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# **1. Chemistry Methods**

## 1.1 General

Unless otherwise specified, all solvents and reagents were obtained from commercial suppliers and used without further drying or purification. SiliaMetS®Thiol SiliCYCLE thiol metal scavenger – (R51030B, particle size: 40-63 µm) was obtained from SiliCYCLE. Normal-phase flash chromatography was run on silica gel using prepacked columns: pre-packed RediSep Rf cartridges, or SNAP cartridges. 1H NMR and 13C spectra were recorded on a Bruker Ultrashield™400 (400 MHz), Bruker Ultrashield™600 (600 MHz) and Bruker AscendTM400 (400 MHz) spectrometers, both with and without tetramethylsilane as an internal standard. High-resolution mass spectra (HRMS) measurements: LC/ESI-MS and LC-UV data were recorded using a Thermo Scientific Q Exactive Plus mass spectrometer equipped with an electrospray ionization source and coupled to a Thermo Ultimate 3000 liquid chromatograph equipped with a diode array detector. The instrument was lock mass calibrated with the protonated di-octylphtalate ion (m/z 391.28429). The accurate mass was obtained by averaging 6 scans at a mass resolution of ca 70000 (FWHM definition). The mass accuracy of the system has been found to be better than 2 ppm. The chromatography was performed at 150 µL/min flow rate (1 mm C18-column) with a polar gradient from 5% to 100% acetonitrile in 5 min. 0.05 % and 0.04% formic acid was used as the modifier additive in the mobile phases water and acetonitrile, respectively. Liquid chromatography mass spectra (LC−MS) were determined by using electrospray ionization in positive and negative ion modus using a Waters Acquity UPLC instrument with Waters SQ detector. Purity was determined by integration of the area under the UV absorption curve at λ 210 to 450 nm and by 1H NMR, all final compounds reported were ≥95%. Rt refers to retention time. The following conditions (UPLC-MS-1 to UPLC-MS-5) were used for analytical UPLC: UPLC-MS-1: Acquity HSS T3; particle size: 1.8 µm; column size: 2.1 x 50 mm; eluent A: H2O + 0.05% HCOOH + 3.75 mM ammonium acetate; eluent B: CH3CN + 0.04% HCOOH; gradient: 5 to 98% B in 1.40 min then 98% B for 0.40 min; flow rate: 1 mL/min; column temperature: 60°C; UPLC-MS-2: Acquity HSS T3; particle size: 1.8 µm; column size: 2.1 x 100 mm; eluent A: H2O + 0.05% HCOOH + 3.75 mM ammonium acetate; eluent B: CH3CN + 0.04% HCOOH; gradient: 5 to 98% B in 9.4 min then 98% B for 0.40 min; flow rate: 1.0 mL/min; column temperature: 60°C; UPLC-MS-3: Acquity BEH C18; particle size: 1.7 µm; column size: 2.1 x 50 mm; eluent A: H2O + 4.76% isopropanol + 0.05% HCOOH + 3.75 mM ammonium acetate; eluent B: isopropanol + 0.05% HCOOH; gradient: 1 to 98% B in 1.7 min then 98% B for 0.1 min; flow rate: 0.6 mL/min; column temperature: 80°C; UPLC-MS-4: Acquity BEH C18; particle size: 1.7 µm; column size: 2.1 x 100 mm; eluent A: H2O + 4.76% isopropanol + 0.05% HCOOH + 3.75 mM ammonium acetate; eluent B: isopropanol + 0.05% HCOOH; gradient: 1 to 60% B in 8.4 min then 60 to 98% B in 1 min; flow rate: 0.4 mL/min; column temperature: 80°C; UPLC-MS-5: Acquity BEH C18; particle size: 1.7 µm; column size: 2.1 x 50 mm; eluent A: H2O + 0.05% HCOOH + 3.75 mM ammonium acetate; eluent B: isopropanol + 0.05% HCOOH; gradient: 5 to 98% B in 1.7 min then 98% B for 0.1 min; flow rate: 0.6 mL/min; column temperature: 80°C.

## 1.2 Discovery of covalent KRAS inhibitors by RapidFire MS screening

### 1.2.1 Primary screening

Test compound solutions (100 µM) were prepared in 384-well plates and incubated with 1 µM KRASG12C cysteine light-GDP in 20 mM Tris pH 7.5, 150 mM NaCl, 100 µM MgCl2, 1% DMSO at room temperature. Reactions were stopped after two or 24 hours by addition of formic acid to 1%. MS measurements were carried out using an Agilent 6530 quadrupole time-of-flight (QToF) MS system coupled to an Agilent RapidFire autosampler RF360 device, resulting in % modification values for each well. Hit compounds with a significantly stronger protein modification at two hours incubation than at 24 hours were regarded as artifacts and removed from the hit set. Similarly, compounds leading to a strong loss of total protein signals (<10% total protein signal) were also removed.

### 1.2.2 Secondary confirmation screening

Serial four-point dilutions of the test compounds (50 µM, ½ dilutions) were prepared in 384-well plates and incubated with 1 µM KRAS G12C cysteine light-GDP or wild-type (WT) KRAS-GDP in 20 mM Tris pH 7.5, 150 mM NaCl, 100 µM MgCl2, 1% DMSO at room temperature. Reactions were stopped after two or 24 hours incubation time by addition of formic acid to 1%. MS measurements were carried out using an Agilent 6530 quadrupole time-of-flight (QToF) MS system coupled to an Agilent RapidFire autosampler RF360 device, resulting in % modification values for each well. Hit compounds also modifying KRAS WT protein were not further pursued. Confirmed hits with dose-responsive single KRASG12C cysteine light-GDP target modification were prioritized for further kinetic profiling if they showed >50% modification after two hours at 50 µM compound concentration.

## 1.3 Molecular modelling

Ligands were manually designed keeping the methyl indazole of **[1]** and the phenyl acrylamide of **[2]** (**Supplementary Figure S1**). Various linkers evaluated including 5 or 6 membered aromatic as well as 5-6 fused aromatic moieties. The designed compounds were covalently docked (Schrodinger Glide covalent docking suite) (1) into the SWII binding site using the protein conformation of compound [1] with KRASG12C (PDB code:5v6s). Evaluation of ligand conformations was carried out using the in house developed ReScoSS tool (2). The minimum ligand conformation in solution (water) was rigidly aligned to the bound ligand conformation and the similarity between the two assessed by visual inspection.

## 1.4 Synthesis of intermediates

### 5-Methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-tosyl-1*H*-indazole



*4-Bromo-5-methyl-1-tosyl-1*H*-indazole*

To an ice-cooled solution of 4-bromo-5-methyl-1*H*-indazole (CAS [926922-40-9], 5.00 g, 23.7 mmol) in THF (50 mL) under inert atmosphere was added NaH (1.90 g, 47.4 mmol), followed by toluene-4-sulfonyl chloride (4.97 g, 26.1 mmol) and the reaction mixture was stirred at RT for 1 h. The reaction mixture was quenched carefully at 0°C with water and extracted with CH2Cl2. The organic phase was dried (Na2SO4), filtered and concentrated under reduced pressure. The crude residue was triturated with Et2O and the white precipitate was filtered, washed with cold Et2O and dried under high vacuum to give the title compound as a white solid (7.94 g, 91% yield). UPLC-MS-1: Rt = 1.34 min; MS m/z [M+H]+: 365 / 367; purity: 100%; 1H NMR (600 MHz, DMSO-d6) δ 8.48 (s, 1H), 8.05 (d, *J* = 8.5 Hz, 1H), 7.81 (d, *J* = 8.2 Hz, 2H), 7.64 (d, *J* = 8.5 Hz, 1H), 7.40 (d, *J* = 8.2 Hz, 2H), 2.45 (s, 3H), 2.33 (s, 3H).

*5-Methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-tosyl-1*H*-indazole*

To a solution of 4-bromo-5-methyl-1-tosyl-1*H*-indazole (7.94 g, 21.7 mmol) in 1,4-dioxane (80 mL) under inert atmosphere were added bis(pinacolato)diboron (11.0 g, 43.5 mmol), PdCl2(dppf).CH2Cl2 adduct (0.89 g, 1.09 mmol) and KOAc (6.40 g, 65.2 mmol). The reaction mixture was degassed then stirred at 100°C for 16 h. The reaction mixture was quenched with water and extracted with CH2Cl2. The combined organic extracts were washed with brine, dried (Na2SO4), filtered and concentrated under reduced pressure. The crude residue was triturated with Et2O, the white precipitate was filtered, washed with cold Et2O and dried under high vacuum. The crude product was purified by normal phase chromatography (eluent: EtOAc in cyclohexane from 0 to 40%) to give the title compound (5.60 g, 61% yield). UPLC-MS-1: Rt = 1.48 min; MS m/z [M+H]+: 413; purity: 95%; 1H NMR (600 MHz, DMSO-d6) δ 8.54 (s, 1H), 8.11 (d, *J* = 8.7 Hz, 1H), 7.76 (d, *J* = 8.2 Hz, 2H), 7.50 (d, *J* = 8.7 Hz, 1H), 7.37 (d, *J* = 8.2 Hz, 2H), 2.57 (s, 3H), 2.31 (s, 3H), 1.17 (s, 12H).

### 5-Chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indazole



*1-Chloro-2,5-dimethyl-4-nitrobenzene*

To an ice-cooled solution of 2-chloro-1,4-dimethylbenzene (3.40 kg, 24.2 mol) in acetic acid (20.0 L) was added H2SO4 (4.74 kg, 48.4.mol, 2.58 L) followed by a dropwise addition (dropping funnel) of a cold solution of HNO3 (3.41 kg, 36.3 mol, 2.44 L, 67.0% purity) in H2SO4 (19.0 kg, 193.mol, 10.3 L). The reaction mixture was then allowed to stir at 0 - 5°C for 0.5 h. The reaction mixture was poured slowly into crushed ice (35.0 L) and the yellow solid precipitated out. The suspension was filtered and the cake was washed with water (5.00 L x 5) to give a yellow solid which was suspended in methyl tert-butyl ether (2.00 L) for 1 h, filtered, and dried to give the title compound as a yellow solid (2.00 kg, 44.6% yield). 1H NMR (400 MHz, CDCl3) δ 7.90 (s, 1H), 7.34 (s, 1H), 2.57 (s, 3H), 2.42 (s, 3H).

*3-Bromo-2-chloro-1,4-dimethyl-5-nitrobenzene*

 To a cooled solution of 1-chloro-2,5-dimethyl-4-nitrobenzene (2.00 kg, 10.8 mol) in TFA (10.5 L) was slowly added concentrated H2SO4 (4.23 kg, 43.1 mol, 2.30 L) and the reaction mixture was stirred at 20°C. *N*-Bromosuccinimide (1.92 kg, 10.8 mol) was added in small portions and the reaction mixture was heated at 55°C for 2 h. The reaction mixture was cooled to 25°C, then poured into crushed ice solution to obtain a pale white precipitate which was filtered, washed with cold water and dried under vacuum to give the title compound as a yellow solid (2.75 kg, 97% yield) which was used without further purification in the next step. 1H NMR (400 MHz, CDCl3) δ 7.65 (s, 1H), 2.60 (s, 3H), 2.49 (s, 3H).

