Supporting Information:

A Codrug Approach for the Potential Treatment of EML4-ALK Positive Lung Cancer.

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CONTENTS OF SUPPORTING INFORMATION

SYNTHETIC PROCEDURES AND COMPOUND CHARACTERIZATION

General

Reagents were purchased from commercial sources (typically, Sigma Aldrich, Alpha Aesar, Fisher Scientific, Fluorochem or Activate Scientific), and were used without further purification. Anhydrous solvents were purchased from Fisher scientific and were used without further distillation. All reactions with air- and moisture-sensitive reagents were carried out under an atmosphere of nitrogen. The flasks were flushed with nitrogen. The liquids were added using plastic syringes.

Where thin-layer chromatography (TLC) was performed, UV light and standard TLC stains were used to visualise the Merck Silica gel 60 Å F_{254} plates. Compounds were purified via column chromatography using either a Thompson pump and normal phase Interchim Puriflash pre-packed cartridges consisting of 50 µM silica, or a glass column using Merck Geduran silica gel 60 Å (230-240 µm) Column size selected was generally 40-60 times the loading amount. STX cartridges

NMR Spectroscopy

Routine ^{1}H and ^{13}C, experiments were attained on a Bruker AV400, spectrometer operating at 400 MHz, where coupling constants (J) are reported to the nearest 0.1 Hz. Additionally, a Bruker AV(III)500 operating at 500 MHz was used for lower concentration samples, and a Bruker AV(III)800 operating at 800 MHz, was used for kinetic experiments and exceptionally low concentration samples. Measurements were recorded at 21 °C unless otherwise stated. All ^{13}C NMR are ^{1}H- broadband-decoupled. The chemical shifts δ are given in [ppm] and all coupling constants J are given in [Hz] and are reported to the nearest 0.1 Hz. The spectra are referenced to the signal of the deuterated solvent. The following abbreviations were used to describe signal shapes and multiplicity: s (singlet), s. br (broad singlet), d (doublet), dd (doublet of doublet), ddd (doublet of a doublet of a doublet), dddd (doublet of a doublet of a doublet of a doublet), t (triplet), dt (doublet of a triplet), q (quartet), dq (doublet of a quartet) and m (multiplet). The processing of the NMR data was carried out using the NMR software Mestrenova (Mnova).

Mass Spectrometry

Liquid chromatography mass spectrometry (LCMS) data was recorded on a Shimadzu UFLCXR (High performance liquid chromatography (HPLC) system coupled to an Applied Biosystems API2000 electrospray ionization mass spectrometer (ESI-MS). The column used was a Phenomenex Gemini-NX 3µm 110 Å C-18, 50 x 2 mm thermostated at 40 °C. The flow rate was 0.5 mL / min of a solvent system of increasing gradient of acetonitrile (5-95%) in water, each containing 0.1% formic acid. UV detection was at 220 nm and 254 nm. m/z values are reported in Daltons to one decimal place and retention times (r.t.) are provided in minutes to two decimal places.

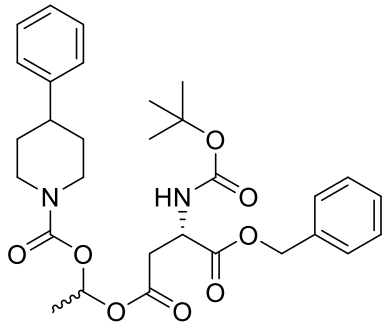
All high-resolution mass spectra (HRMS) time of flight electrospray were recorded on a Bruker Daltonics micrOTOF Electrospray ionisation- time of flight mass spectrometer (ESI-TOF MS). Measured (m/z) is within 6 ppm of the theoretical (m/z).

Preparative HPLC

Preparative RP-HPLC was performed on a Waters 2767 sample manager coupled to Waters 2525 binary gradient module and a Waters 2457 dual wavelength absorbance detector. The column used was a Phenomenex Gemini-NX 5µm-110 Å C18, 150x21 mm at ambient temperature. The flow rate was 40 mL/min, the UV detection was at 254 nm and unless otherwise stated the sample injections were 500 µL in DMF.

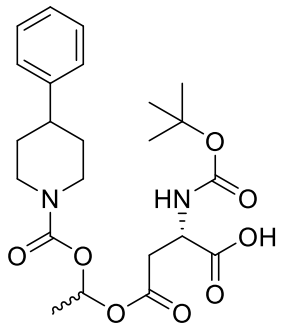
Model System

1-Benzyl 4-(1-((4-phenylpiperidine-1-carbonyl)oxy)ethyl) (tert-butoxycarbonyl)aspartate



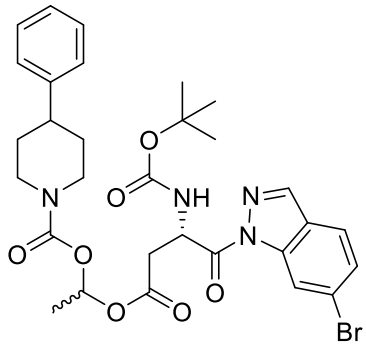
To a precooled (-10 °C) solution of 4-phenylpiperidine (1.0 g, 6.20 mmol) in CH_2Cl_2 (~100 mL) was added NMM (1.5 mL, 13.6 mmol) then ACE-Cl (0.73 mL, 6.8 mmol). The reaction was allowed to warm to room temperature and stirred for a further 1.5 h after which time the reaction mixture was poured onto HCl (2 M, 30 mL) and extracted with dichloromethane (3 x 30 mL). The combined organics were dried ($MgSO_4$), filtered, and solvent removed *in vacuo* to give an orange oil which was taken up in toluene (~100 mL). 4-(benzyloxy)-3-((tert-butoxycarbonyl)amino)-4-oxobutanoic acid (1.34 g, 5.02 mmol), Ag_2O (1.40 g, 6.02 mmol) and tetrabutyl ammonium bromide (0.32 g, 1.00 mmol) were added, and the reaction stirred at room temperature for 1 h before heating at 65 °C overnight. The reaction mixture was then absorbed onto silica gel and purified by flash column chromatography, eluting with 10 - 50% EtOAc in petroleum ether to give a yellow oil (1.59 g, 48%), NMR (400 MHz, MeOD) δ 7.43 – 7.14 (m, 10H), 6.90 – 6.74 (m, 1H), 5.23 – 5.08 (m, 2H), 4.77 – 4.72 (m, 1H), 4.68 – 4.57 (m, 1H), 4.21 (s, 2H), 3.02 – 2.77 (m, 4H), 2.75 – 2.61 (m, 1H), 2.01 (s, 3H), 1.82 – 1.71 (m, 2H), 1.67 – 1.52 (m, 1H), 1.49 – 1.36 (m, 9H); LCMS (m/z): calc. for $C_{30}H_{38}N_2O_8$ [M + H]+ 555.3 found 555.6, r.t. 3.29.

2-((tert-Butoxycarbonyl)amino)-4-oxo-4-(1-((4-phenylpiperidine-1-carbonyl)oxy)ethoxy)butanoic acid



To a solution of 1-benzyl 4-(1-((4-phenylpiperidine-1-carbonyl)oxy)ethyl) (tert-butoxycarbonyl)aspartate (1.39 g, 2.51 mmol) in EtOAc (25 mL) was added Pd/C (139 mg, 10% wt) as a suspension in EtOAc. The reaction was stirred under an atmosphere of hydrogen for 3 h after which time the reaction was filtered and solvent removed *in vacuo* to give a white foam (1.06 g, 76%). The compound was used without further purification. NMR (400 MHz, MeOD) δ 7.26 (ddd, J = 25.5, 16.3, 7.3 Hz, 5H), 6.83 – 6.78 (m, 1H), 4.57 – 4.47 (m, 1H), 4.29 – 4.21 (m, 2H), 3.06 – 2.83 (m, 5H), 2.76 (tt, J = 12.1, 3.7 Hz), 1.89 – 1.84 (m, 1H), 1.66 (tt, J = 12.6, 6.4 Hz, 1H), 1.52 (d, J = 5.4, 3H), 1.50 – 1.42 (m, 10H). LCMS (m/z): calc. for $C_{23}H_{32}N_2O_8$ [M + H]+ 465.5, found 465.4, r.t. 2.97.

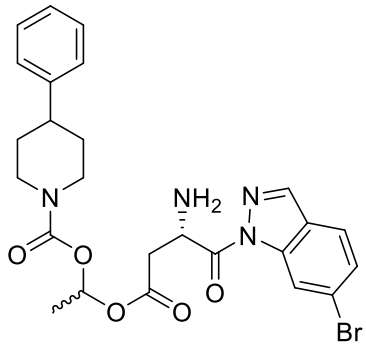
1-((4-(6-Bromo-1H-indazol-1-yl)-3-((tert-butoxycarbonyl)amino)-4-oxobutanoyl)oxy)ethyl 4-phenylpiperidine-1-carboxylate



To a stirred solution of 2-((tert-butoxycarbonyl)amino)-4-oxo-4-(1-((4-phenylpiperidine-1-carbonyl)oxy)ethoxy)butanoic acid (0.97 g, 1.75 mmol) in CH_2Cl_2 (50 mL) was added HATU (730 mg, 1.92 mmol), 5-bromoindazole (344 mg, 1.75 mmol),and DIPEA (0.91 mL, 5.24 mmol). After 2.5 h the reaction mixture was absorbed onto silica and purified by flash column chromatography, eluting with 0-15% EtOAc in petroleum ether to give a colourless gum (989 mg, 88%). 1H NMR (400 MHz, CDCl3) δ 8.37 – 8.30 (m, 1H), 8.14 – 8.10 (m, 1H), 7.93 – 7.88 (m, 1H), 7.70 – 7.65 (m, 1H), 7.38 – 7.29 (m, 2H), 7.29 – 7.18 (m, 3H), 6.82 (q, J = 5.4, 1H), 5.97 – 5.92 (m, 1H), 5.82 – 5.74 (m, 1H), 5.32 (1H, s), 4.38 – 4.25

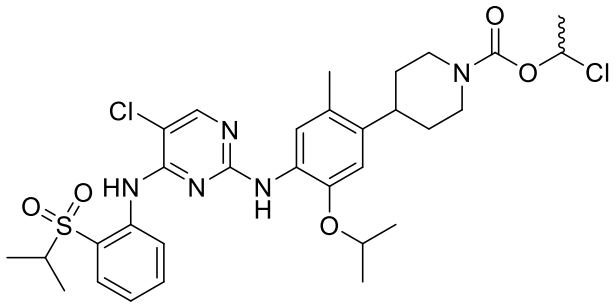
(1H, m), 4.22 – 4.09 (1H, m), 3.30 – 3.21 (m, 1H), 3.17 – 3.07 (m, 1H), 2.94 – 2.85 (m, 2H), 2.75 – 2.62 (m, 1H), 1.86 (d, J = 11.4, 3H), 1.79 – 1.57 (m, 2H), 1.47 (d, J = 6.3, 9H) LCMS (m/z): calc. for $C_{30}H_{35}BrN_4O_7$ [M + H]+ 643.5, found 643.5, r.t. 3.42.

