ahx-EQEDEPEGIYGVLF-OH), and ATP at 40 μM (the apparent Km). The reaction was allowed to proceed for 90 minutes at 23 °C and was then terminated with 20 mM EDTA in 50 mM Tris (pH 7.5), 100 mM NaCl, 0.05% BSA, and 0.1% Tween-20. Phosphorylation of the peptide substrate was detected using the Lance® detection reagents streptavidin-allophycocyanin (SA-APC) and Eu-W1024 anti-phosphotyrosine antibody (PT66) from PerkinElmer Life Sciences (Waltham, MA). The plates were read on a RUBYstar plate reader (BMG LABTECH, Cary, NC) with an excitation wavelength of 320 nm. Emission was monitored at 615 nm and 665 nm, with increased emission at 665 nm indicating peptide phosphorylation. IC_{50} values for compounds were calculated from the magnitude of signal in the 665 nm emission channel.

IGF1R inhibition in enzyme assay. The cytoplasmic domain (amino acids 960-1367) of wild-type human IGF-1R (NP_000866.1) was expressed in High Five (Hi5) cells as an N-terminal GST fusion protein. Kinase activity of the purified protein was assessed using a Lance® TR-FRET assay. The kinase reaction was performed in a 384-well microtiter plate using 15 pM enzyme in 50 mM HEPES (pH 7.5), 0.05% BSA, 2 mM DTT, 10 mM $MgCl_2$, 1 μM peptide substrate (Biotin-Ahx- EEEEAYGWLDF-OH), and ATP at 60 μM (the apparent Km). The reaction was allowed to

proceed for 90 minutes at 23 °C and was then terminated with 20 mM EDTA in 50 mM Tris (pH 7.5), 100 mM NaCl, 0.05% BSA, and 0.1% Tween-20. Phosphorylation of the peptide substrate was detected using the Lance® detection reagents streptavidin-allophycocyanin (SA-APC) and Eu-W1024 anti-phosphotyrosine antibody (PT66) from PerkinElmer Life Sciences (Waltham, MA). The plates were read on a RUBYstar plate reader (BMG LABTECH, Cary, NC) with an excitation wavelength of 320 nm. Emission was monitored at 615 nm and 665 nm, with increased emission at 665 nm indicating peptide phosphorylation. IC_{50} values for compounds were calculated from the ratio of the emission at 665nm/615nm.

Cell culture. ALK-positive Karpas-299 cells (DSMZ, Braunschweig, Germany) and ALK-negative HT cells (ATCC, Manassas, VA) were maintained at a cell density below 2x106 cells/mL in RPMI-1640 medium supplemented with 1X penicillin-streptomycin-glutamine (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum from Sigma-Aldrich (St Louis, MO) for the AlphaScreen® assay or Thermo Fisher Scientific (Waltham, MA) for phosflow. Assays were performed on cells in log-phase growth (density between 0.8x106 and 1.2x106cells/mL).

AlphaScreen® Surefire® cell-based pY^{1604} ALK assay. Cells (10,000 per well) were dispensed in 3 µL of assay buffer (HBSS, 0.1%BSA, 5 mM HEPES, pH 7) into a 384-well low volume white-walled polystyrene Proxiplate (PerkinElmer Life Sciences) containing 1 µL of compound in 2% DMSO (98% assay buffer) per well for a final reaction concentration of 0.5% DMSO. Cells were incubated with compound at 23 °C for 1 hour prior to analysis of pY^{1604} ALK. Measurement of pY^{1604} ALK was performed with an AlphaScreen® SureFire® assay kit (PerkinElmer Life Sciences). Cells were lysed with 1 µL of 5X lysis buffer and incubated at 23 °C for 10 minutes. Next, 4.3 µL of a mix containing antibodies, reaction buffer, activation buffer and protein A acceptor beads

(PerkinElmer Life Sciences) was added as per the manufacturer's protocol. Plates were incubated overnight in the dark at 23 °C before the addition of 1.8 µL of streptavadin donor bead mix and an additional hour of incubation at 23 °C. Automated addition of cells, lysis buffer, and detection reagents was performed with a FlexDrop bulk liquid dispenser (PerkinElmer Life Sciences). Plates were read on an EnVision Reader (PerkinElmer Life Sciences) using the AlphaScreen® assay setting. IC_{50} values for compounds were calculated from the magnitude of signal in the 570 nm emission channel and were expressed as the mean of two or three replicates.

Data analysis. The amount of signal generated in the presence of compounds versus that in the presence of DMSO vehicle alone (high control) was calculated using the formula: % of control (POC) = (signal with compound – average low) / (average high – average low) x 100 where the low control was defined by the response observed with 1 µM (proliferation and phosflow assays) or 50 µM (enzyme and AlphaScreen® assays) of a benchmark ALK inhibitor. For IC_{50} determination, POC values were fitted to a 4-parameter equation (minimum POC, maximum POC, IC_{50} compound concentration, and slope factor) using the mFit non-linear regression algorithm (Fomenko et al. 2006). The potency of most compounds was assessed once in each assay, with 2 or 3 replicates per point in each experiment. In instances in which a compound was run more than once in an assay, its mean IC_{50} value was used for further analyses. Recombinant ALK enzyme and AlphaScreen® assay results were analyzed using Screener® version 7.0 from Genedata AG (Basel, Switzerland). Phosflow and cellular proliferation data were analyzed using ActivityBase XE from IDBS (Guildford, United Kingdom).

Table S1. Data collection and refinement statistics

	ALK + 1
Data Collection	
Space group	$P2_1$
Unit cell dimensions	
a, b, c (Å)	51.6, 104.8, 57.8
α, β, γ (°)	90, 90.2, 90
Resolution (Å)	50-2.03 (2.10-2.03)
Total reflections	89382
Unique reflections	39292
Completeness (%)[1]	98.5 (90.3)
R_{merge}[1]	0.048 (0.409)
$I/\sigma(I)$[1]	18.6 (1.8)
Refinement	
Reflections used	37208
R / R_{free}	0.211/0.256
Average B-value (Å^2)	34.4
Number of atoms	

Protein	4499
Ligand	72
Solvent	287
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (º)	1.03
PDB ID code	4DCE

[1] Numbers in parentheses are for the highest resolution shell.

Table S2. Ambit Biosciences KINOME*scan* Results

Ambit Gene Symbol	Entrez Gene Symbol	Compound 1 POC at 1 µM	Compound 11w POC at 1 µM
ALK	ALK	1	0.8
FLT3	FLT3	8.1	7.5
KIT	KIT	1.5	1
TRKA	NTRK1	19	3.9
PAK2	PAK2	1.3	100
MEK5	MAP2K5	9.4	34
IGF1R	IGF1R	91	100