*3-Bromo-4-chloro-2,5-dimethylaniline*

To a ice-cooled solution of 3-bromo-2-chloro-1,4-dimethyl-5-nitrobenzene (2.75 kg, 10.4 mol) in THF (27.5 L) was added HCl (4M, 15.6 L) then zinc (2.72 kg, 41.6 mol) in small portions. The reaction mixture was allowed to stir at 25°C for 2 h. The reaction mixture was basified by addition of a saturated aqueous NaHCO3 solution (until pH = 8). The mixture was diluted with EtOAc (2.50 L) and stirred vigorously for 10 min and then filtered through a pad of celite. The organic layer was separated and the aqueous layer was re-extracted with EtOAc (3.00 L x 4). The combined organic layers were washed with brine (10.0 L), dried (Na2SO4), filtered and concentrated under vacuum to give the title compound as a yellow solid (2.20 kg, 91%) which was used without further purification in the next step. 1H NMR (400 MHz, DMSO-*d*6) δ 6.59 (s, 1H), 5.23 (s, 2H), 2.22 (s, 3H), 2.18 (s, 3H).

*3-Bromo-4-chloro-2,5-dimethylbenzenediazonium tetrafluoroborate*

 BF3.Et2O (2.00 kg, 14.1 mol, 1.74 L) was dissolved in CH2Cl2 (20.0 L) and cooled to -5 to -10°C under nitrogen atmosphere. A solution of 3-bromo-4-chloro-2,5-dimethylaniline (2.20 kg, 9.38 mol) in CH2Cl2 (5.00 L) was added to above mixture and stirred for 0.5 h. *Tert*-butyl nitrite (1.16 kg, 11.3 mol, 1.34 L) was added dropwise and the reaction mixture was stirred at the same temperature for 1.5 h. TLC (petroleum ether : EtOAc = 5:1) showed that starting material(Rf = 0.45) was consumed completely. Methyl *tert-*butyl ether (3.00 L) was added to the reaction mixture to give a yellow precipitate, which was filtered and washed with cold methyl *tert*-butyl ether (1.50 L x 2) to give the title compound as a yellow solid (3.13 kg, quantitative yield) which was used without further purification in the next step.

*4-Bromo-5-chloro-6-methyl-1*H*-indazole*

To 18-Crown-6 ether (744 g, 2.82 mol) in chloroform (20.0 L) was added KOAc (1.29 kg, 13.2 mol) and the reaction mixture was cooled to 20°C. Then 3-bromo-4-chloro-2,5-dimethylbenzenediazonium tetrafluoroborate(3.13 kg, 9.39 mol) was added slowly. The reaction mixture was then allowed to stir at 25°C for 5 h. After completion of the reaction, the reaction mixture was poured into ice cold water (10.0 L), and the aqueous layer was extracted with CH2Cl2 (5.00 L x 3). The combined organic layers were washed with a saturated aqueous NaHCO3 solution (5.00 L), brine (5.00 L), dried (Na2SO4), filtered and concentrated under vacuum to give the title compound as a yellow solid (1.28 kg, 55% yield). 1H NMR (600 MHz, CDCl3) δ 10.42 (br s, 1H), 8.04 (s, 1H), 7.35 (s, 1H), 2.58 (s, 3H). UPLC-MS-1: Rt = 1.02 min; MS m/z [M+H]+: 243/ 245/ 247.

*4-Bromo-5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole*

To a solution of p-toluene sulfonic acid (89.8 g, 521 mmol) and 4-bromo-5-chloro-6-methyl-1*H*-indazole (1.28 kg, 5.21 mol) in CH2Cl2 (12.0 L) was added 3,4-dihydro-2*H*-pyran (658 g, 7.82 mol, 715 mL) dropwise at 25°C. The mixture was stirred at 25°C for 1 h. After completion the reaction, the reaction mixture was diluted with water (5.00 L) and the organic layer was separated. The aqueous layer was re-extracted with CH2Cl2 (2.00 L). The combined organic layers were washed with a saturated aqueous NaHCO3 solution (1.50 L), brine (1.50 L), dried (Na2SO4), filtered and concentrated under vacuum. The crude residue was purified by normal phase chromatography (eluent: Petroleum ether/ EtOAc from 100/1 to 10/1) to give the title compound as a yellow solid (750 g, 43% yield). 1H NMR (600 MHz, DMSO-*d*6) δ 8.04 (s, 1H), 7.81 (s, 1H), 5.88 - 5.79 (m, 1H), 3.92 - 3.83 (m, 1H), 3.80 - 3.68 (m, 1H), 2.53 (s, 3H), 2.40 - 2.32 (m, 1H), 2.06 - 1.99 (m, 1H), 1.99 - 1.93 (m, 1H), 1.77 - 1.69 (m, 1H), 1.60 - 1.56 (m, 2H). UPLC-MS-5: Rt = 1.32 min; MS m/z [M+H]+: 329.0/ 331.0/ 333.0

*5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indazole*

A suspension of 4-bromo-5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole(450 g, 1.37 mol), KOAc (401 g, 4.10 mol) and B2Pin2 (520 g, 2.05 mol) in 1,4-dioxane (3.60 L) was degassed with nitrogen for 0.5 h. Pd(dppf)Cl2.CH2Cl2 (55.7 g, 68.3 mmol) was added and the reaction mixture was stirred at 90°C for 6 h. The reaction mixture was filtered through diatomite and the filter cake was washed with EtOAc (1.50 L x 3). The mixture was concentrated under vacuum to give a black oil which was purified by normal phase chromatography (eluent: Petroleum ether/ EtOAc from 100/1 to 10/1) to give the desired product as brown oil. The residue was suspended in petroleum ether (250 mL) for 1 h to obtain a white precipitate. The suspension was filtered, dried under vacuum to give the title compound as a white solid (315 g, 49% yield). 1H NMR (400 MHz, CDCl3) δ 8.17 (s, 1H), 7.52 (s, 1H), 5.69 - 5.66 (m, 1H), 3.99 - 3.96 (m, 1H), 3.75 - 3.70 (m, 1H), 2.60 - 2.45 (m, 1H), 2.51 (s, 3H), 2.21 - 2.10 (m, 1H), 2.09 - 1.99 (m, 1H), 1.84 - 1.61 (m, 3H), 1.44 (s, 12H); UPLC-MS-5: Rt = 1.29 min; MS m/z [M+H]+: 377.1/ 379.

### *Tert*-butyl 6-(tosyloxy)-2-azaspiro[3.3]heptane-2-carboxylate



To a solution of *tert*-butyl 6-hydroxy-2-azaspiro[3.3]heptane-2-carboxylate (CAS [1147557-97-8], 2.92 kg, 12.94 mmol) in CH2Cl2 (16.5 L) were added *N*,*N*-dimethylpyridin-4-amine (316.1 g, 2.59 mol) and tosyl chloride (2.96 kg, 15.52 mol) at 20°C - 25ºC. To the reaction mixture was added dropwise Et3N (2.62 kg, 25.88 mol) at 10ºC - 20°C. The reaction mixture was stirred 0.5 h at 5ºC - 15°C and then was stirred 1.5 h at 18ºC - 28°C. After completion of the reaction, the reaction mixture was concentrated under vacuum. To the residue was added NaCl (5% in water, 23 L) followed by extraction with EtOAc (23 L). The combined aqueous layers were extracted with EtOAc (10 L x 2). The combined organic layers were washed with NaHCO3 (3% in water, 10 L x 2)) and concentrated under vacuum to give the title compound (4.46 kg, 92% yield). 1H NMR (400 MHz, DMSO-*d*6) δ 7.81 - 7.70 (m, 2H), 7.53 - 7.36 (m, 2H), 4.79 - 4.62 (m, 1H), 3.84 - 3.68 (m, 4H), 2.46 - 2.38 (m, 5H), 2.26 - 2.16 (m, 2H), 1.33 (s, 9H). UPLC-MS-1: Rt = 1.18 min; MS m/z [M+H]+: 368.2.

### *Tert*-butyl 6-(3-bromo-4-iodo-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate



*3,5-Dibromo-1*H*-pyrazole*

To a solution of 3,4,5-tribromo-1*H*-pyrazole (55.0 g, 182.2 mmol) in anhydrous THF (550 mL) was added at -78°C *n*-BuLi (145.8 mL, 364.5 mmol) dropwise over 20 min maintaining the internal temperature at -78°C /-60°C. The reaction mixture was stirred at this temperature for 45 min. Then the reaction mixture was carefully quenched with MeOH (109 mL) at -78°C and stirred at this temperature for 30 min. The mixture was allowed to reach to 0°C and stirred for 1 h. Then, the mixture was diluted with EtOAc (750 mL) and HCl (0.5 N, 300 mL) was added. The layers were separated and the organic layer was washed with brine (350 mL), dried (Na2SO4), filtered, and concentrated under vacuum. The crude residue was dissolved in CH2Cl2 (100 mL), cooled to -50°C and petroleum ether (400 mL) was added. The precipitated solid was filtered, washed with *n*-hexane (250 mL x2) and dried under vacuum to give the title compound (26.6 g, 66% yield). 1H NMR (400 MHz, DMSO-*d*6) δ 13.5 (br s, 1H), 6.58 (s, 1H).

Tert*-butyl 6-(3,5-dibromo-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

To a solution of *tert*-butyl 6-(tosyloxy)-2-azaspiro[3.3]heptane-2-carboxylate (900 g, 2.40 mol) in DMF (10.8 L) was added Cs2CO3 (1988 g, 6.10 mol) and 3,5-dibromo-1*H*-pyrazole (606 g, 2.68 mol) at 15°C. The reaction mixture was stirred at 90°C for 16 h. The reaction mixture was poured into ice-water/brine (80 L) and extracted with EtOAc (20 L). The aqueous layer was re-extracted with EtOAc (10 L x 2). The combined organic layers were washed with brine (10 L), dried (Na2SO4), filtered and concentrated under vacuum. The residue was triturated with 1,4-dioxane (1.8 L) and dissolved at 60°C. To the light yellow solution was slowly added water (2.2 L) and recrystallization started after addition of 900 mL of water. The resulting suspension was cooled down to 0°C, filtered, and washed with cold water. The filtered cake was triturated with *n*-heptane, filtered, then dried under vacuum at 40°C to give the title compound (920 g, 88% yield). 1H NMR (400 MHz, DMSO-*d*6) δ 6.66 (s, 1H), 4.86 - 4.82 (m, 1H), 3.96 - 3.85 (m, 4H), 2.69 - 2.62 (m, 4H), 1.37 (s, 9H); UPLC-MS-3: Rt = 1.19 min; MS m/z [M+H]+: 420.0/ 422.0/ 424.0.

Tert*-butyl 6-(3-bromo-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

To a solution of *tert*-butyl 6-(3,5-dibromo-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (960 g, 2.3 mol) in THF (9.6 L) was added *n*-BuLi (1.2 L, 2.50 mol) dropwise at -80°C under an inert atmosphere. The reaction mixture was stirred 10 min at -80°C. To the reaction mixture was then added dropwise iodomethane (1633 g, 11.5 mol) at -80°C. After stirring for 5 min at -80°C, the reaction mixture was allowed to warm up to 18°C. The reaction mixture was poured into a saturated aqueous NH4Cl solution (4 L) and extracted with CH2Cl2 (10 L). The separated aqueous layer was re-extracted with CH2Cl2 (5 L) and the combined organic layers were concentrated under vacuum. The crude product was dissolved in 1,4-dioxane (4.8 L) at 60°C, then water (8.00 L) was added dropwise slowly. The resulting suspension was cooled to 17°C and stirred for 30 min. The solid was filtered, washed with water, and dried under vacuum to give the title compound (702 g, 86 % yield). 1H NMR (400 MHz, DMSO-*d*6) δ 6.14 (s, 1H), 4.74 - 4.66 (m, 1H), 3.95 - 3.84 (m, 4H), 2.61 - 2.58 (m, 4H), 2.20 (s, 3H), 1.37 (s, 9H); UPLC-MS-1: Rt = 1.18 min; MS m/z [M+H]+: 356.1/ 358.1.