1-((3-Amino-4-(6-bromo-1H-indazol-1-yl)-4-oxobutanoyl)oxy)ethyl 4-phenylpiperidine-1-carboxylate (5)



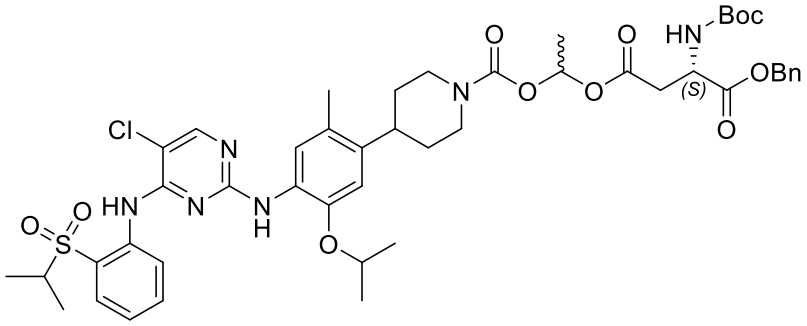
To a stirred solution of 4-benzyl 1-(1-((4-phenylpiperidine-1-carbonyl)oxy)ethyl) (tert-butoxycarbonyl)-L-aspartate (1.28 g, 0.21 mmol) in CH_2Cl_2 (1 mL) was added TFA (1 mL). After 1 h, the solvent and TFA were removed *in vacuo* and the residual solid was triturated with diethyl ether to give a white solid. 1H NMR (400 MHz, DMSO) δ 8.63 – 8.58 (m, 1H), 8.28 – 8.24 (m, 3H), 7.36 – 7.27 (m, 2H), 7.27 – 7.20 (m, 5H), 6.68 (dq, J = 11.0, 5.4 Hz, 1H), 5.33 – 5.26 (m, 1H), 4.02 – 3.98 (m, 3H), 3.25 – 2.60 (m, 2H), 1.78 – 1.73 (m, 3H), 1.51 – 1.46 (m, 3H), 1.43 (t, J = 5.1 Hz, 3H), LCMS (m/z): calc. for $C_{25}H_{27}BrN_4O_5$ [M + H]+ 543.4, found 543.3 , r.t. 2.51.

1-Chloroethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (6)



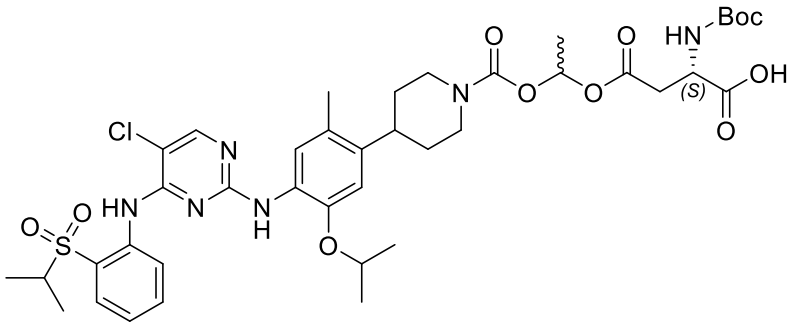
To a precooled (-10 °C) solution of 5- chloro-N2-(2-isopropoxy-5-methyl-4-(piperidin-4-yl)phenyl)-N4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (170 mg , 0.30 mmol) in CH_2Cl_2 (4 mL) was added NMM (134 µL, 1.22 mmol) then ACE-Cl (40 µL, 0.37 mmol). The reaction was allowed to warm to room temperature and stirred for a further 1.5 h after which time the reaction mixture was poured onto HCl (2 M, 30 mL) and extracted with CH_2Cl_2 (3 x 30 mL). The combined organics were dried ($MgSO_4$), filtered, and solvent removed in vacuo to give a pale yellow solid (197 mg, 100%) which was used without further purification. (m/z): calc. for $C_{31}H_{39}Cl_2N_5O_5S$ $[M + H]^+$ 664.2 found 664.0, r.t 3.43.

1-Benzyl 4-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethyl) (tert-butoxycarbonyl)-L-aspartate (10)



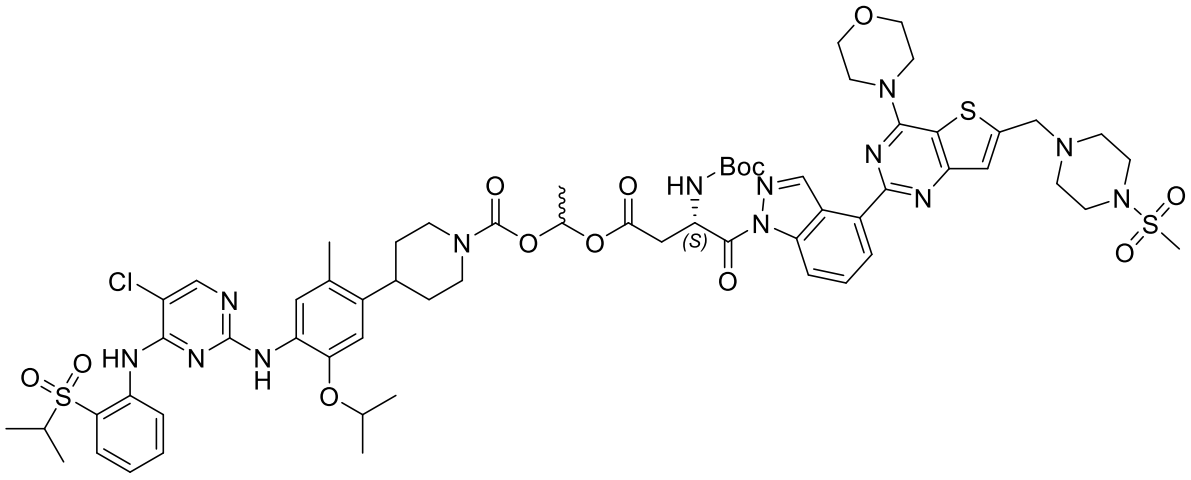
To a stirred solution of 1-chloroethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methyl-phenyl)piperidine-1-carboxylate (100 mg, 0.15 mmol) was added 4- (benzyloxy)-3-((tert-butoxycarbonyl)amino)-4-oxobutanoic acid (58 mg, 0.18 mmol), Ag_2O (42 mg, 0.18 mmol) and tetrabutyl ammonium bromide (10 mg, 0.03 mmol) were added, and the reaction stirred at room temperature for 1 h before heating at 65 °C overnight. The reaction mixture was then absorbed onto silica gel and purified by flash column chromatography, eluting with 10 - 50% ethyl acetate in petroleum ether to give a yellow oil (110 mg, 77 %). 1H NMR (400 MHz, DMSO) δ 9.47 (s, 1H), 8.47 (d, J = 8.4 Hz, 1H), 8.26 (s, 1H), 8.05 (s, 1H), 7.85 (dd, J = 7.9, 1.7 Hz, 1H), 7.63 (t, J = 7.6 Hz, 1H), 7.54 (s, 1H), 7.41 – 7.30 (m, 6H), 6.70 (s, 1H), 5.13 (s, 2H), 4.69 – 4.51 (m, 1H), 4.49 – 4.39 (m, 1H), 4.19 – 4.01 (m, 3H), 3.45 (p, J = 6.7 Hz, 1H), 3.07 – 2.63 (m, 3H), 2.15 (s, 3H), 1.70 – 1.65 (m, 2H), 1.62 – 1.55 (m, 2H), 1.46 – 1.40 (m, 2H), 1.40 – 1.35 (m, 9H), 1.32 – 1.23 (m, 4H), 1.23 – 1.14 (m, 12H). LCMS (m/z): calc. for $C_{47}H_{59}ClN_6O_{11}S$ $[M + H]^+$ 951.4 found 951.1, rt 3.56.

(2S)-2-((tert-butoxycarbonyl)amino)-4-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethoxy)-4-oxobutanoic acid (**13**)

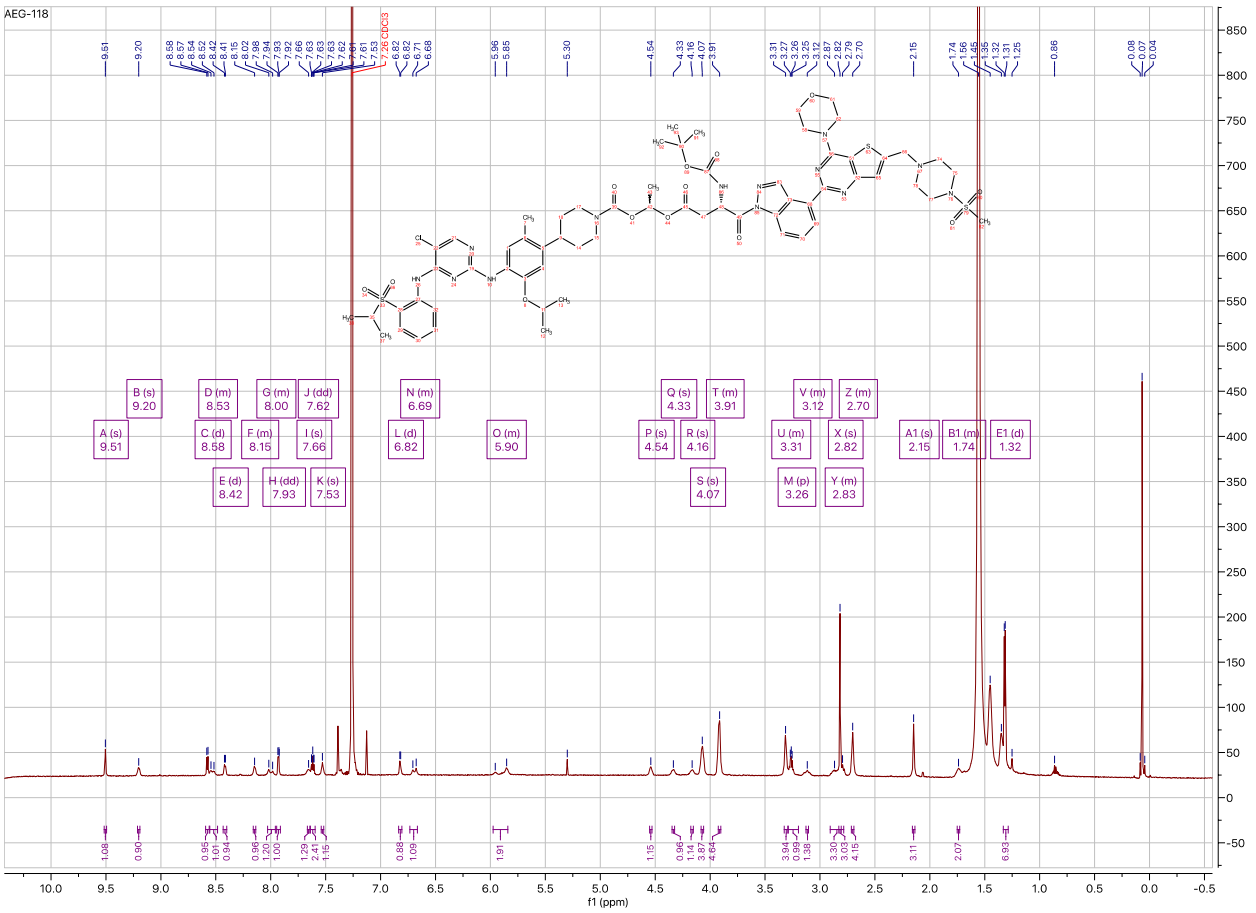


To a solution of 1-benzyl 4-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl) phenyl) amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl) piperidine-1-carbonyl)oxy)ethyl) (tertbutoxycarbonyl)aspartate. (110 mg, 0.12 mmol) in ethyl acetate (3 mL) was added Pd/C (22 mg, 20% wt) as a suspension in EtOAc. The reaction was stirred under an atmosphere of hydrogen for 3 h after which time the reaction was filtered and solvent removed in vacuo to give a white foam (104 mg, 100%). The compound was used without further purification. (m/z): calc. for $C_{40}H_{53}ClN_6O_{11}S$ $[M + H]^+$ 861.3 found 861.0, r.t. 3.29.

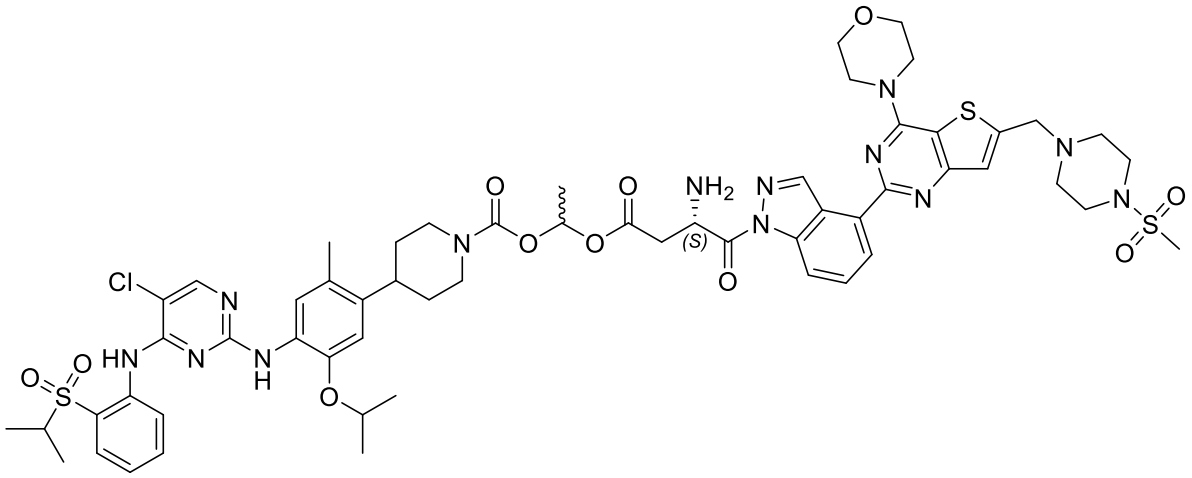
1-(((S)-3-((tert-butoxycarbonyl)amino)-4-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-4-oxobutanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (16)



To a stirred solution of 2-((tert-butoxycarbonyl)amino)-4-(1-((4-(4-((5- chloro-4-((2-(isopropyl-sulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-cabonyl)oxy)ethoxy)-4-oxobutanoic acid (104 mg, 0.12 mmol) in DMF (4 mL) was added HATU (50 mg, 0.13 mmol), 4-(2-(1H-in- dazol-4-yl)-6-((4-(methylsulfonyl)piperazin-1-yl)methyl)thieno[3,2- d]py-rimidin-4-yl)morpholine (62 mg, 0.12 mmol), and DIPEA (63 µL, 0.36 mmol). After 2.5 h the reaction mixture was directly purified by preparative HPLC, method A, and was freeze dried to give (20) as a white solid (2.5 mg, 3%). ^{1}H NMR (800 MHz, CDCl3) δ 9.51 (s, 1H), 9.20 (s, 1H), 8.58 (d, J = 8.3 Hz, 1H), 8.55 – 8.48 (m, 1H), 8.42 (d, J = 7.5 Hz, 1H), 8.16 – 8.14 (m, 1H), 8.03 – 7.96 (m, 1H), 7.93 (dd, J = 7.9, 1.5 Hz, 1H), 7.66 (s, 1H), 7.62 (dd, J = 8.6, 1.4 Hz, 2H), 7.53 (s, 1H), 6.82 (d, J = 5.7 Hz, 1H), 6.73 – 6.67 (m, 1H), 5.98 – 5.84 (m, 2H), 4.54 (s, 1H), 4.33 (s, 1H), 4.16 (s, 1H), 4.07 (s, 4H), 3.93 – 3.90 (m, 5H), 3.32 – 3.29 (m, 4H), 3.26 (p, J = 6.8 Hz, 1H), 3.13 – 3.10 (m, 1H), 2.91 – 2.78 (m, 3H), 2.82 (s, 3H), 2.71 – 2.69 (m, 4H), 2.15 (s, 3H), 1.75 – 1.73 (m, 2H), 1.70 – 1.38 (m, 24H), 1.32 (d, J = 6.7 Hz, 6H). LCMS (m/z): calc. for $C_{63}H_{78}ClN_{13}O_{13}S_3$ 1355.5 found 1356.3 [M + H]+ , r.t. 3.52, 99% purity. HRMS (m/z): calc. for $C_{63}H_{78}ClN_{13}O_{13}S_3$ $[M + H]^+$ 1355.4687 found 1356. 4712.



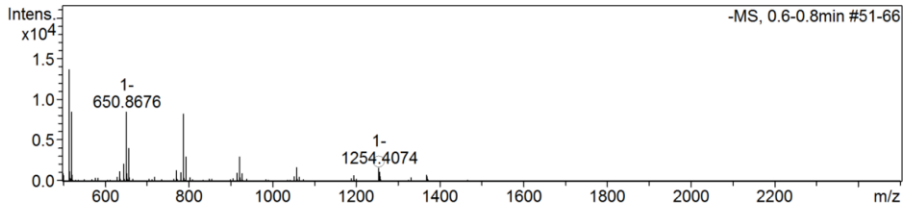
1-(((S)-3-amino-4-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-4-oxobutanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (19)



1-(((S)-3-((tert-butoxycarbonyl)amino)-4-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-4-oxobutanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (1 mg, 0.74 μmol) was taken up in TFA (20 μL, 20% v:v in CH_2Cl_2) and sonicated. After 5 mins, the solvent and TFA were removed in vacuo and the residual solid was triturated with diethyl ether to give a white solid (930 μg, 100%). LCMS (m/z): calc. for $C_{58}H_{70}ClN_{13}O_{11}S_3$ 1255.4 found 1256.6, r.t. 2.68, 100% purity. HRMS (m/z): calc. for $C_{58}H_{70}ClN_{13}O_{11}S_3$ $[M + H]^+$ 1255.4163 found 1255.4456.

Ionisation Mode ESI Negative Instrument Bruker MicroTOF

-MS, 0.6-0.8min #51-66



#	m/z	I %
1	514.8933	100.0
2	515.8974	9.0
3	520.9097	62.1
4	634.8904	8.9
5	644.8513	15.9
6	650.8676	62.2
7	651.8705	7.2
8	656.8831	29.6
9	770.8643	9.9
10	780.8266	8.3
11	786.8421	60.4
12	787.8473	7.9
13	792.8582	22.3
14	916.7998	7.6
15	922.8158	22.2
16	928.8309	7.3
17	1058.7923	12.4
18	1254.4074	12.0
19	1255.4073	8.6
20	1256.4059	8.5

Generate Molecular Formula Parameters

Charge	Tolerance	sigma limit	H/C Ratio	Electron Conf.	Nitrogen Rule	Chrom.BackGround	Calibration
-1	6 ppm	0.08	3 - 0	both	false	false	TRUE

Expected Formula C58 H70 Cl1 N13 O11 S3 **Adduct(s):** H, radical

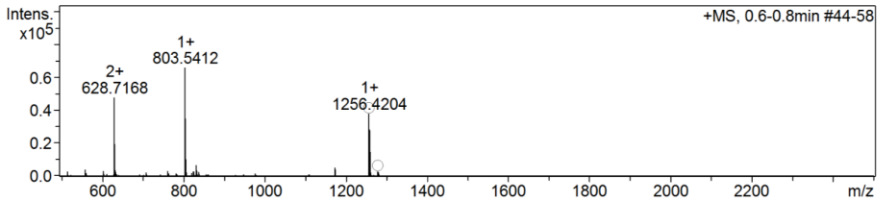
#	meas. m/z	theo. m/z	|Err|[ppm]	Sigma	Formula	Adduct	Adduct Mass
1	1 254.4074	1 254.4096	1.70	0.0204	C58H69ClN13O11S3	M- H	1.0078

Note: Sigma fits < 0.05 indicates high probability of correct MF

HRMS for **19** run in the negative mode

Ionisation Mode ESI Positive Instrument Bruker MicroTOF

+MS, 0.6-0.8min #44-58



#	m/z	I %
1	558.2283	6.5
2	628.7168	72.4
3	629.2173	52.2
4	629.7166	50.9
5	630.2168	29.9
6	630.7164	13.4
7	631.2134	5.8
8	761.2689	5.2
9	803.5412	100.0
10	804.5451	53.3
11	805.5482	15.9
12	831.5724	10.2
13	832.5739	5.6
14	1173.1689	8.2
15	1173.6691	6.5
16	1256.4204	57.8
17	1257.4226	43.0
18	1258.4184	42.2
19	1259.4177	22.4
20	1260.4163	10.0

Generate Molecular Formula Parameters

Charge	Tolerance	sigma limit	H/C Ratio	Electron Conf.	Nitrogen Rule	Chrom.BackGround	Calibration
+1	6 ppm	0.08	3 - 0	both	false	false	TRUE

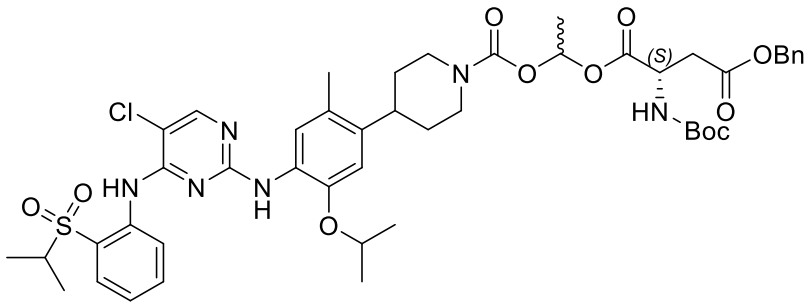
Expected Formula C58 H70 Cl1 N13 O11 S3 **Adduct(s):** H, Na, NH4, C3H5N2, radical

#	meas. m/z	theo. m/z	|Err|[ppm]	Sigma	Formula	Adduct	Adduct Mass
1	1 256.4204	1 256.4241	3.00	0.0144	C58H71ClN13O11S3	M+H	1.0078
1	1 278.4000	1 278.4061	4.80	0.0663	C58H70ClN13NaO11S3	M+Na	22.9898