Tert*-butyl 6-(3-bromo-4-iodo-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

 To a solution of *tert*-butyl 6-(3-bromo-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (350 g, 0.980 mol) in acetonitrile (3.5 L) was added *N*-iodosuccinimide (332 g, 1.47 mol) at 15°C. The reaction mixture was stirred at 40°C for 6 h. After completion of the reaction, the reaction mixture was diluted with EtOAc (3 L) and washed with water (5 L x 2). The organic layer was washed with Na2SO3 (10% in water, 2 L), with brine (2 L), was dried (Na2SO4), filtered, and concentrated under vacuum to give the title compound (350 g, 73% yield). 1H NMR (400 MHz, DMSO-*d*6) δ 4.81 - 4.77 (m, 1H), 3.94 - 3.83 (m, 4H), 2.61 - 5.59 (m, 4H), 2.26 (s, 3H), 1.37 (s, 9H); UPLC-MS-1: Rt = 1.31 min; MS m/z [M+H]+: 482.0/ 484.0.

## 1.5 Synthesis of compounds

### **[2]:** *N*-(4-((2-Bromo-6-((2-hydroxyethyl)amino)pyridin-4-yl)thio)phenyl)acrylamide



*2,6-Dibromo-4-((4-nitrophenyl)thio)pyridine*

To a solution of 2,6-dibromo-4-nitropyridine (CAS [175422-04-5], 14.0 g, 49.7 mmol) in DMF (140 mL) and cesium carbonate (24.3 g, 74.5 mmol) was added portion-wise 4-nitrobenzenethiol (CAS [1849-36-1], 8.48 g, 54.6 mmol, content 80%) and the reaction mixture was stirred at RT for 2 h. EtOAc and a saturated aqueous solution of sodium bicarbonate were added, the layers were separated and the aqueous layer was back-extracted with EtOAc. The combined organic extracts were washed with brine. A precipitate formed, was filtered, washed with EtOAc and dried under vacuum at 50°C. The filtrate was dried (Na2SO4), filtered and concentrated. The resulting solid residue was taken-up in EtOAc, filtered and dried under vacuum at 50°C. The solids were combined to give the title product as a light yellow solid (16.0 g, 79% yield). UPLC-MS-1: Rt = 1.29 min; MS m/z [M+H]+: 389.0/ 391.0/ 393.0; purity: 94.9%; 1H NMR (400 MHz, DMSO-d6) δ 8.30 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 8.8 Hz, 2H), 7.52 (s, 2H).

*4-((2,6-Dibromopyridin-4-yl)thio)aniline*

To a solution of 2,6-dibromo-4-((4-nitrophenyl)thio)pyridine (3.08 g, 7.50 mmol) in THF (75 mL) was added Sn (2.67 g, 22.5 mmol) and aqueous HCl (4M, 15.0 mL, 60.0 mmol) and the mixture stirred at RT for 20 min. Aqueous NaOH (1M, 65 mL) was added and the mixture was filtered over celite. EtOAc was added to the filtrate, the layers were separated, the aqueous layer was back-extracted with EtOAc and the combined organic extracts were washed with brine, dried (Na2SO4), filtered and concentrated under reduced pressure. The crude residue was purified by normal phase column chromatography (eluent: EtOAc/ MeOH 20/1) to give the title compound (2.37 g, 82% yield). UPLC-MS-1: Rt = 1.23 min; MS m/z [M+H]+: 359.0/ 361.0 /362.9; purity: 94.4%; 1H NMR (400 MHz, DMSO-*d*6) δ 7.22 (d, *J* = 8.5 Hz, 2H), 7.12 (s, 2H), 6.68 (d, *J* = 8.5 Hz, 2H), 5.79 (s, 2H).

N*-(4-((2,6-Dibromopyridin-4-yl)thio)phenyl)acrylamide*

To a solution of 4-((2,6-dibromopyridin-4-yl)thio)aniline (2.35 g, 6.13 mmol) in CH2Cl2 (61.3 mL) was added DIPEA (3.21 mL, 18.4 mmol) under nitrogen atmosphere. The mixture was cooled with an ice-bath and acryloyl chloride (6.23 mL, 7.36 mmol) was added. The reaction mixture was stirred for 15 min. An aqueous solution of NaHCO3 (1M) was added, the layers were separated and the aqueous layer was back-extracted with EtOAc. The combined organic extracts were washed with brine, dried (Na2SO4), filtered, concentrated under reduced pressure and the crude residue was purified by normal phase column chromatography (eluent: EtOAc in heptane from 0 to 100 %) and then was crystallized from CH2Cl2/ heptane (1/3) to give the title compound as a light yellow solid (1.77 g, 65% yield). UPLC-MS-1: Rt = 1.18 min; MS m/z [M+H]+: 413.0/ 414.0 /415.0; purity: 93.5%; 1H NMR (400 MHz, DMSO-*d*6) δ 10.5 (s, 1H), 7.90 (d, *J* = 8.5 Hz, 2H), 7.63 (d, *J* = 8.5 Hz, 2H), 7.23 (s, 2H), 6.48 (dd, *J* = 16.9 Hz, 10.0 Hz, 1H), 6.40 (dd, *J* = 16.9 Hz, 2.00 Hz, 1H), 5.80 (dd, *J* = 10.0 Hz, 2.00 Hz, 1H).

N*-(4-((2-Bromo-6-((2-hydroxyethyl)amino)pyridin-4-yl)thio)phenyl)acrylamide*

To an argon-purged solution of 2-aminoethanol (15.5 mg, 0.25 mmol), *N*-(4-((2,6-dibromopyridin-4-yl)thio)phenyl)acrylamide (100 mg, 0.24 mmol) and tBuXPhos-Pd-G3 (19.2 mg, 0.024 mmol) in THF (1.61 mL) was added LiHMDS (1M in toluene, 1.21 mL, 1.21 mmol) dropwise at RT. The resulting solution was heated at 40°C and stirred for 15 h. The reaction mixture was quenched with a saturated aqueous NH4Cl solution and the aqueous layer was separated and extracted with CH2Cl2. The combined organic extracts were dried (MgSO4), filtered, concentrated under reduced pressure and the crude residue was purified by reverse phase preparative HPLC (eluent: 5 to 100% CH3CN in water containing 0.1% TFA) to afford **[2]** (48 mg, 37% yield) as a white solid after lyophilisation. UPLC-MS-1: Rt = 0.97 min; MS m/z [M+H]+: 394.0/ 396.0; purity: 99%; HRMS [M+H]+ calcd for C16H16BrN3O2S [M+H]+ 394.02194, found 394.02222; 1H NMR (400 MHz, DMSO-*d*6) δ 10.4 (s, 1H), 7.78 (d, *J* = 8.5 Hz, 2H), 7.49 (d, *J* = 8.5 Hz, 2H), 6.42 (dd, *J* = 16.9, 10.0 Hz, 1H), 6.30 - 6.18 (m, 2H), 5.99 (s, 1H), 5.77 (dd, *J* = 10.0, 2.00 Hz, 1H), 3.40 (m, 2H), 3.15 (m, 2H).

### **[3]**: *N*-(4-(3,5-dimethyl-4-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)phenyl)acrylamide



*4-Bromo-3,5-dimethyl-1-(4-nitrophenyl)-1*H*-pyrazole*

To a solution of 1-fluoro-4-nitrobenzene (CAS [350-46-9],525 mg, 3.72 mmol) and 4-bromo-3,5-dimethyl-1*H*-pyrazole (CAS [3398-16-1],651 mg, 3.72 mmol) in DMF (5.00 mL) under nitrogen atmosphere at RT was added sodium hydride (60% in mineral oil, 149 mg, 3.72 mmol). The reaction mixture was stirred at RT for 4 h and quenched by addition of water. EtOAc was added, the layers were separated and the aqueous layer was back-extracted with EtOAc. The combined organic extracts were washed with brine, dried (MgSO4), filtered and concentrated under reduced pressure. The crude residue was triturated in Et2O, filtered and washed with Et2O to give the title compound as a white powder (1.10 g, 98% yield). UPLC-MS-1: Rt = 1.17 min; MS m/z [M+H]+: 296.1/ 298.1; purity: 98%; 1H NMR (400 MHz, DMSO-*d*6) δ 8.33 (d, *J* = 9.1 Hz, 2H), 7.81 (d, *J* = 9.1 Hz, 2H), 2.38 (s, 3H), 2.20 (s, 3H).

*4-(4-Bromo-3,5-dimethyl-1*H*-pyrazol-1-yl)aniline*

To a solution of 4-bromo-3,5-dimethyl-1-(4-nitrophenyl)-1*H*-pyrazole (530 mg, 1.79 mmol) in EtOH (12.0 mL) were added iron (400 mg, 7.16 mmol) and a solution of ammonium chloride (8M in water, 6.71 mL, 53.7 mmol). The reaction mixture was stirred at 65°C for 1 h. Water and EtOAc were added, the layers were separated and the aqueous layer was back-extracted with EtOAc. The combined organic extracts were washed with brine, dried (MgSO4), filtered and concentrated under reduced pressure to give the title compound as a beige solid (472 mg, 98% yield). UPLC-MS-1: Rt = 0.94 min; MS m/z [M+H]+: 266.2/ 268.2; purity: 98%; 1H NMR (400 MHz, DMSO-*d*6) δ 7.06 (d, *J* = 8.65 Hz, 2H), 6.61 (d, *J* = 8.65 Hz, 2H), 5.38 (s, 2H), 2.15 (s, 3H), 2.14 (s, 3H).

N-*(4-(4-Bromo-3,5-dimethyl-1*H*-pyrazol-1-yl)phenyl)acrylamide*

To a solution of 4-(4-bromo-3,5-dimethyl-1*H*-pyrazol-1-yl)aniline (470 mg, 1.77 mmol) in CH2Cl2 (9.00 mL) were added DIPEA (0.92 mL, 5.30 mmol) and acryloyl chloride (0.16 mL, 1.94 mmol) at 0°C under a nitrogen atmosphere. The reaction mixture was stirred at 0°C for 30 min. MeOH was added and mixture was evaporated to dryness. Then EtOAc and a saturated aqueous solution of NaHCO3 were added. The layers were separated and the aqueous layer was back-extracted with EtOAc. The combined organic extracts were washed with brine, dried (MgSO4), filtered and concentrated under reduced pressure. The crude residue was purified by normal phase column chromatography (eluent: EtOAc in cyclohexane from 0 to 70%) to give the title compound as a white powder (492 mg, 86% yield). UPLC-MS-1: Rt = 0.99 min; MS m/z [M+H]+: 320.1/ 322.1; purity: 99%; 1H NMR (400 MHz, DMSO-*d*6) δ 10.3 (s, 1H), 7.80 (d, *J* = 8.85 Hz, 2H), 7.45 (d, *J* = 8.85 Hz, 2H), 6.45 (dd, *J* = 16.9, 10.0 Hz, 1H), 6.29 (dd, *J* = 16.9, 2.0 Hz, 1H), 5.79 (dd, *J* = 10.0, 2.0 Hz, 1H), 2.26 (s, 3H), 2.18 (s, 3H).