Note: Sigma fits < 0.05 indicates high probability of correct MF

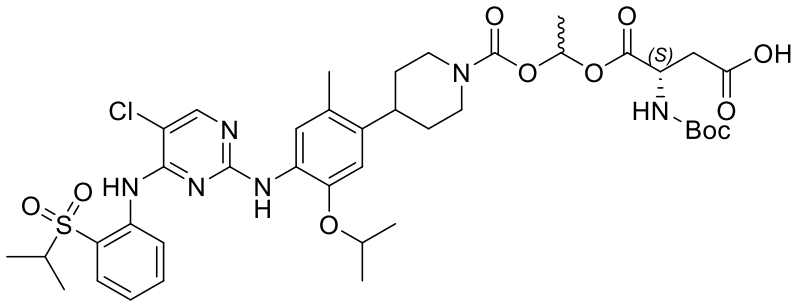
HRMS for **19** run in the positive mode

4-Benzyl 1-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethyl) (tert-butoxycarbonyl)-L-aspartate (11)



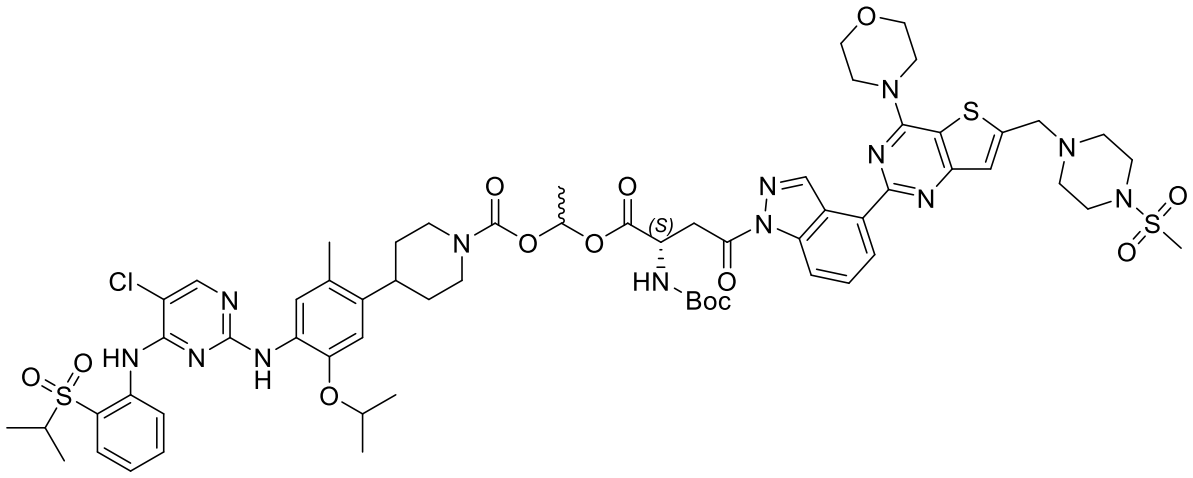
To a precooled (-10 °C) solution of 5-chloro-N2-(2-isopropoxy-5-methyl-4-(piperidin-4-yl)phenyl)-N4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (150 mg , 0.27 mmol) in CH_2Cl_2 (4 mL) was added NMM (67 µL, 0.60 mmol) then ACE-Cl (32 µL, 0.30 mmol). The reaction was allowed to warm to room temperature and stirred for a further 1.5 h after which time the reaction mixture was poured onto HCl (2 M, 30 mL) and extracted with CH_2Cl_2 (3 x 30 mL). The combined organics were dried (MgSO4), filtered, and solvent removed in vacuo to give an orange residue which was taken up in toluene. (S)-4-(benzyloxy)-3-((tert-butoxycarbonyl)amino)-4-oxobutanoic acid (105 mg, 0.32 mmol), Ag_2O (75 mg, 0.32 mmol) and tetrabutyl ammonium bromide (18 mg, 0.05 mmol) were added, and the reaction stirred at room temperature for 1 h before heating at 65 °C overnight. The reaction mixture was then absorbed onto silica gel and purified by flash column chromatography, eluting with 10 - 50% ethyl acetate in petroleum ether to give the product as a beige oil (88 mg, 34%). LCMS (m/z): calc. for $C_{47}H_{59}ClN_6O_{11}S$ $[M + H]^+$ 951.4 found 951.7, rt 3.50.

(3S)-3-((tert-Butoxycarbonyl)amino)-4-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethoxy)-4-oxobutanoic acid (14)



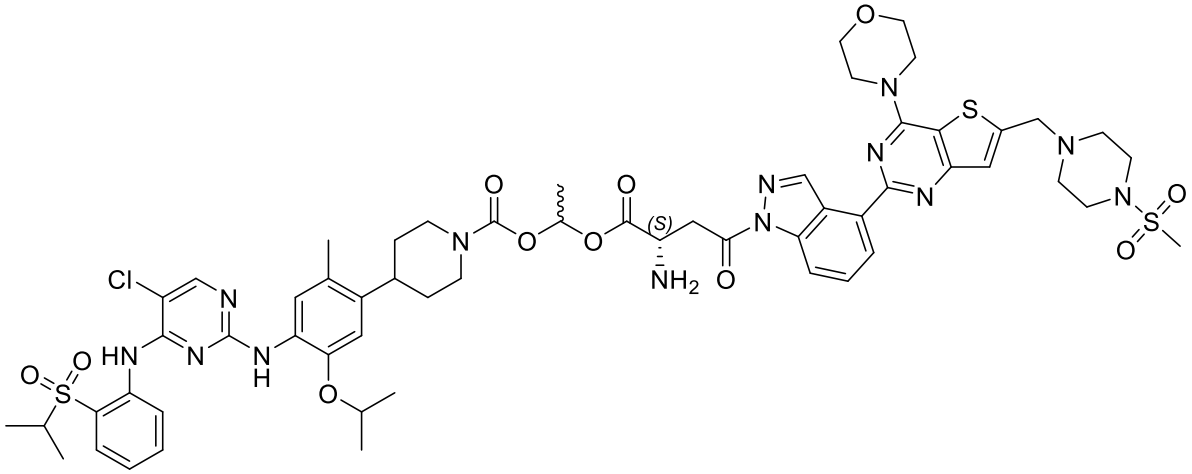
To a solution of 4-benzyl 1-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethyl)(tert-butoxycarbonyl)-L-aspartate (88 mg, 93 µmol) in ethyl acetate (3 mL) was added Pd/C (17 mg, 20% wt) as a suspension in ethyl acetate. The reaction was stirred under an atmosphere of hydrogen for 3 h after which time the reaction was filtered and solvent removed in vacuo to give a white foam (80 mg, 100%). The compound was used without further purification.

1-(((S)-2-((tert-Butoxycarbonyl)amino)-4-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-4-oxobutanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (17)

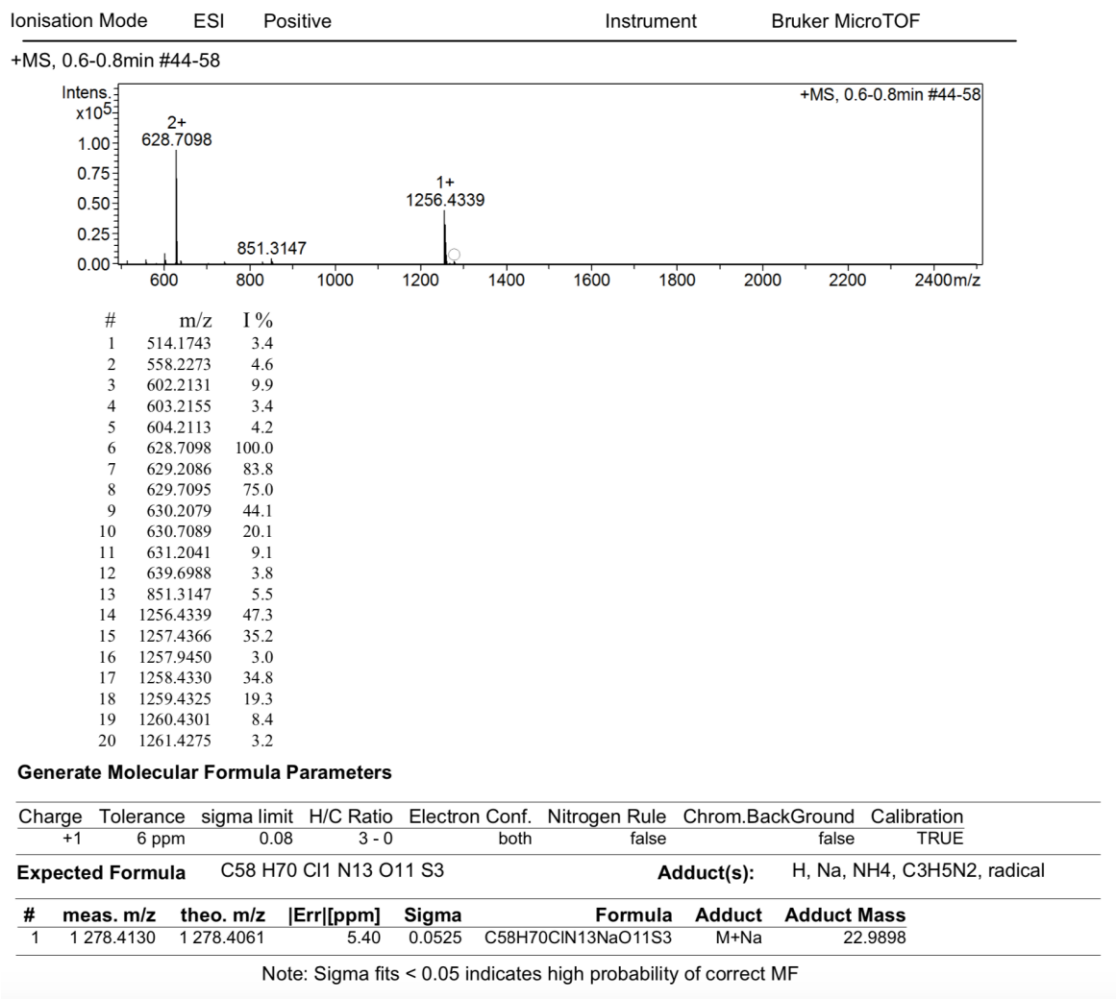


To a stirred solution of (3S)-3-((tert-butoxycarbonyl)amino)-4-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethoxy)-4-oxobutanoic acid (80 mg, 93 µmol) in DMF (3 mL) was added HATU (39 mg, 102 µmol), 4-(2-(1H-in- dazol-4-yl)-6-((4-(methylsulfonyl)piperazin-1-yl)methyl)thieno[3,2- d]pyrimidin-4-yl)morpholine (48 mg, 93 µmol), and DIPEA (49 µL, 0.28 mmol). After 2.5 h the reaction mixture was directly purified by preparative HPLC, method A, and was freeze dried to give a white solid (14 mg, 11%). 1H NMR (800 MHz, $CDCl_3$) δ 9.55 – 9.46 (m, 1H), 9.24 – 9.16 (m, 1H), 8.61 (d, J = 8.3 Hz, 1H), 8.58 – 8.50 (m, 1H), 8.51 – 8.35 (m, 2H), 8.19 (s, 1H), 8.07 – 7.99 (m, 1H), 7.96 (dd, J = 7.5, 1.4 Hz, 1H), 7.65 (dd, J = 8.6, 1.2 Hz, 1H), 7.64 – 7.48 (m, 2H), 6.97 – 6.95 (m, 1H), 6.81 – 6.60 (m, 1H), 5.76 – 5.67 (m, 1H), 4.91 – 4.88 (m, 1H), 4.82 – 4.55 (m, 2H), 4.38 – 4.34 (m, 1H), 4.25 (s, 1H), 4.08 (d, J = 33.7 Hz, 4H), 3.93 (s, 5H), 3.78 (s, 1H), 3.35 – 3.33 (m, 4H), 3.32 – 3.27 (m, 1H), 2.85 (s, 3H), 2.74 – 2.72 (m, 5H), 2.22 – 2.02 (m, 3H), 1.88 – 1.16 (m, 32H). LCMS (m/z): calc. for $C_{63}H_{78}ClN_{13}O_{13}S_3$ 1355.5 found 1356.5 $[M + H]^+$, r.t. 3.46, 100% purity. HRMS (m/z): calc. for $C_{63}H_{78}ClN_{13}O_{13}S_3$ $[M + H]^+$ 1356.4687 found 1356.4679.

1-(((S)-2-Amino-4-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-4-oxobutanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (20)



1-(((S)-2-((tert-butoxycarbonyl)amino)-4-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-4-oxobutanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (250 μg, 0.18 μmol) was taken up in TFA (20 μL, 20% v:v in CH_2Cl_2) and sonicated. After 5 mins, the solvent and TFA were removed in vacuo and the residual solid was triturated with diethyl ether to give a white solid (213 μg, 100%). LCMS (m/z): calc. for $C_{58}H_{70}ClN_{13}O_{11}S_3$ 1255.4 found 1256.5 $[M+H]^+$, r.t. 2.55, 100% purity. HRMS (m/z): calc. for $C_{58}H_{70}ClN_{13}O_{11}S_3$ $[M + H]^+$ 1256.4247 found 1256.4339.



#	m/z	I %
1	514.1743	3.4
2	558.2273	4.6
3	602.2131	9.9
4	603.2155	3.4
5	604.2113	4.2
6	628.7098	100.0
7	629.2086	83.8
8	629.7095	75.0
9	630.2079	44.1
10	630.7089	20.1
11	631.2041	9.1
12	639.6988	3.8
13	851.3147	5.5
14	1256.4339	47.3
15	1257.4366	35.2
16	1257.9450	3.0
17	1258.4330	34.8
18	1259.4325	19.3
19	1260.4301	8.4
20	1261.4275	3.2

Generate Molecular Formula Parameters

Charge	Tolerance	sigma limit	H/C Ratio	Electron Conf.	Nitrogen Rule	Chrom.BackGround	Calibration
+1	6 ppm	0.08	3 - 0	both	false	false	TRUE

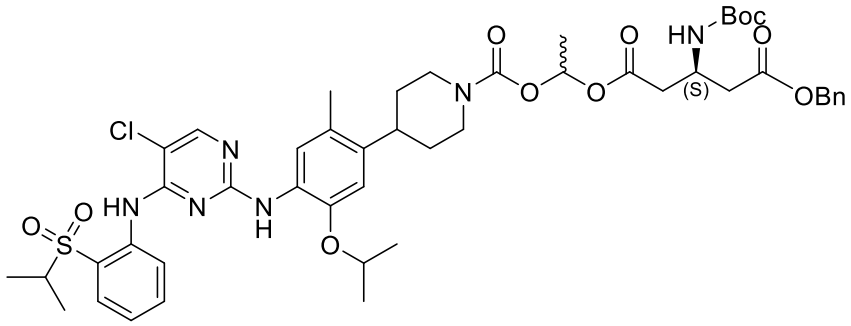
Expected Formula C58 H70 Cl1 N13 O11 S3 **Adduct(s):** H, Na, NH4, C3H5N2, radical

#	meas. m/z	theo. m/z	|Err|[ppm]	Sigma	Formula	Adduct	Adduct Mass
1	1 278.4130	1 278.4061	5.40	0.0525	C58H70ClN13NaO11S3	M+Na	22.9898

Note: Sigma fits < 0.05 indicates high probability of correct MF

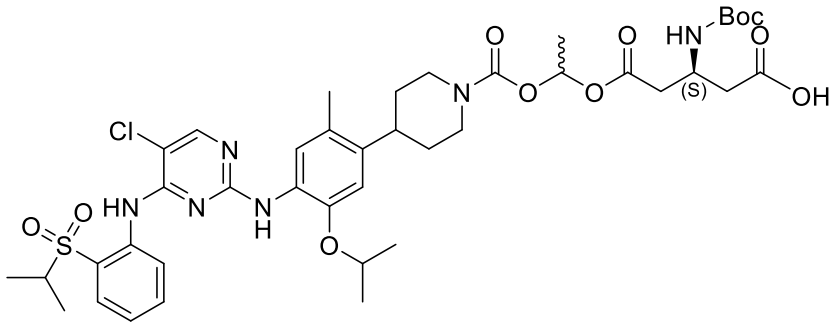
Figure 1. HRMS of **20** run in the positive

1-benzyl 5-(1-((4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethyl) (3S)-3-((tert-butoxycarbonyl)amino)pentanedioate (12)



To a precooled (-10 °C) solution of 5-chloro-N2-(2-isopropoxy-5-methyl-4-(piperidin-4-yl)phenyl)-N4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (150 mg , 0.27 mmol) in CH_2Cl_2 (4 mL) was added NMM (67 µL, 0.60 mmol) then ACE-Cl (32 µL, 0.30 mmol). The reaction was allowed to warm to room temperature and stirred for a further 1.5 h after which time the reaction mixture was poured onto HCl (2 M, 30 mL) and extracted with CH_2Cl_2 (3 x 30 mL). The combined organics were dried ($MgSO_4$), filtered, and solvent removed in vacuo to give an orange residue which was taken up in toluene. (R)-5-(benzyloxy)-3-((tert-butoxycarbonyl)amino)-5-oxopentanoic acid (109 mg, 0.32 mmol), Ag_2O (75 mg, 0.32 mmol) and tetrabutyl ammonium bromide (18 mg, 0.05 mmol) were added, and the reaction stirred at room temperature for 1 h before heating at 65 °C overnight. The reaction mixture was then absorbed onto silica gel and purified by flash column chromatography, eluting with 10 - 50% ethyl acetate in petroleum ether to give the product as a beige oil (67 mg, 26%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.53 (s, 1H), 8.60 (d, J = 8.5 Hz, 1H), 8.18 (s, 1H), 8.05 (s, 1H), 7.96 (d, J = 8.1 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.59 – 7.54 (m, 1H), 7.37 (s, 6H), 6.89 – 6.84 (m, 1H), 6.75 – 6.70 (m, 1H), 5.32 (s, 1H), 5.15 (s, 2H), 4.60 – 4.55 (m, 1H), 4.44 – 4.16 (m, 3H), 3.68 – 3.64 (m, 1H), 3.49 – 3.45 (m, 1H), 3.29 (p, J = 7.0 Hz, 1H), 2.86 – 2.81 (m, 6H), 2.80 – 2.67 (m, 4H), 2.19 (s, 3H), 1.79 – 1.75 (m, 3H), 1.68 – 1.02 (m, 20H). LCMS (m/z): calc. for $C_{48}H_{61}ClN_6O_{11}S$ $[M + H]^+$ 965.4 found 965.8, r.t. 3.50.

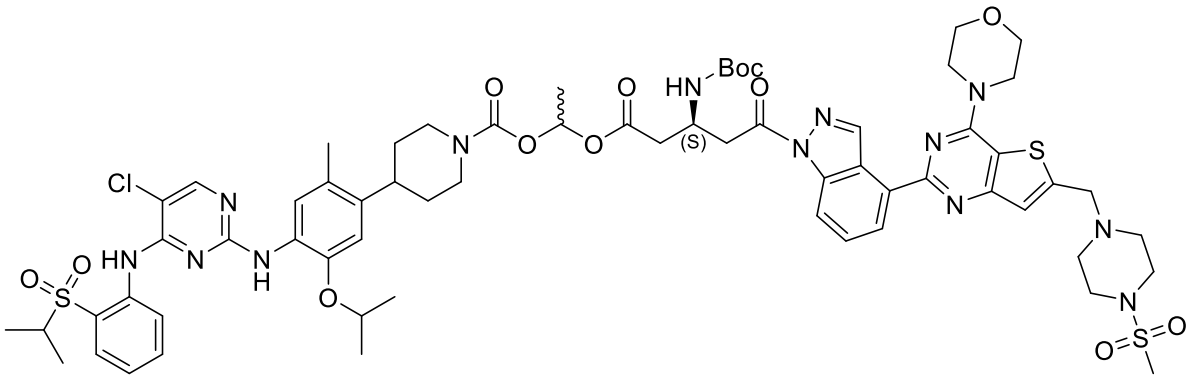
(3S)-3-((tert-Butoxycarbonyl)amino)-5-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethoxy)-5-oxopentanoic acid (15)



To a solution of 1-benzyl 5-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethyl) (3S)-3-((tert-butoxycarbonyl)amino)pentanedioate (67 mg, 69 µmol) in ethyl acetate (3 mL) was added Pd/C (15 mg) as a suspension in ethyl acetate. The reaction was stirred under an atmosphere of hydrogen for 3 h after which time the reaction was filtered and solvent removed in vacuo to give a white foam (50 mg, 83%). The

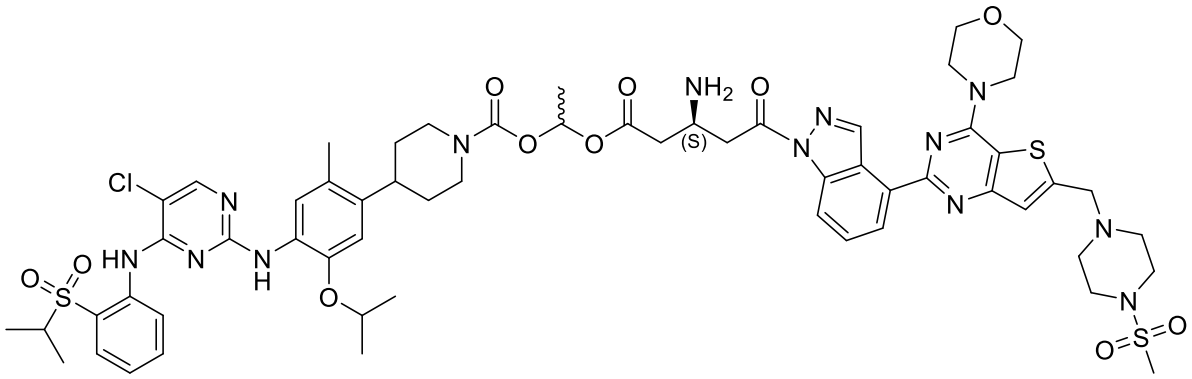
compound was used without further purification. LCMS (m/z): calc. for $C_{41}H_{55}ClN_6O_{11}S$ $[M + H]^+$ 875.3 found 875.5, r.t. 3.50.