N*-(4-(3,5-Dimethyl-4-(5-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)phenyl)acrylamide*

To a suspension of *N*-(4-(4-bromo-3,5-dimethyl-1*H*-pyrazol-1-yl)phenyl)acrylamide (130 mg, 0.41 mmol), 5-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indazole (CAS [1698028-42-0], 278 mg, 0.81 mmol), K3PO4 (259 mg, 1.22 mmol) in 1,4-dioxane (4.00 mL) and water (1.00 mL) were added under nitrogen atmosphere RuPhos (18.9 mg, 0.041 mmol) and RuPhos-Pd-G3 (34.0 mg, 0.041 mmol). The reaction mixture was stirred for 2 h at 80°C. A saturated aqueous solution of NaHCO3 and EtOAc were added, the layers were separated and the aqueous layer was back-extracted with EtOAc. The combined organic extracts were washed with brine, dried (MgSO4), filtered and concentrated under reduced pressure. The crude residue was purified by normal phase column chromatography (eluent: EtOAc in cyclohexane from 0 to 60%) to give the title compound as a beige solid (169 mg, 65.8% yield). UPLC-MS-1: Rt = 1.13 min; MS m/z [M+H]+: 456.4; purity: 72%.

N*-(4-(3,5-Dimethyl-4-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)phenyl)acrylamide*

To a solution of *N*-(4-(3,5-dimethyl-4-(5-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)phenyl)acrylamide (168 mg, 0.27 mmol, purity 72%) in CH2Cl2 (2.50 mL) at 0°C, was added TFA (0.41 mL, 5.31 mmol) and the reaction mixture was stirred for 1 h at RT. The reaction mixture was evaporated to dryness, CH2Cl2 and a saturated aqueous solution of NaHCO3 were added. The layers were separated and the aqueous layer was back extracted with CH2Cl2. The combined organic extracts were dried (MgSO4), filtered and concentrated under reduced pressure. The crude residue was purified by preparative SFC (column: Viridis 2EP 130A 5 m, column temperature 50°C, back pressure 120 bars, eluent 14 to 22% MeOH in CO2) to give the title compound as a racemic mixture. The atropisomers were separated by preparative chiral SFC (column: Chiralpak IG 5 µm; 250 x 30 mm; flow rate: 80 mL/min; column temperature: 40°C; back pressure: 120 bars; mobile phase: CO2/isopropanol 60/40) to give **[3]** as the second eluting peak (white powder, 21 mg, 21% yield): UPLC-MS-1: Rt = 0.88 min; MS m/z [M+H]+: 372.2; purity 99%; HRMS [M+H]+ calcd for C22H21N5O [M+H]+ 372.18189, found 372.18192; Rt (chiral HPLC: column: Chiralpak IG 5 µm; 100 x 4.6 mm; flow rate: 3 mL/min; column temperature: 40°C; back pressure: 1800 psi; mobile phase: CO2/isopropanol 60/40): 2.20 min, ee: 99.5%; 1H NMR (600 MHz, DMSO-*d*6) δ 13.0 (m, 1H), 10.33 (s, 1H), 7.82 (d, *J* = 8.9 Hz, 2H), 7.63 (m, 1H), 7.60 (d, *J* = 8.9 Hz, 2H), 7.45 (d, *J* = 8.4 Hz, 1H), 7.32 (d, *J* = 8.4 Hz, 1H), 6.47 (dd, *J* = 16.9, 10.2 Hz, 1H), 6.30 (dd, *J* = 16.9, 1.9 Hz, 1H), 5.79 (dd, J = 10.2, 1.9 Hz, 1H), 2.22 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H). The other isomer was obtained as the first eluting peak: Rt (chiral HPLC: column: Chiralpak IG 5 µm; 100 x 4.6 mm; flow rate: 3 mL/min; column temperature: 40°C; back pressure: 1800 psi; mobile phase: CO2/isopranol 60/40): 1.38 min.

### **[4]**: 1-(6-(3-Isopropyl-5-methyl-4-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptan-2-yl)prop-2-en-1-one



Tert*-butyl 6-(4-bromo-3-isopropyl-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

To a solution of 4-bromo-3-isopropyl-5-methyl-1*H*-pyrazole (CAS [60061-69-0], 400 mg, 1.67 mmol) in DMF (6.00 mL) at RT was added NaH (60% in mineral oil, 140 mg, 3.51 mmol) and the mixture was stirred for 15 min. A solution of *tert*-butyl 6-(tosyloxy)-2-azaspiro[3.3]heptane-2-carboxylate (736 mg, 2.00 mmol) in DMF (2.00 mL) was added and the reaction mixture was stirred at 65°C for 24 h. A solution of *tert*-butyl 6-(tosyloxy)-2-azaspiro[3.3]heptane-2-carboxylate (736 mg, 2.00 mmol) in DMF (2.00 mL) was added again and the reaction mixture was further stirred at 65°C for 24 h. The reaction mixture was allowed to cool to RT, water was slowly added and the mixture was extracted with EtOAc. The combined organic extracts were dried (Na2SO4), filtered and concentrated under reduced pressure. The crude residue was purified by normal phase column chromatography (eluent: EtOAc in cyclohexane 0 to 40%) to give a mixture of isomers which were separated by preparative HPLC (column: XBridge 50 x 250 mm 5 μM, flow: 100 mL/min, mobile phase: CH3CN in water (containing 0.1% NH4OH) 65 to 85% in 21 min) to give *ter*t-butyl 6-(4-bromo-5-isopropyl-3-methyl-1H-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (137 mg, 20% yield) as the first eluting isomer as a light yellow solid (UPLC-MS-1: Rt = 1.41 min; MS m/z [M+H]+: 398.2/ 400.2; purity 99%) and the title compound: *tert*-butyl 6-(4-bromo-3-isopropyl-5-methyl-1H-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (395 mg, 58.8%) as the second eluting isomer a light yellow solid: UPLC-MS-1: Rt = 1.46 min; MS m/z [M+H]+: 398.2/ 400.2; purity 99%; 1H NMR (600 MHz, DMSO) δ ppm 4.67 (m, 1 H), 3.96 (m, 2H), 3.84 (m, 2H), 2.89 (hept, *J* = 6.9 Hz, 1H), 2.57 (m, 2H), 2.51 (m, 2H), 2.15 (s, 3H), 1.37 (s, 9H), 1.19 (d, *J* = 6.9 Hz, 6H).

Tert*-butyl 6-(3-isopropyl-5-methyl-4-(5-methyl-1-tosyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

To a solution of *tert*-butyl 6-(4-bromo-3-isopropyl-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (390 mg, 0.98 mmol) and 5-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-tosyl-1H-indazole (565 mg, 1.37 mmol) in 1,4-dioxane (2.75 mL) and water (550 µL) were added K3PO4 (416 mg, 1.96 mmol) and Pd-XPhos-G3 (83 mg, 0.098 mmol). The reaction mixture was evacuated and filled with argon and stirred at 80°C for 1 h. Water was added and the mixture was extracted with EtOAc. The combined organic extracts were dried (Na2SO4), filtered and concentrated under reduced pressure. The crude residue was purified by normal phase column chromatography (eluent: EtOAc in cyclohexane from 0 to 40%) to give the title compound as a yellow gum (395 mg, 66.2% yield). UPLC-MS-1: Rt = 1.50 min; MS m/z [M+H]+: 604.4; purity: 99%; 1H NMR (400 MHz, DMSO) δ 8.00 (d, *J* = 8.6 Hz, 1H), 7.94 (m, 1H), 7.81 (m, 2H), 7.60 (d, *J* = 8.6 Hz, 1H), 7.40 (d, *J* = 8.6 Hz, 2H), 4.68 (m, 1H), 3.96 (m, 2H), 3.88 (m, 2H), 2.73 (m, 1H), 2.67 (hept, *J* = 6.9 Hz, 1H), 2.66 - 2.62 (m, 3H), 2.34 (s, 3H), 2.12 (s, 3H), 1.83 (s, 3H), 1.38 (s, 9H), 0.93 (d, *J* = 6.9 Hz, 3H), 0.90 (d, *J* = 6.9 Hz, 3H).

Tert*-butyl 6-(3-isopropyl-5-methyl-4-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

To a solution of *tert*-butyl 6-(3-isopropyl-5-methyl-4-(5-methyl-1-tosyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (393 mg, 0.65 mmol) in 1,4-dioxane (4.50 mL) was added NaOH (2N, 0.98 mL, 1.96 mmol) and the reaction mixture was stirred at 90°C for 1 h. A saturated aqueous solution of NaHCO3 and EtOAc were added, the layers were separated and the aqueous layer was back-extracted with EtOAc. The combined organic extracts were dried (Na2SO4), filtered and concentrated under reduced pressure. The crude residue was purified by normal phase chromatography (eluent: AcOEt in cyclohexane from 0 to 100%) to give the title compound (288 mg, 96% yield) as a light yellow gum. UPLC-MS-1: Rt = 1.26 min; MS m/z [M+H]+: 450.4; purity 98%; 1H NMR (400 MHz, DMSO) δ 12.9 (s, 1H), 7.45 (s, 1H), 7.40 (d, *J* = 8.5 Hz, 1H), 7.28 (d, *J* = 8.5 Hz, 1H), 4.70 (m, 1H), 3.97 (m, 2H), 3.90 (m, 2H), 2.75 (dd, *J* = 12.4, 8.0 Hz, 1H), 2.70-2.63 (m, 3H), 2.58 (hept, *J* = 6.9 Hz, 1H), 2.11 (s, 3H), 1.89 (s, 3H), 1.38 (s, 9H), 1.01 (d, *J* = 6.9 Hz, 3H), 0.98 (d, *J* = 6.9 Hz, 3H).

*4-(3-Isopropyl-5-methyl-1-(2-azaspiro[3.3]heptan-6-yl)-1*H*-pyrazol-4-yl)-5-methyl-1*H*-indazole*

To a solution of *tert-*butyl 6-(3-isopropyl-5-methyl-4-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (50 mg, 0.11 mmol) in CH2Cl2 (2 mL) at 0°C, was added TFA (0.26 mL, 3.34 mmol) and the reaction mixture was stirred for 1 h at RT. The reaction mixture was concentrated under reduced pressure, the residue was taken up in CH3CN and water frozen and lyophilized to the title compound as a trifluoroacetate salt (0.11 mmol, quantitative yield) which was used in the next step without purification. UPLC-MS-1: Rt = 0.68 min; MS m/z [M+H]+: 350.4; purity 96%.