1-(((S)-3-((tert-Butoxycarbonyl)amino)-5-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-5-oxopentanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (18)



To a stirred solution of 2(3S)-3-((tert-butoxycarbonyl)amino)-5-(1-((4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carbonyl)oxy)ethoxy)-5-oxopentanoic acid (50 mg, 57 µmol) in DMF (2 mL) was added HATU (24 mg, 63 µmol), 4-(2-(1H-in- dazol-4-yl)-6-((4-(methylsulfonyl)piperazin-1-yl)methyl)thieno[3,2-d]pyrimidin-4-yl)morpholine (30 mg, 57 µmol), and DIPEA (30 µL, 171 mmol). After 2.5 h the reaction mixture was directly purified by preparative HPLC and was freeze dried to give a white solid (4.5 mg, 5%). ^{1}H NMR (800 MHz, $CDCl_3$) δ 9.54 (s, 1H), 9.21 – 9.19 (m, 1H), 8.62 – 8.58 (m, 1H), 8.58 – 8.56 (m, 1H), 8.45 – 8.43 (m, 1H), 8.18 (s, 1H), 8.05 – 8.02 (m, 1H), 7.96 (d, J = 7.7 Hz, 1H), 7.69 – 7.67 (m, 1H), 7.66 – 7.61 (m, 1H), 7.58 – 7.56 (m, 1H), 6.92 – 6.89 (m, 1H), 6.78 – 6.70 (m, 1H), 5.67 – 5.51 (m, 1H), 4.66 – 4.63 (m, 1H), 4.58 – 4.56 (m, 1H), 4.34 – 4.31 (m, 1H), 4.27 – 4.25 (m, 1H), 4.12 – 4.08 (m, 3H), 3.97 – 3.92 (m, 6H), 3.74 – 3.72 (m, 1H), 3.60 – 3.58 (m, 1H), 3.35 – 3.33 (m, 4H), 3.29 (p, J = 6.9 Hz, 1H), 2.94 – 2.92 (m, 2H), 2.85 (s, 3H), 2.74 – 2.71 (m, 4H), 2.18 (d, J = 6.0 Hz, 3H), 1.78 – 1.75 (m, 2H), 1.71 – 1.12 (m, 32H). LCMS (m/z): calc. for $C_{64}H_{80}ClN_{13}O_{13}S_3$ $[M + H]^+$ 1370.5 found 1370.5, r.t. 3.44. HRMS (m/z): calc. for $C_{64}H_{80}ClN_{13}O_{13}S_3$ $[M – H]^-$ 1368.4771 found 1368.4768.

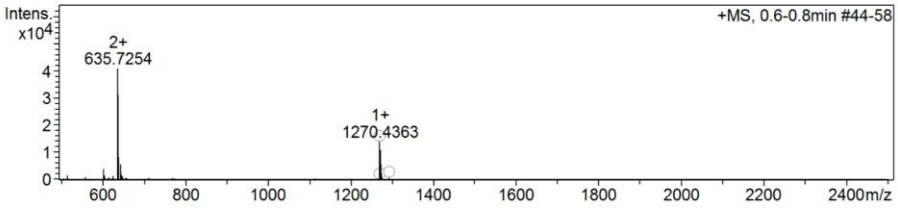
1-(((S)-3-Amino-5-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-5-oxopentanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (21)



1-(((S)-3-((tert-butoxycarbonyl)amino)-5-(4-(6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-1H-indazol-1-yl)-5-oxopentanoyl)oxy)ethyl 4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidine-1-carboxylate (250 μg, 0.18 μmol) was taken up in TFA (20 μL, 20% v:v in CH_2Cl_2) and sonicated. After 5 mins, the solvent and TFA were removed in vacuo and the residual solid was triturated with diethyl ether to give a white solid (232 μg, 100%). LCMS (m/z): calc. for $C_{59}H_{72}ClN_{13}O_{13}S_3$ 1270.5 $[M + H]^+$ found 1270.6 , r.t. 2.58, 100% purity. HRMS (m/z): calc. for $C_{59}H_{72}ClN_{13}O_{13}S_3$ $[M + H]^+$ 1270.4325 found 1270.4363.

Ionisation Mode ESI Positive Instrument Bruker MicroTOF

+MS, 0.6-0.8min #44-58



#	m/z	I %
1	514.1680	4.0
2	602.2187	9.9
3	603.2207	3.5
4	604.2179	4.3
5	635.7254	100.0
6	636.2268	75.5
7	636.7252	76.7
8	637.2252	42.1
9	637.7242	20.2
10	638.2233	7.9
11	643.2105	13.8
12	644.2138	5.6
13	646.7156	3.8
14	1270.4363	34.0
15	1270.9409	3.7
16	1271.4389	27.2
17	1271.9399	4.7
18	1272.4347	26.6
19	1273.4339	13.8
20	1274.4318	6.1

Generate Molecular Formula Parameters

Charge	Tolerance	sigma limit	H/C Ratio	Electron Conf.	Nitrogen Rule	Chrom.BackGround	Calibration
+1	6 ppm	0.08	3 - 0	both	false	false	TRUE

Expected Formula C59 H72 Cl1 N13 O11 S3 **Adduct(s):** H, Na, NH4, C3H5N2, radical

#	meas. m/z	theo. m/z	|Err|[ppm]	Sigma	Formula	Adduct	Adduct Mass
1	1 270.4363	1 270.4398	2.70	0.0348	C59H73ClN13O11S3	M+H	1.0078

Note: Sigma fits < 0.05 indicates high probability of correct MF

Figure 2. HRMS of **21** run in the positive

Mammalian Cell Tissue Culture and Equipment

A549 and ATCC® CCL-185IG™ (A549 EML4-ALK$^+$), H226 and H460 cell lines were obtained from American Type Culture Collection (ATTC). Cell lines were incubated at 37 °C under 5% CO_2 and cultured in RPMI 1640 culture medium supplemented with 10% heat deactivated foetal bovine serum (FBS). All cell culture procedures were performed in a class II laminar flow hood using sterile techniques.

Cell lines were passaged in the growth phase before reaching confluence. To passage, RPMI medium was aspirated and the cells washed with warm phosphate buffered saline (PBS), (~3 mL). The PBS was aspirated and the cells incubated with trypsin/EDTA (1 mL) until the cells detached from the flask. Once detached, the trypsin was deactivated with RPMI medium (6 mL), and an aliquot (500 µL for A549 and H460, 1.5 mL for H226) dispensed into flasks containing culture medium (13 mL).

Stock cell lines were preserved cryogenically in a liquid nitrogen cell bank. To generate stocks of cells, the procedure for trypsinisation above was followed, however following deactivation of the trypsin the cells were pelleted using a centrifuge. The supernatant was aspirated and the pellet was dispersed in 1 mL of freezing medium (5% DMSO in FBS), which was dispensed into a cryovial. The cells were then stored overnight in the standard lab freezer (~ -20 °C) and then next day placed in a -80 °C freezer. After 24 h the cells were moved to the liquid nitrogen cell bank (-196 °C). When required, cells were thawed by placing the cryovial in a water bath set to 37°C. Once thawed the cell suspension was transferred into a flask containing RPMI medium supplemented with 10% FBS. After 24 h the medium was aspirated and replaced. Cells were passaged at least twice before they were used for assays. Passage numbers did not exceed 30. Cell culture and assays were performed without antibiotics.

Standard equipment used for mammalian cell culture included a Beckham-Coulter allegro centrifuge, a Perkin Elmer Envsion plate reader, and a FC-500 Beckham-Coulter flow cytometer.

MTT Assay: Inhibition of Cell Growth and Metabolic Activity Assay

3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; AlfaAesar) was used to quantify an inhibitor's ability to inhibit growth and metabolic activity of human-derived lung cancer cell lines. Cells were plated in 96-well microtiter plates at a density of 3 x 10^3 (A549, ATCC® CCL-185IG™, H460) and 1 x 10^4 (H226) cells per well in 180 µL for single agents and 160 µL for combination assays.

Cells were allowed to attach for 24 h before test agent was introduced. Serial dilutions from a 10 mM top stock solution in DMSO were prepared in RPMI medium before each assay and were introduced to the assay in 20 µL aliquots, in a minimum of 3 wells per concentration (n = 3 - 6). Measurements representative of viable cells were performed at the time of test agent addition (T_{zero}) and after 72 h treatment by adding 50 µL of MTT to each well followed by 2.5 h incubation at 37 °C, allowing time for MTT reduction by viable cell dehydrogenases to violet formazan. The supernatant of each well was then aspirated followed by addition of 150 µL 100 % DMSO (Sigma Aldrich) for formazan solubilisation. Optical density values were retrieved at 570 nm. There were a minimum of three replicates per assay (n = 3).

The concentration required to inhibit cell proliferation by 50 % (GI_{50}) was calculated by:

$$Abs.of\ GI_{50} = \left[\frac{Abs.of\ Control - Abs.of\ Tzero}{2}\right] + Abs.of\ Tzero$$

and

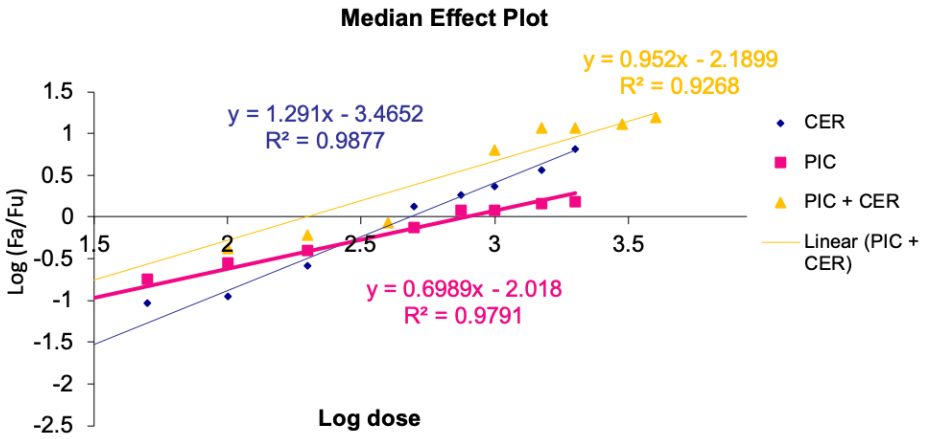
$$GI_{50} = \left[\frac{\left(\begin{matrix}Highest\ Abs.\\ where\ GI_{50}\ falls\end{matrix}\right) - (Abs.of\ GI_{50})}{\left(\begin{matrix}Highest\ Abs.\\ where\ GI_{50}\ falls\end{matrix}\right) - \left(\begin{matrix}Lowest\ Abs.\\ where\ GI_{50}\ falls\end{matrix}\right)}\right] \times \left[\left(\begin{matrix}Highest\ Conc.\\ where\\ GI_{50}\ falls\end{matrix}\right) - \left(\begin{matrix}Lowest\ Conc.\\ where\\ GI_{50}\ falls\end{matrix}\right)\right] + \left[\begin{matrix}Lowest\ Conc.\\ where\\ GI_{50}\ falls\end{matrix}\right]$$

The GI_{50} is expressed as the mean value ± the standard error of the mean (SEM).

Graphs and statistical analyses were generated and performed using GraphPad Prism. Statistical significance was determined using t-tests, one-way analysis of variance (ANOVA) or two-way ANOVA.