*1-(6-(3-Isopropyl-5-methyl-4-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptan-2-yl)prop-2-en-1-one*

To a solution of 4-(3-isopropyl-5-methyl-1-(2-azaspiro[3.3]heptan-6-yl)-1*H*-pyrazol-4-yl)-5-methyl-1*H*-indazole (0.11 mmol) in CH2Cl2 (2 mL) at 0°C, were successively added DIPEA (108 µL, 0.62 mmol) and a solution of acryloyl chloride (10.3 µL, 0.12 mmol) in CH2Cl2 (200 µL) and the reaction mixture was stirred for 1 h at 0°C. MeOH (1.00 mL) was added, the mixture was stirred for 10 min and was concentrated under reduced pressure. The crude residue was purified by reverse phase preparative HPLC (Waters X-Bridge C18 OBD 30 x 100mm 5 µm; eluent: CH3CN in H2O containing NH4OH (7.3 mM) from 15 to 100% in 20 min; flow 40 mL/min). The purified fractions were frozen and lyophilized to give the title compound as a racemic mixture. The atropisomers were separated by preparative chiral HPLC (column: Chiralpak AD 5 µm; 250 x 25 mm; flow rate: 15 mL/min; column temperature: RT; mobile phase: heptane/ isopropanol 85/15 containing 0.05% Et3N) to give **[4]** as the second eluting peak (white powder, 5.30 mg, 15% yield): UPLC-MS-2: Rt = 4.56 min; MS m/z [M+H]+: 404.4; purity 100%; HRMS [M+H]+ calcd for C24H29N5O [M+H]+ 404.24454, found 404.24449; Rt (chiral HPLC: column: Chiralpak AD 5 µm; 250 x 4.6 mm; flow rate: 1 mL/min; mobile phase: heptane/ isopropanol 85/15): 11.8 min, ee: 100%; 1H NMR (400 MHz, DMSO) δ 12.9 (s, 1H), 7.46 (s, 1H), 7.40 (d, *J* = 8.5 Hz, 1H), 7.28 (d, *J* = 8.5 Hz,1H), 6.31 (m, 1H), 6.10 (m, 1H), 5.67 (m, 1H), 4.75 (m, 1H), 4.35 (s, 1H), 4.29 (s, 1H), 4.07 (s, 1H), 4.00 (s, 1H), 2.67 - 2.85 (m, 4H), 2.60 (hept, *J* = 6.9 Hz, 1H), 2.10 (s, 3H), 1.89 (s, 3H), 1.02 (d, *J* = 6.9 Hz, 3H), 0.98 (d, *J* = 6.9 Hz, 3H). The other isomer was obtained as the first eluting peak Rt (chiral HPLC: column: Chiralpak AD 5 µm; 250 x 4.6 mm; flow rate: 1 mL/min; mobile phase: heptane/ isopropanol 85/15): 9.97 min.

### General synthetic scheme for the preparation of **JDQ443 [5]**



### **JDQ443 [5]**: a(*R*)-1-(6-(4-(5-Chloro-6-methyl-1*H*-indazol-4-yl)-5-methyl-3-(1-methyl-1*H*-indazol-5-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptan-2-yl)prop-2-en-1-one



Tert*-butyl 6-(3-bromo-4-(5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-4-yl)-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

 To a stirred suspension of *tert*-butyl 6-(3-bromo-4-iodo-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (400 g, 829 mmol) and 5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indazole (343 g, 912 mmol) in 1,4-dioxane (2000 mL) was added aqueous K3PO4 (528 g, 2.49 mol) in water (1200 mL) followed by RuPhos (38.7 g, 82.9 mmol) and RuPhos-Pd-G3 (41.6 g, 49.7 mmol). The reaction mixture was stirred at 80°C for 1 h under inert atmosphere. After completion of the reaction, the reaction mixture was poured into a saturated aqueous NaHCO3 solution (1300 mL) and extracted with EtOAc (1500 mL x 3). The combined organic layers were washed with brine (1200 mL x3), dried (Na2SO4), filtered, and concentrated under reduced pressure. The crude residue was purified by normal phase chromatography (eluent: Petroleum ether / EtOAc from 1/0 to 0/1) to give the title compound (563 g, 97% yield). 1H NMR (400 MHz, DMSO-*d*6) δ 7.81 (s, 1H), 7.66 (s, 1H), 5.94 - 5.81 (m, 1H), 4.90 - 4.78 (m, 1H), 3.99 (br s, 2H), 3.93 - 3.84 (m, 3H), 3.81 - 3.70 (m, 1H), 2.81 - 2.64 (m, 4H), 2.52 (s, 3H), 2.46 - 2.31 (m, 1H), 2.11 - 1.92 (m, 5H), 1.82 - 1.67 (m, 1H), 1.64 - 1.52 (m, 2H), 1.38 (s, 9H); UPLC-MS-3: Rt = 1.30 min; MS m/z [M+H]+: 604.1 / 606.1.

Tert*-butyl 6-(4-(5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-4-yl)-5-methyl-3-(1-methyl-1*H*-indazol-5-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate*

In a 500 mL flask, *tert*-butyl 6-(3-bromo-4-(5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-4-yl)-5-methyl-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (10.0 g, 16.5 mmol), (1-methyl-1*H*-indazol-5-yl)boronic acid (6.12 g, 33.1 mmol), RuPhos (1.16 g, 2.48 mmol) and RuPhos-Pd-G3 (1.66 g, 1.98 mmol) were suspended in toluene (165 mL) under argon. K3PO4 (2M, 24.8 mL, 49.6 mmol) was added and the reaction mixture was placed in a preheated oil bath (95°C) and stirred for 45 min. The reaction mixture was poured into a saturated aqueous NH4Cl solution and was extracted with EtOAc (x3). The combined organic extracts were washed with a saturated aqueous NaHCO3 solution, dried (Na2SO4), filtered and concentrated under reduced pressure. The crude residue was diluted with THF (50 mL), SiliaMetS®Thiol (15.9 mmol) was added and the mixture swirled for 1 h at 40°C. The mixture was filtered, the filtrate was concentrated and the crude residue was purified by normal phase chromatography (eluent: MeOH in CH2Cl2 from 0 to 2%), the purified fractions were again purified by normal phase chromatography (eluent: MeOH in CH2Cl2 from 0 to 2%) to give the title compound as a beige foam (10.7 g, 90% yield). UPLC-MS-3: Rt = 1.23 min; MS m/z [M+H]+: 656.3/ 658.3, purity: 92%; 1H NMR (600 MHz, DMSO-*d*6) δ 7.88 (s, 1H), 7.76 (s, 1H), 7.58 (m, 1H), 7.45 (m, 1H), 7.41 (m, 1H), 7.26 (m, 1H), 5.80 (m, 1H), 4.87 (m, 1H), 4.04 - 3.98 (m, 2H), 3.94 - 3.91 (m, 5H), 3.87 (m, 1H), 3.73 (m, 1H), 2.92 - 2.78 (m, 2H), 2.79 - 2.69 (m, 2H), 2.48 (s, 3H), 2.32 (m, 1H), 2.04 - 1.89 (m, 5H), 1.72 (m, 1H), 1.60 - 1.51 (m, 2H), 1.39 (1, 9H).

*5-Chloro-6-methyl-4-(5-methyl-3-(1-methyl-1*H*-indazol-5-yl)-1-(2-azaspiro[3.3]heptan-6-yl)-1*H*-pyrazol-4-yl)-1*H*-indazole*

TFA (19.4 mL, 251 mmol) was added to a solution of *tert*-butyl 6-(4-(5-chloro-6-methyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-4-yl)-5-methyl-3-(1-methyl-1*H*-indazol-5-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptane-2-carboxylate (7.17 g, 10.0 mmol) in CH2Cl2 (33 mL). The reaction mixture was stirred at RT under nitrogen for 1.5 h. The reaction mixture was concentrated under reduced pressure to give the title compound as a trifluoroacetate salt (10.0 mmol, quantitative yield) which was used without purification in the next step. UPLC-MS-3: Rt = 0.74 min; MS m/z [M+H]+: 472.3 / 474.3; purity: 92%.

*a(*R*)-1-(6-(4-(5-Chloro-6-methyl-1*H*-indazol-4-yl)-5-methyl-3-(1-methyl-1*H*-indazol-5-yl)-1*H*-pyrazol-1-yl)-2-azaspiro[3.3]heptan-2-yl)prop-2-en-1-one*

A mixture of acrylic acid (0.69 mL, 10.1 mmol), propylphosphonic anhydride (50% in EtOAc, 5.94 mL, 7.53 mmol) and DIPEA (21.6 mL, 126 mmol) in CH2Cl2 (80 mL) was stirred for 20 min at RT and then was added (dropping funnel) to an ice-cooled solution of 5-chloro-6-methyl-4-(5-methyl-3-(1-methyl-1*H*-indazol-5-yl)-1-(2-azaspiro[3.3]heptan-6-yl)-1*H*-pyrazol-4-yl)-1*H*-indazole trifluoroacetate (6.30 mmol) in CH2Cl2 (40 mL). The reaction mixture was stirred at RT under nitrogen for 15 min. The reaction mixture was poured into a saturated aqueous NaHCO3 solution and extracted with CH2Cl2 (x3). The combined organic layers were dried (phase separator) and concentrated. The crude residue was diluted with THF (60 mL) and LiOH (2N, 15.7 mL, 31.5 mmol) was added. The mixture was stirred at RT for 30 min until disappearance (UPLC) of the side product resulting from the reaction of acryloyl chloride with the indazole NH, then the mixture was poured into a saturated aqueous NaHCO3 solution and extracted with CH2Cl2 (3x). The combined organic layers were dried (phase separator) and concentrated under reduced pressure. The crude residue was purified by normal phase chromatography (eluent: MeOH in CH2Cl2 from 0 to 5%) to give the title compound as a racemic mixture (2.27 g, 60% yield). The enantiomers (805 mg, 1.45 mmol) were separated by chiral SFC (column: Amylose-C NEO 5 µm; 250 x 30 mm; mobile phase CO2/[isopropanol+0.1% Et3N]: 69/31; flow rate: 80 mL/min; column temperature: 40°C; back pressure: 120 bars) to give JDQ443 **[5]** as the second eluting peak as a white powder (340 mg, 42% yield): UPLC-MS-3: Rt = 0.91 min; MS m/z [M+H]+: 526.3/ 528.3, purity: 100%; UPLC-MS-4: Rt = 4.22 min; MS m/z [M+H]+: 526.3/ 528.3, purity: 99%; HRMS [M+H]+ calcd for C29H28ClN7O [M+H]+ 526.21204, found 526.21166; Rt (chiral SFC; column: Chiralpak AD-H 5 µm; 100 x 4.6 mm; mobile phase: CO2/[isopropanol+0.1% Et3N]: 67/33; flow rate: 3 mL/min; column temperature: 40°C; back pressure: 1800 psi): 2.23 min, ee: 99.5%; 1H NMR (600 MHz, DMSO-*d*6) δ 13.1 (s, 1H), 7.89 (s, 1H), 7.57 (m, 1H), 7.54 (m, 1H), 7.42 - 7.39 (m, 2H), 7.28 (dt, *J* = 8.7, 1.9 Hz, 1H), 6.32 (m, 1H), 6.10 (m, 1H), 5.67 (m, 1H), 4.90 (m, 1H), 4.39 (s, 1H), 4.32 (s, 1H), 4.10 (s, 1H), 4.03 (s, 1H), 3.94 (s, 3H), 2.94-2.85 (m, 2H), 2.83-2.76 (m, 2H), 2.48 (s, 3H), 2.03 (s, 3H); 13C NMR (151 MHz, DMSO) δ 164.40, 147.47, 138.83, 138.26, 137.78, 137.75, 134.15, 133.08, 132.60, 127.09, 127.08, 126.78, 126.56, 126.37, 126.34, 125.10, 124.91, 123.39, 123.32, 117.79, 117.78, 112.84, 112.83, 110.91, 109.32, 61.91, 60.63, 59.77, 58.45, 47.04, 46.93, 39.86, 35.29, 31.54, 31.35, 21.62, 9.75. The absolute stereochemistry of JDQ443 has been determined by analysis of its X-ray crystal structure bound to KRasG12C. The other isomer was obtained as the first eluting peak: Rt (chiral SFC; column: Chiralpak AD-H 5 µm; 100 x 4.6 mm; mobile phase: CO2/[isopropanol+0.1% Et3N]: 67/33; flow rate: 3 mL/min; column temperature: 40°C; back pressure: 1800 psi): 1.55 min.

# **2. X-Ray Crystal Structure Determination**

KRAS protein (1-169) G12C, C51S, C80L, C118S (KRASG12C cysteine light-GDP) in the GDP loaded form was pre-incubated with **[2]**, **[3]** or JDQ443 (**[5]**) overnight to allow for covalent bond formation between Cys12 and the reactive group of the respective inhibitors. The covalent inhibitor complexes were co-crystallized by the sitting drop vapor diffusion method. 0.2 L protein solution (20.6 mg/ml KRASG12C cysteine light–GDP-compound complex, 50 mM Hepes pH 7.5, 150 mM sodium chloride, 5 mM MgCl2) was mixed with 0.2 L reservoir solution (33% PEG 6000 and 0.01 M sodium citrate for compound 2; 25% PEG 4000, 0.1 M Tris HCl pH 8.5 and 0.2 M CaCl2 for compound 3; 30% PEG 4000, 0.1 M Tris HCl pH 7.5 and 0.2 M CaCl2 for JDQ443) and equilibrated against 80 L reservoir solution. For data collection crystals were directly flash cooled in liquid nitrogen.

X-ray diffraction data were collected from single crystals at the Swiss Light Source, beamline X10SA equipped with a PILATUS detector for the KRASG12C-**[2]** and KRASG12C-**[3]** complexes and with an EIGER detector for KRASG12C-JDQ443complex. The diffraction data were processed and scaled with XDS and XSCALE (3), respectively. The structures were solved by molecular replacement using the coordinates of PDB code 4LRW as search model and the program MOLREP (4). Structures were built using the program COOT (5) and refined using the program BUSTER (6). Images were generated using the program PyMOL (<http://www.pymol.org>).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **[2]** | **[3]** | JDQ443 |
| **Data collection** |  |  |  |
| Space group | P212121 | P1 | P1 |
| Cell dimensions |  |  |  |
|  *a*, *b*, *c* (Å) | 41.4, 59.2, 65.2 | 33.2, 39.8, 62.8 | 33.7, 39.5, 62.4 |
| ****** ()  | 90, 90, 90 | 76.9, 81.2, 77.3 | 77.2, 80.9, 77.2 |
| Resolution (Å) | 1.2 (1.23-1.2)\* | 1.88 (1.93-1.88) \* | 1.61 (1.8-1.61) \* |
| *R*sym or *R*merge | 3.9 (69.6) | 6.8 (70.0) | 7.6 (55.5) |
| *I*/*I* | 19.8 (2.2) | 7.6 (2.2) | 6.1 (1.3) |
| Completeness (%) (ellipsoidal) | 99.9 (99.9) | 92.8 (90.1) | 83.6 (44.5) |
| Redundancy | 6.2 (5.6) | 1.7 (1.8) | 2.8 (3.1) |
|  |  |  |  |
| **Refinement** |  |  |  |
| Resolution (Å) | 1.2 | 1.95 | 1.61 |
| No. reflections | 50770 | 20598 | 23415 |
| *R*work/ *R*free | 20.2 / 22.2 | 23.7 / 29.3 | 19.5 (24.9) |
| No. atoms |  |  |  |
|  Protein | 1335 | 2677 | 2690 |
|  Ligand/GDP/ion | 23 / 28 / 1 | 56 / 56 / 3 | 76 / 56 / 4 |
|  Water | 186 | 135 | 261 |
| B-factors (Å2) | 13.3 | 24.9 | 16.5 |
| R.m.s deviations |  |  |  |
|  Bond lengths (Å)  | 0.008 | 0.008 | 0.008 |
|  Bond angles (º) | 0.95 | 0.90 | 0.85 |

\*Highest resolution shell is shown in parenthesis.

# **3. Biochemical and Biophysical Methods**

## 3.1 Cloning, expression and purification of RAS protein constructs

The *E. coli* expression constructs used were based on the pET system and generated using standard molecular cloning techniques. Following the cleavable N-terminal his affinity purification tag the cDNA encoding KRAS, NRAS, and HRAS comprised aa 1-169 and was codon-optimized and synthesized by GeneArt (Thermo Fisher Scientific). Point mutations were introduced with the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent). All final expression constructs were sequence verified by Sanger sequencing.

Two liters of culture medium were inoculated with a pre-culture of E. coli BL21(DE3) freshly transformed with the expression plasmid and protein expression induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) for 16 hours at 18 °C. Proteins with an avi-tag were transformed into *E. coli* harboring a compatible plasmid expressing the biotin ligase BirA and the culture medium was supplemented with 135 µM d-biotin (Sigma).

Cell pellets were resuspended in buffer A (20 mM Tris, 500 mM NaCl, 5 mM imidazole, 2 mM TCEP, 10 % glycerol, pH 8.0) supplemented with Turbonuclease (Merck) and cOmplete protease inhibitor tablets (Roche). The cells were lysed by three passages through a homogenizer (Avestin) at 800-1000 bar and the lysate clarified by centrifugation at 40000 g for 40 min.

The lysate was loaded onto a HisTrap HP 5 ml column (Cytiva) mounted on an ÄKTA Pure 25 chromatography system (Cytiva). Contaminating proteins were washed away with buffer A and bound protein was eluted with a linear gradient to buffer B (buffer A supplemented with 200 mM imidazole). During dialysis O/N the N-terminal His affinity purification tags on the non-tagged and avi-tagged proteins were cleaved off by TEV or HRV3C protease, respectively. The protein solution was re-loaded onto a HisTrap column and the flow through containing the target protein collected.

Guanosine 5’-diphosphate sodium salt (GDP, Sigma) or GppNHp-Tetralithium salt (Jena Bioscience) was added to a 24-32x molar excess over protein. EDTA (pH adjusted to 8) was added to a final concentration of 25 mM. After 1 hour at room temperature the buffer was exchanged on a PD-10 desalting column (Cytiva) against 40 mM Tris, 200 mM (NH4)2SO4, 0.1 mM ZnCl2, pH 8.0. GDP (for KRASG12C resistance mutants H95Q/D/R, Y96D/C and R68S) or GppNHp was added to a 24-32x molar excess over protein to the eluted protein. 40 U Shrimp Alkaline Phosphatase (New England Biolabs) was added to GppNHp containing samples only. The sample was then incubated for 1 hour at 5°C. Finally, MgCl2 was added to a concentration of about 30 mM. The protein was then further purified over a HiLoad 16/600 Superdex 200 pg column (Cytiva) pre-equilibrated with 20 mM HEPES, 150 mM NaCl, 5 mM MgCl2, 2 mM TCEP, pH 7.5. The purity and concentration of the protein was determined by RP-HPLC, its identity was confirmed by LC-MS. Present nucleotide was determined by ion-pairing chromatography (7).

## 3.2 Determination of covalent rate constants by RapidFire MS

### 3.2.1 Assay and curve fitting

Serial dilutions of the test compounds (50 µM, ½ dilutions) were prepared in 384-well plates and incubated with 1 µM KRAS G12C (with/without additional mutants) in 20mM Tris pH7.5, 150mM NaCl, 100 µM MgCl2, 1% DMSO at room temperature. Reactions were stopped at different time points by addition of formic acid to 1%. MS measurements were carried out using an Agilent 6530 quadrupole time-of-flight (QToF) MS system coupled to an Agilent RapidFire autosampler RF360 device, resulting in % modification values for each well. In parallel, compound solubility was assessed by nephelometry and compound concentrations resulting in measurable turbidity were excluded from curve fitting.

Plotting the % modification vs. time allowed for extraction of kobs values for the different compound concentrations. In a second step, the obtained kobs values were plotted against the compound concentrations. Rate constants (i.e. kinact/KI) were derived from the initial linear part of the resulting curves.

### 3.2.2 MS measurements

The RapidFire autosampler RF 360 was used to perform the injections. Solvents were delivered by Agilent 1200 pumps. A C18 Solid Phase Extraction (SPE) cartridge was used for all experiments.

A volume of 30 μL was aspirated from each well of a 384-well plate. The sample load/wash time was 3000 ms at a flow rate of 1.5 mL/min (H2O, 0.1% formic acid); elution time was 3000 ms (acetonitrile, 0.1% formic acid); reequilibration time was 500 ms at a flow rate of 1.25 mL/min (H2O, 0.1% formic acid).

 Mass spectrometry (MS) data were acquired on an Agilent 6530 quadrupole time-of-flight (QToF) MS system, coupled to a dual Electrospray (AJS) ion source, in positive mode. The instrument parameters were as follows: gas temperature 350 °C, drying gas 10 L/min, nebulizer 45 psi, sheath gas 350 °C, sheath gas flow 11 L/min, capillary 4000 V, nozzle 1000 V, fragmentor 250 V, skimmer 65 V, octapole RF 750 V. Data were acquired at the rate of 6 spectra/s. The mass calibration was performed over the 300–3200 m/z range.

All data processing was performed using a combination of Agilent MassHunter Qualitative Analysis, Agilent Rapid-Fire control software, and the Agilent DA Reprocessor Offline Utilities. A Maximum Entropy algorithm produced zero-charge spectra in separate files per injection. A batch processing generated a single file incorporating all mass spectra in a text format as x,y coordinates. This file was used to calculate the % of protein modification in each well.

## 3.3 Determination of covalent rate constants by competition with covalent probe in a scintillation proximity assay (SPA)

### 3.3.1 Probe synthesis

####

#### 3.3.1.1 Synthesis of the unlabelled probe **[6]**: N-(3-fluoro-4-(2-methyl-3-(5-methyl-1H-indazol-4-yl)-1H-pyrrolo[2,3-b]pyridin-1-yl)phenyl)acrylamide

**Synthesis scheme**



***Step 1:*** *3-Bromo-2-methyl-1*H*-pyrrolo[2,3-b]pyridine*

To a solution of 2-methyl-1*H*-pyrrolo[2,3-b]pyridine (1.63 g, 12.3 mmol) in acetonitrile (50 mL) was added NBS (2.19 g, 12.3 mmol) at RT. The reaction mixture was stirred at RT for 1 h. The reaction was quenched by addition of a 10% solution of sodium thiosulfate (10 mL). The solution was then extracted with EtOAc (20 mL). The organic phase was washed with water (3x), brine, then dried over MgSO4, filtered and evaporated. UPLC-MS-1: Rt = 0.88 min; MS m/z [M+H]+: 211 / 213.