Chou & Talalay Method of drug combinations analyses

The Chou and Talalay method is widely used to determine whether drug combinations are additive, antagonistic or synergistic. It is based on the median-effect equation and can be used in any system that follows the physiochemical principle of the mass action law, regardless of the dynamics, whether it be first or higher order, i.e. regardless of the mechanism of action. Synergy is ultimately determined by calculation of the Combination Index (CI), where a CI of 1 is considered additive, a CI greater than 1 is antagonistic, and a CI less than 1 is synergistic. Chou and Talalay recommend that the combination assay be performed in constant ratio as this allows for a range of CI index values to be computed.



An example median effect plot. Ceritinib, pictilisib and their combination against the ALK+ cell line.

The median effect equation is used to linearise dose-response curves, the result being the median-effect plot, a plot of Log [Dose] vs Log [Fa/(1-Fa)]. The anti-log of the x-intercept gives the median dose (D_m) value, (the dose required to achieve 50% effect) and the gradient gives the m value, a Hill-type coefficient, describing the shape of the dose-response curve. When m = 1, the dose-response curve is hyperbolic, where m is greater than 1 the dose-response curve is sigmoidal and the greater the value, the greater its sigmoidicity. When the dose-effect relationships of drug A, drug B and the combination are all parallel in the median-effect plot, the effects of drug A and drug B are mutually exclusive. If the plots of drugs A and B are parallel but the plot of the combination tends to intersect the plot of the more potent of the two drugs, their effects are mutually non-exclusive. If the plots for drugs A and B and the combination are not parallel to one another, then the exclusivity effects cannot be determined.

The median-effect equation:

$$\frac{F_a}{(1-F_a)} = \left(\frac{D}{D_m}\right)^m \quad \Rightarrow \quad Log\left(\frac{F_a}{(1-F_a)}\right) = mLog(D) - mLog(D_m)$$

Once the m-value and the D_m value have been obtained from the median-effect plot, D_x values can be calculated for each drug and drug combination at different Fa values. These are the doses of each drug required to achieve the desired percentage effect. From this, the D_A, D_B... values can be obtained by factoring in the ratio (A : B) of the individual drug concentrations in the drug combinations.

Calculation of D_x values:

$$D_x = D_m\left(\frac{F_a}{(1-F_a)}\right)^{m^{-1}}$$

Calculation of D_A, D_B values:

$$(D)_A = (D_x)_{A,B} \times \left(\frac{A}{A+B}\right) \qquad (D)_B = (D_x)_{A,B} \times \left(\frac{B}{A+B}\right)$$

Once the D_A and D_B doses are known to cause a % effect, Fa, it is then possible to calculate the CI, and plot the CI plot. The equations below are used to calculate the CI for mutually exclusive and mutually non-exclusive combinations respectively.

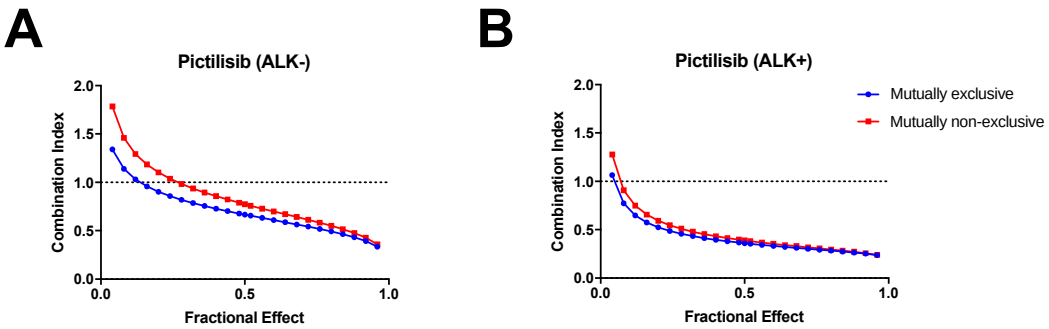
CI (mutually exclusive):

$$CI = \left(\frac{(D)_A}{(D_x)_A}\right) + \left(\frac{(D)_B}{(D_x)_B}\right)$$

CI (mutually non-exclusive):

$$CI = \left(\frac{(D)_A}{(D_x)_A}\right) + \left(\frac{(D)_B}{(D_x)_B}\right) + \frac{(D)_A(D)_B}{(D_x)_A(D_x)_B}$$

The combination index for each fractional effect is represented on a CI plot where a CI of 1 is additive, >1 is antagonistic, and <1 is synergic.

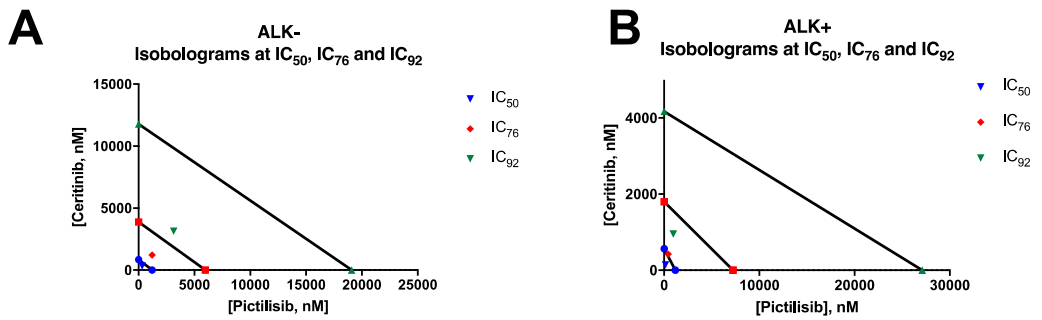


CI plots generated using the Chou and Talalay method for a 1:1 molar ratio of pictilisib and ceritinib against the ALK- cell line (A) and the ALK+ cell line (B). A combination index at a given fractional effect of 1 is an additive effect,>1 is antagonistic and <1 is synergistic.

The D_m value and m-value were calculated from the median effect plot using linear regression and are expressed as the mean value ± SEM. The combination index is calculated from the means of the D_m and m-values.

Linearization of the dose response curve by generation of the median-effect plot for pictilisib gave a D_m value of 1.22 µM ± 0.09 for the ALK^- cell line, and a D_m value of 1.12 µM ± 0.1 for the ALK^+ cell line. Additionally, the m-value obtained for pictilisib against the ALK^- cell line was 0.72 ± 0.09, and the m-value obtained for pictilisib was 0.64 ± 0.08. Ceritinib gave D_m values of 0.85 µM ± 0.09 against the ALK^- cell line and 0.57 µM ± 0.1 against the ALK^+ cell line. The corresponding m-values obtained were 0.76 ± 0.07 and 0.76 ± 0.07 respectively. For the combination of pictilisib with ceritinib, the D_m values obtained were 0.67 µM ± 0.05 against the ALK^- cell line and 0.28 µM ± 0.05 against the ALK^- cell line. The corresponding m-values were 0.88 ± 0.07 and 0.28 ± 0.05 for the ALK^- and ALK^+ cell lines respectively. The D_m and m-values allowed calculation of the combination indices (CI) across a range of concentrations (Table 2, main manuscript).

Dm values refer to the median dose effect – or drug potency. The m value refers to the shape of the concentration-response curve. Knowledge of the Dm and m values allows determination of combination indices (CI).

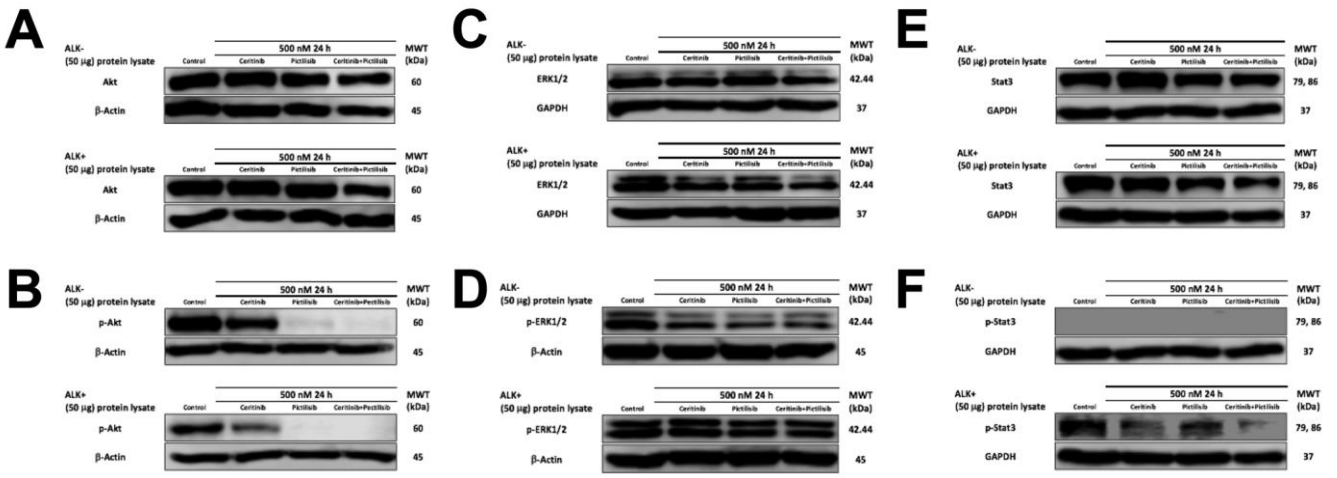


Isobolograms generated from the Chou and Talalay method IC_{50}, IC_{75} and IC_{90}.

Immunoblot analysis

After observing substantial synergy between ALK and PI3K inhibitors, the investigation progressed to explore the effect of pictilisib/ceritinib combination on signal transduction activity using immunoblot analysis of: (a) PI3K/AKT/mTOR pathway activation through phosphorylation of AKT, (b) Ras/Raf/MEK/ERK activity through phosphorylation of ERK1/2 and (c) JAK/STAT pathway signalling through STAT3 phosphorylation. In each experiment, cells were treated with ceritinib (500 nM, 24 h), pictilisib (500 nM, 24 h), and their combination (500 nM of each inhibitor, 24 h).

Following desired treatments, whole cell lysates were prepared, and protein content determined by Bradford assay. Proteins were separated by sodium dodecyl sulphate poly- acrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Detection of proteins was performed by immunoblotting28 using C-DiGitTM Blot Scanner. All antibodies were purchased from Cell Signaling Technologies: ALK, P-ALK (Tyr1604), Stat3, P-Stat3 (Tyr705), p44/42 MAPK (Erk1/2), P-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), P-Akt (Ser473), Akt, GAPDH and β-Actin. Both anti-mouse and anti-rabbit immunoglobulin G (IgG) horseradish peroxidase-antibodies were obtained from Dako.