***Step 2****: 3-Bromo-1-(2-fluoro-4-nitrophenyl)-2-methyl-1*H*-pyrrolo[2,3-b]pyridine*

To a solution of 3-bromo-2-methyl-1*H*-pyrrolo[2,3-b]pyridine (step 1) (2.4 g, 11.4 mmol) in dry DMSO (20 mL) was added under inert atmosphere potassium *tert*-butoxide (1.40 g, 12.5 mmol) and 1,2-difluoro-4-nitrobenzene (1.81 g, 11.4 mmol). The reaction mixture was stirred at 80°C for 2h. The reaction was quenched by addition of water. The solution was then extracted with EtOAc (3x). The organic phase was washed with brine, then dried over MgSO4, filtered and evaporated. The crude product was purified by normal phase chromatography (eluent: EtOAc in Heptane 0 to 30%) to give the title compound. UPLC-MS-1 : Rt = 1.24 min; MS m/z [M+H]+; 350.0 / 352.1.

***Step 3:*** *4-(1-(2-Fluoro-4-nitrophenyl)-2-methyl-1*H*-pyrrolo[2,3-b]pyridin-3-yl)-5-methyl-1-tosyl-1*H*-indazole*

To a solution of 3-bromo-1-(2-fluoro-4-nitrophenyl)-2-methyl-1*H*-pyrrolo[2,3-b]pyridine (step 3) (1.00 g, 2.86 mmol) in 1,4-dioxane (10 mL) / water (2.8 mL) was added under inert atmosphere 5-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-tosyl-1*H*-indazole (1.41 g, 3.43 mmol) followed by K3PO4 (1.21 g, 5.71 mmol) and then Pd XPhos-G3 (0.24 g, 0.29 mmol). The reaction mixture was stirred 3 h at 80°C. The reaction was quenched by addition of saturated solution of NaHCO3. The solution was then extracted with EtOAc. The organic phase was washed with brine, then dried over MgSO4, filtered and evaporated. The crude product was purified by normal phase chromatography (eluent: EtOAc in Heptane 0 to 50%) to give the title compound. UPLC-MS-1: Rt = 1.38 min; MS m/z [M+H]+: 556.2.

***Step 4****: 4-(1-(2-Fluoro-4-nitrophenyl)-2-methyl-1*H*-pyrrolo[2,3-b]pyridin-3-yl)-5-methyl-1*H*-indazole*

To a stirred solution of 4-(1-(2-fluoro-4-nitrophenyl)-2-methyl-1*H*-pyrrolo[2,3-b]pyridin-3-yl)-5-methyl-1-tosyl-1*H*-indazole (step 3) (1.34 g, 2.41 mmol) in 1,4-dioxane (12 mL) was added NaOH (6.03 mL, 12.1 mmol) at RT. The mixture was stirred at 60°C for 3 h. The reaction was quenched by addition of water. The solution was then extracted with EtOAc. The organic phase was washed with brine, then dried over MgSO4, filtered and evaporated. The crude product was purified by normal phase chromatography (eluent: EtOAc in Heptane 0 to 100%) to give the title compound. UPLC-MS-1: Rt = 1.11 min; MS m/z [M+H]+: 402.2.

***Step 5****: 3-Fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)aniline*

To a solution of 4-(1-(2-fluoro-4-nitrophenyl)-2-methyl-1*H*-pyrrolo[2,3-b]pyridin-3-yl)-5-methyl-1*H*-indazole (step 4) (780 mg, 1.94 mmol) in THF (10 mL) were added Tin powder (807 mg, 6.80 mmol) and HCl conc (0.59 mL, 19.4 mmol). The solution was stirred at 70°C for 4 h. The reaction was quenched by addition of sodium hydroxide and water. The solution was then extracted with EtOAc. The organic phase was washed with brine, then dried over MgSO4, filtered and evaporated to give the title compound. UPLC-MS-1: Rt = 0.93 min; MS m/z [M+H]+: 372.4.

***Step 6****: Isomers of 3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)aniline*

3-Fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)aniline as mixture of isomers (454 mg) in iPrOH (30 mg/mL) were separated by chiral HPLC (column: Lux IC 5 µm; 250 x 21.2 mm; mobile phase: CO2/iPrOH 55/45; flow rate: 50 mL/min; column temperature: 40°C; back pressure: 105 bars) to give 3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)aniline as first eluting isomer (analytical chiral HPLC: (column: Chiralpak AD-H 5 µm; 250 x 4.6 mm; mobile phase: CO2/[iPrOH+1% isopropylamine]: 50/50; flow rate: 3 mL/min; column temperature: 40°C; back pressure: 120 bars): Rt = 2.99 min) and 3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)aniline second eluting isomer (analytical chiral HPLC: (column: Chiralpak AD-H 5 µm; 250 x 4.6 mm; mobile phase: CO2/[iPrOH+1% isopropylamine]: 50/50; flow rate: 3 mL/min; column temperature: 40°C; back pressure: 120 bars): Rt = 5.79 min). UPLC-MS-1: Rt = 0.92 min; MS m/z [M+H]+: 372.2.

***Step 7****:* N*-(3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)phenyl)acrylamide*

To a solution of 3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)aniline (step 6, second eluting peak) (19 mg, 0.05 mmol) in CH2Cl2 (1.5 mL) were added DIPEA (0.03 mL, 0.15 mmol) and acryloyl chloride (4.57 µl, 0.06 mmol) at 0°C. The reaction mixture was stirred at 0°C for 4 h. MeOH was added to the mixture and evaporated to dryness. The reaction was quenched by addition of saturated solution of NaHCO3. The solution was then extracted with CH2Cl2. The organic phase was washed with brine, then dried over MgSO4, filtered and evaporated. The crude product was purified by normal phase chromatography (eluent: EtOAc in Heptane 0 to 100%) to give the title compound. 1H NMR (600 MHz, DMSO-d6) δ 13.12 – 13.03 (m, 1H), 10.63 (s, 1H), 8.21 – 8.16 (m, 1H), 8.03 – 7.96 (m, 1H), 7.79 – 7.45 (m, 5H), 7.43 – 7.37 (m, 1H), 7.18 – 7.10 (m, 1H), 6.54 – 6.45 (m, 1H), 6.40 – 6.32 (m, 1H), 5.90 – 5.83 (m, 1H), 2.29 – 2.22 (m, 3H), 2.09 (s, 3H); UPLC-MS-1: Rt = 0.98 min; MS m/z [M+H]+: 426.4.

#### 3.3.1.2 Synthesis of the tritiated probe Compound **[7]**

***Step 1****:* N*-(3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)phenyl)propiolamide*

To an ice-cooled solution of 3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)aniline (**[6]**, step 6 second eluting peak) (50 mg, 0.14 mmol) in DMF (1.5 mL) was added a mixture of DIPEA (0.09 mL, 0.54 mmol), propiolic acid (9.43 mg, 0.14 mmol) and propylphosphonic anhydride (50% in DMF, 0.16 mL, 0.27 mmol). The reaction mixture was stirred at RT under nitrogen for 15 min. The reaction mixture was poured into a saturated aqueous NaHCO3 solution and extracted with EtOAc (x3). The combined organic layers were dried over MgSO4, and concentrated. The crude product was purified by achiral SFC (column: Princeton PPU 5 µm; 250 x 30 mm; mobile phase: CO2/ MeOH: gradient with 24-29% MeOH in CO2 over 9.8 min; flow rate: 30 mL/min; column temperature: 36°C; back pressure: 120 bars) and re-purified by achiral SFC (column: Princeton PPU 5 µm; 250 x 30 mm; mobile phase: CO2/ MeOH: gradient with 20-26% MeOH in CO2 over 9.8 min; flow rate: 30 mL/min; column temperature: 36°C; back pressure: 120 bars) to give the title compound. UPLC-MS-1: Rt = 0.97 min; MS m/z [M+H]+: 424.4.

***Step 2****: [acrylamide-2,3-3*H*2]-*N*-(3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)phenyl)acrylamide*

*N*-(3-fluoro-4-(2-methyl-3-(5-methyl-1*H*-indazol-4-yl)-1*H*-pyrrolo[2,3-b]pyridin-1-yl)phenyl)propiolamide (step 1) (3.20 mg, 7.56 μmol), Lindlar catalyst (6.57 mg), quinoline (11.8 μL, 12.9 mg, 99.6 μmol) were suspended in DMF (0.60 mL). The suspension was degassed three times at the high vacuum manifold and stirred under an atmosphere of tritium gas (355 GBq, 508 mbar initial pressure) for 80 min at room temperature (end pressure was 505 mbar, no more tritium gas consumption was observed). The solvent was removed *in vacuo*, and labile tritium was exchanged by adding methanol (0.70 mL), stirring the solution, and removing the solvent again under vacuo. This process was repeated two times. Finally, the well dried solid was extracted with 5 mL of ethanol and the suspension was filtered through a 0.2 μm nylon membrane obtaining a clear and colourless solution. The radiochemical purity was determined to 86% by HPLC (Waters Sunfire HPLC with UV detector; column: C18 5 µm; 250 x 4.6 mm; mobile phase: A:water / B:acetonitrile, 0 min 10% B, 10 min 95% B, 14.5 min 95% B, 15 min 10% B; flow rate: 1 mL/min; column temperature: 30°C). Purification of the crude product was carried out by reverse phase HPLC (Waters Sunfire; column: C18 5 µm; 250 x 10 mm; detection UV 254 nM; mobile phase: A: water / B: MeOH, isocratic 62% B; flow rate: 4.7 mL/min; column temperature: 25°C). The target compound eluted at 19.1 min. The combined HPLC fractions were partially reduced at the rotary evaporator at 40°C. Then, the product was extracted with a Phenomenex StrataX cartridge (33 μm Polymeric Reversed Phase, 100 mg, 3 mL; 8B-S100-EB) which was eluted with 5 mL of ethanol. The extracted product contained the title compound with an activity of 2.61 GBq and a radiochemical purity of >99%. The molar activity was determined to be 2.12 TBq/mmol.

### 3.3.2 Assay methodology

Scintillation proximity assays (SPA) were run using 384-well plates (781207, Greiner) in which one column was designated as the high signal (no inhibition) control, and contained DMSO with no test compound, and another column was designated as the low signal control (maximal inhibition), and contained no protein. Serial dilutions of compounds were added to the assay plate (resulting in duplicate 11-point dose response with semi-log compound dilutions from 50 µM to 0.5 nM or 5 µM to 0.05 nM for the most potent compounds). A 1/20 isotopic dilution of labeled and non-labeled covalent probe was prepared and added to all wells on the plate. KRASG12C (aa1-169, avi-tagged and biotinylated on the N-terminus) was added to the compounds and incubated at room temperature for 2 h with continuous agitation allowing for full modification of KRASG12C with probe or test compounds. Final concentrations in an assay volume of 40 µL were 10 nM KRASG12C, 25 nM radio-labelled probe and 475 nM unlabelled probe. The assay buffer contained 20 mM Tris-HCl pH 7.5 (Invitrogen), 150 mM NaCl (Sigma Aldrich), 0.1 mM MgCl2 (Sigma Aldrich), and 0.01% Tween-20 (Sigma Aldrich). Following addition of 50 uL streptavidin-coated YSi SPA beads (Perkin Elmer) suspended in buffer to 400 ug/mL, plates were incubated further 30 min incubation with continuous agitation before reading the plates on a Topcount NXT 384 (Packard).