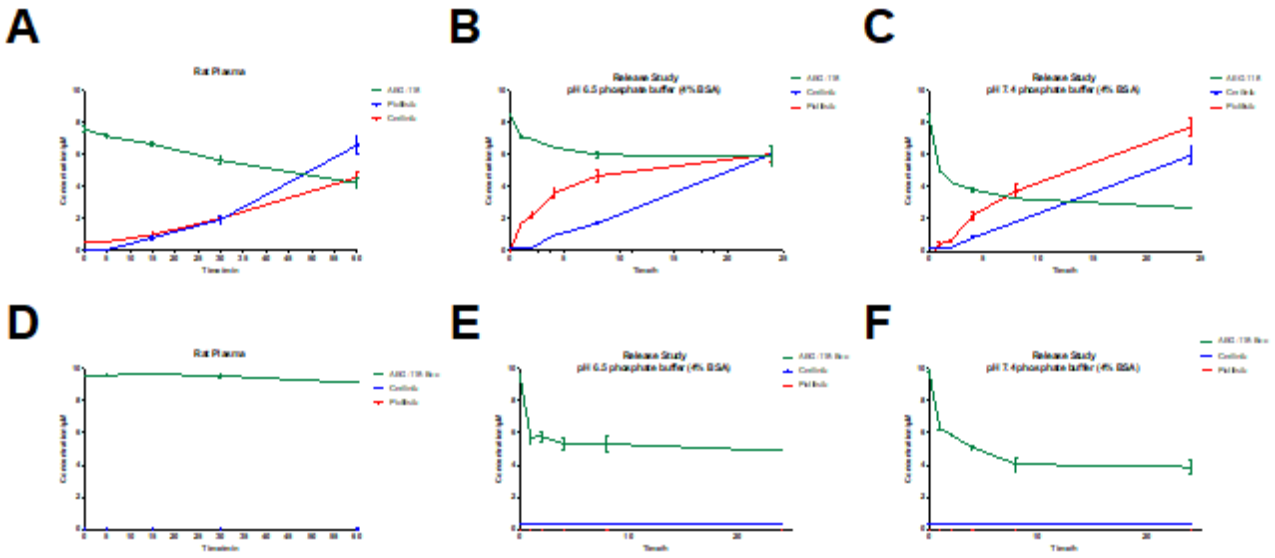


Immunoblot analysis of total AKT (A) and phosphorylated AKT (B) against the ALK^- and ALK^+ cell lines after 24 h treatment of pictilisib, ceritinib and their combination. Immunoblot analysis of total ERK1/2 (C) and phosphorylated ERK1/2 (D) against the ALK^- and ALK+ cell lines after 24 h treatment of pictilisib, ceritinib and their combination. Immunoblot analysis of total Stat3 (E) and phosphorylated Stat3 (F) against the ALK^+ and ALK^- cell lines after 24 h treatment of pictilisib, ceritinib and their combination.

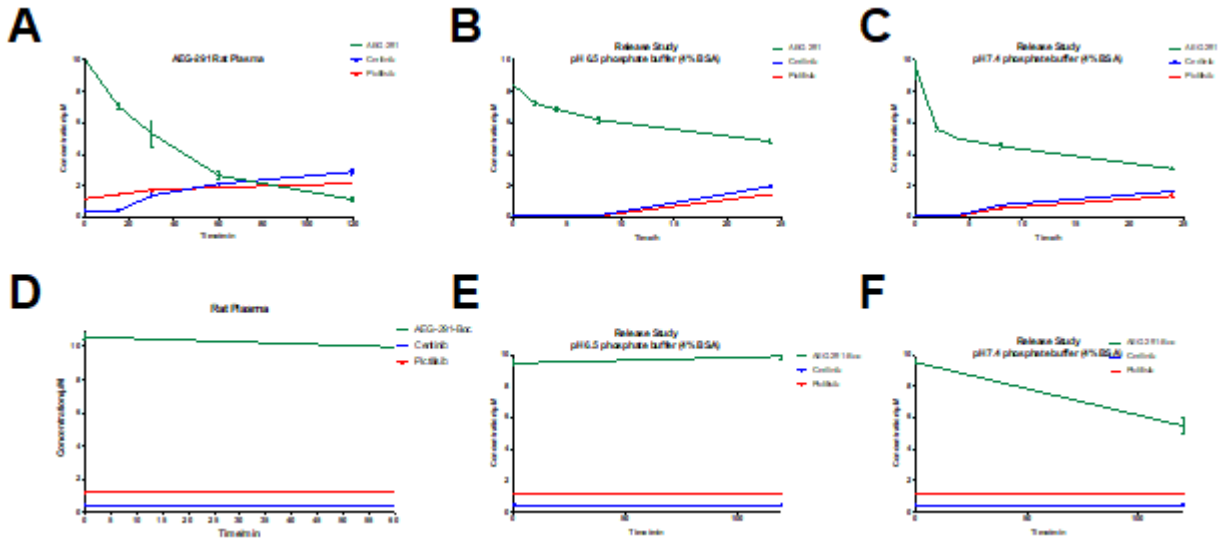
Invitro Stability Studies

After synthesis of the prodrug systems the stability was investigated in buffered solutions supplemented with bovine serum albumin (BSA), 4% by HPLC methods. Initially the experiment was attempted in phosphate buffer and DMSO without the supplemented BSA, although solubility issues did not allow for the experiment to be performed. The release study was performed by taking aliquots of the reaction and acidifiying to pH 1 to quench the reaction for HPLC analysis. The results of this experiment at both pH 6.5 and pH 7.4 is shown below:

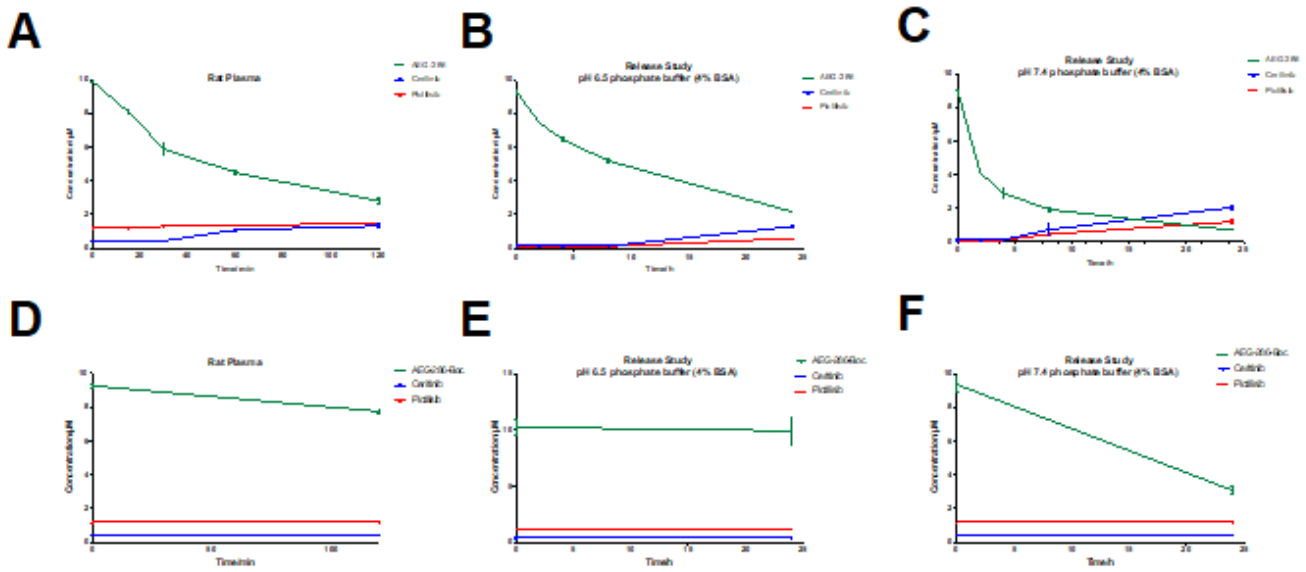
Compound 19:



Compound 20:

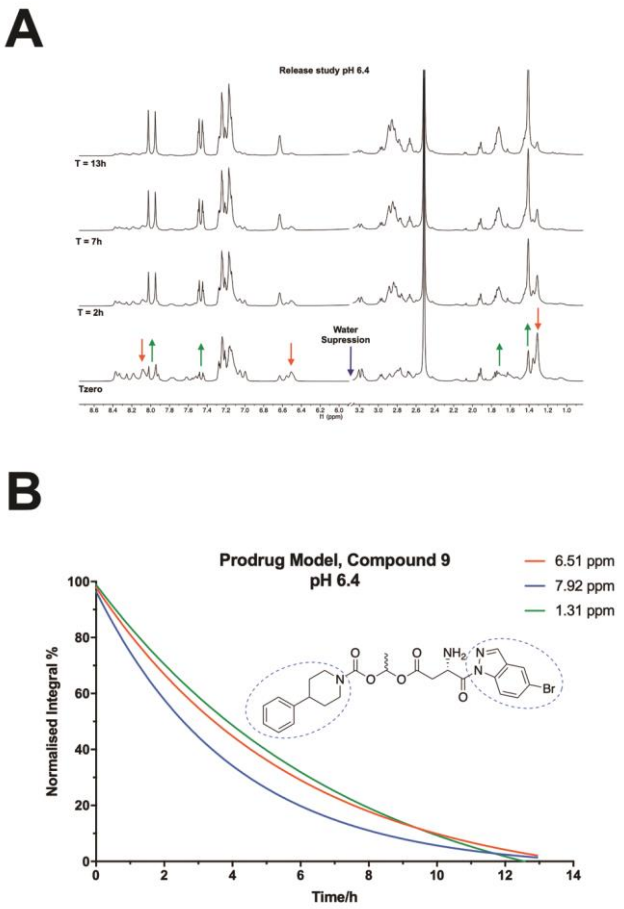


Compound 21:



NMR-Based release study on the codrug model system (5)

A kinetic study was performed using a time-course NMR method. Additionally, since pH is dependent on hydrogen ion concentration, a water-based buffer was used adding deuterated DMSO onto which the spectrometer could lock. Water suppression was applied to the experiment, which causes “baseline wobble” and difficulty phasing the spectrum around 3.5 ppm. This was not a major issue since the peaks of interest were in the aromatic and aliphatic regions of the spectrum.



(A) Spectra at 0 h, 2 h, 7 h, and 13 h showing selected slices of a time course experiment (512 slices) of the α-amino codrug model system performed at 21 °C in pH 6.4 phosphate buffer and d6- DMSO (1:1). Peaks showing the appearance of drug mimetics are shown by green arrows, and peaks showing the disappearance of codrug are shown by green arrows. Note that water suppression was applied, causing "baseline wobble" at approximately 3.5 ppm. Graphs showing the integration of NMR spectra peaks over time. (B) Degradation of the codrug looking at peaks from each component of the codrug. The peak at 1.31 ppm shows disappearance of a peak from the pictilisib mimetic region, and the peak at 7.92 ppm shows disappearance of a peak in the ceritinib mimetic region of the codrug. Additionally, the peak at 6.51 ppm shows disappearance of a peak from the linker region of the codrug.

The kinetics of the reaction mechanism proposed in Figure 7 can be examined by considering the consecutive irreversible reactions:

$$Prodrug \overset{k_1}{\rightarrow} Prodrug\ Carboxylic\ Acid \overset{k_2}{\rightarrow} Ceritinib\ Mimetic$$

Where k_1 is the rate constant for the release of the pictilisib mimetic and k_2 is the rate constant for the release of the ceritinib mimetic. However, since k_2 is much greater than k_1, k_1 is the rate determining step. The reaction is therefore pseudo first order since the overall rate is dependent only on the first, rate determining step - the hydrolysis of the ester. Plotting Ln([codrug]0/[codrug]) *vs* time gives a linear graph with

an r^2 value of >0.99, where the rate constant k_1 can be obtained from the slope. The time course experiment was run at pH 6.4, 6.8 and 7.4 and the corresponding half-lives and rate constants calculated