Evaluation was carried out using the standard Novartis in-house assay data analysis software (Helios software application, Novartis Institutes for BioMedical Research, unpublished): Following normalization of activity values for the wells to % inhibition (%inhibition= [(high control- sample)/ (high control-low control)] x 100), IC50 fitting was carried out from the duplicate determinations present on each plate according to Formenko et al. 2006 (8). The second order rate constants were then extrapolated from the endpoint IC50 determinations according to (kinact/KI)inh = (kinact/KI)probe × [probe]/IC50 with (kinact/KI)probe =5,000 M-1 × s-1 and [probe] = 0.5 µM. For the derivation of the relationship between kinact/KI and IC50 under full modification conditions, see Miyahisa, 2015 (9)

### 3.3.3 Surface Plasmon Resonance (SPR) assessment of ligand affinity

Ligand affinities (KD) were determined by SPR using a BiacoreTM T200 device (GE Healthcare). Biotinylated avi-tagged RAS constructs (1-169) with defined nucleotide loading (GDP or GMPPNP) were immobilized on a Streptavidin-coated sensorchip (GE Healthcare, BR-1005-31) to a density of ~3000 RU. Following immobilization, 2 injections with 25 uM biotin solution (30ul/min, 2x100s) were carried out to reduce unspecific binding. The running buffer contained 50 mM HEPES, 150 mM NaCl, 0.5 mM MgCl2, 0.01 mM GMPPNP or 0.01 mM GDP, 0.1% Tween-20, 2% DMSO, pH 7.5.

Experiments were carried out at 22 °C using a flow-rate of 50 uL/min. An in-house identified LMW binder was included in each run to confirm sensitivity between experiments. In addition, a fixed concentration of this binder was injected between test compounds to assess the stability of the signal throughout the run. Compounds were tested in standard successive injection mode using a maximal test concentration of 200µM. Curve fitting was performed using the Biacore T200 Evaluation software. The sensorgrams were fitted by applying a 1:1 binding model to calculate kinetic rate constants and equilibrium dissociation constants.

# **4. Pharmacological Characterization**

Pharmacological characterization of JDQ443 across selected cell lines (242 solid tumor cell lines) was performed as previously described (10) using final drug concentrations ranging from 30 µM to 9.53 nM (8 point dose response assays by 3.16-fold dilutions). Visualizations for **Fig. 2A-B** were generated using the ggplot2 package in R and a parametric unpaired t-test was used for statistical analysis.

# **5. Cellular Characterization**

## 5.1 Cell-based assay to measure the protein–protein interaction of RAS and cRAF using the NanoBiT technology from Promega

### 5.1.1 Cells and cell culture conditions

HEK293A was obtained from ATCC and is part of the Novartis Cancer Cell Line Encyclopedia (CCLE) (10). The cells were cultivated in RPMI media with Glutamax (#61870036, Gibco) supplemented with 10% heat inactivated FCS (#SH30066.03HI, HyClone), 1mM sodium pyruvate (#5-60F00-H, BioConcept), 10mM HEPES (#5-31F00-H, BioConcept), and 1% Penicillin/Streptomycin (#4-01F00-H, BioConcept).

The cells were stably transfected and single clones were selected for the following SmBiT/LgBiT combinations. LgBiT-KRASWT full length-SmBiT-cRAF full length, LgBiT-KRASG12C full length-SmBiT-cRAF, LgBiT-KRASG12D full length-SmBiT-cRAF, LgBiT-NRASWT full length-SmBiT-cRAF, LgBiT-HRASWT full length-SmBiT-cRAF.

### 5.1.2 Compound dilution

Compound stock solution were prepared in DMSO/H2O (90:10) by compound hub at Novartis Pharma AG. Compounds were tested with a 7-point serial dilution 1:3 with a starting concentration of 1 µM.

### 5.1.3 Measuring NanoBiT® PPI luminescence

To assess protein–protein interaction in cells by NanoBiT technology (11), HEK293A\_SmBiT-cRAF\_LgBiT-RAS clones (KRASG12C, KRASWT, HRASWT and NRASWT) were cultured in RPMI media with Glutamax (#61870036, Gibco) supplemented with 10% heat inactivated FCS (#SH30066.03HI, HyClone), 1 mM sodium pyruvate (#5-60F00-H, BioConcept), 10 mM HEPES (#5-31F00-H, BioConcept), and 1% Penicillin/Streptomycin (#4-01F00-H, BioConcept). Cells were passaged by centrifuging in the respective culture media, and splitted into fresh media at a ratio of 1:10, 2 times per week. All cell lines were diluted in complete culture media and the mix was plated in a white 96-well plate with white bottom (#136102, Thermo Scientific) at a density of 20,000 cells/well in 150 µL media and incubated overnight at 37˚C, 95% CO2. The next day, cells were treated using a HP300 dispenser with increasing concentrations of compound, 7-point serial dilution 1:3 starting from 1 µM to 1.4 nM as the lowest concentration, for 4 hours. After incubation, the medium was replaced by 60 µL of Opti-MEM, adding 15 µL of NanoGlo Live™ substrate (#N2012, Promega) diluted 1:20 in LCS dilution buffer (provided with the kit). The HEK293A lines expressing the WT isoforms of RAS were then stimulated with human EGF for 10 minutes at a final concentration of 100 ng/mL per well. After incubation, luminescence was detected using a Pherastar FSX (integration time 1000 ms, Gain 3600, Lum Plus module).

To analyze the results the software XLfit from Microsoft was used. The IC50 was determined using a 4 parameter sigmoidal dose-response model (Fit Model 205).

$$Fit=A+ \frac{B-A}{1+\left(\frac{C}{x}\right)^{D}}$$

## 5.2 p-ERK1/2 and total ERK1/2 levels

### 5.2.1 Cells and cell culture conditions

The NSCLC lines NCI-H358 (KRASG12C), NCI-H2122 (KRASG12C) and NCI-H1437 (RASWT / MEKQ56P) were obtained from the ATCC (Cat. nos. CRL-5985, CRL-5807, CRL-5872, respectively) and maintained in RPMI1640 media with Glutamax (#61870036, Gibco) supplemented with 10% heat inactivated FCS (#SH30066.03HI, HyClone), 1mM sodium pyruvate (#5-60F00-H, BioConcept), 10mM HEPES (#5-31F00-H, BioConcept), and 1% Penicillin/Streptomycin (#4-01F00-H, BioConcept) at 37°C and 5% CO2 as specified by the provider.

### 5.2.2 Compound dilution

Compound stock solutions were prepared in DMSO/H2O (90:10) by compound hub at Novartis Pharma AG. Compounds were tested with a 7-point serial dilution 1:3 with a starting concentration of 1 µM.

### 5.2.3 Assessment of p-ERK1/2 and total ERK1/2 levels

NCI-H358, NCI-H2122 and NCI-H1437 were plated in a 96-well plate (#92696, TPP) at a density of 25,000 cells/well in 150 µL growth media and incubated overnight at 37˚C, 95% CO2. The next day, cells were treated with the corresponding compounds using a HP300 digital dispenser with increasing concentrations of compound for 6 hours and then lysed using 50 µL 1% NP-40/RIPA lysis buffer (below) per well. The plates were placed on a plate shaker for 20 minutes at 1,000rpm at 4˚C and finally stored at -80˚C for at least 2 hours until further use.

After thawing the samples, the pERK and total ERK levels were quantified using the Phospho/Total ERK1/2 Whole Cell Lysate Kit (K15107D, MesoScale Discovery) according to the manufacturer’s instructions and using buffers provided in the kit. The plate was first blocked for an hour with 150 µL Blocker A diluted in MSD tris wash buffer. After three washing steps, 30 µL of cell lysates were loaded undiluted in each well and incubated for 3 hours at 600 rpm. After another washing cycle, 25 µL of antibody diluted in antibody dilution buffer (for one plate: 150 µL Blocker D-M + 30 µL Blocker D-R + 1 mL Blocking Buffer + 1.82 mL Tris Wash Buffer) was added in each well and the plate was incubated for another hour on the plate shaker. Finally, after three last washing steps, 150 µL per well of 1X read buffer was added and the plate was read using an MSD Sector 600 imager.

**Composition of NP-40/RIPA buffer (stock solution)**

| Product | Reference |
| --- | --- |
|  |  |
| 1 M Tris pH 7.5  | Gibco *#15567-027* |
| 4 M NaCl | Merck #1.06404 |
| 0.5 M NaF | Merck #1.06450 |
| 0.1 M EDTA pH 7.4  | Invitrogen #AM92606 |
| 0.1 M EGTA pH 7-8 | Sigma #E4378 |
| Natriumpyrophosphate (PPi)  | Sigma #32.246-6 |
| Benzamidin | Fluka #12073 |
| 100 mM PMSF (in EtOH, -20°C) | Fluka #78830 |
| 100 mM Vanadate (-20°C) | Sigma #S-6508 |
| 100 % NP-40 | Sigma #74385 |
| H20 dd |  |
| complete Mini protease tablet | Roche *#11 836 153 001* |
| phosSTOP EASYpack phosphatase tablet  | Roche *#04 906 845 001)* |

###

### 5.2.4 Calculation and normalization

Percent phosphoprotein in each sample was calculated using following equation:

$$\% phosphoprotein=\left(\left(\frac{d×phospho-signal}{phospho-signal + total signal}\right)\right)×100$$

d being the distribution factor that needs to be calculated on DMSO control wells using the same formula and representing 100% phosphoprotein. Therefore, data are normalized on both DMSO control wells and total ERK protein detected in each well.

Absolute qualified AC50 were analyzed with the standard Novartis in-house assay data analysis software (Helios software application, Novartis Institutes for BioMedical Research, unpublished) using previously published methods (8,12-16).

## 5.3 Proliferation assay to determine the effect of covalent KRASG12C inhibitors on cell viability

### 5.3.1 Cells and cell culture conditions

The NSCLC lines NCI-H358 (KRASG12C), NCI-H2122 (KRASG12C) and NCI-H1437 (RASWT / MEKQ56P) were obtained from the ATCC (Cat. nos. CRL-5985, CRL-5807, CRL-5872, respectively) and maintained in RPMI1640 media with Glutamax (#61870036, Gibco) supplemented with 10% heat inactivated FCS (#SH30066.03HI, HyClone), 1mM sodium pyruvate (#5-60F00-H, BioConcept), 10mM HEPES (#5-31F00-H, BioConcept), and 1% Penicillin/Streptomycin (#4-01F00-H, BioConcept) at 37°C and 5% CO2 as specified by the provider.

### 5.3.2 Compound dilution

Compound stock solution were prepared in DMSO/H2O (90:10) by the compound hub at Novartis Pharma AG. Compounds were tested with a 7-point serial dilution 1:3 with a starting concentration of 1 µM for NCI-H2122 and 10 µM for NCI-H1437 and NCI-H358.

### 5.3.3 Cell proliferation measurement using the CellTiter-Glo assay (Promega)

Cells were seeded into 96-well plates (#3904, Costar) at a cell density of 500 cells/well (NCI-H2122) and 500 cells/well (NCI-H1437) in 150 µL/well culture medium and incubated overnight at 37°C/ 5% CO2. Compounds were added to the cells by using a Tecan D300e digital dispenser (Tecan, Switzerland), to achieve final concentrations ranging from 10 µM highest and 14 nM lowest concentration for NCI-H1437 and 1 µM highest and 1.4 nM lowest concentration for NCI-H2122 cells. DMSO concentration was normalized to 0.1% (NCI-H1437) or 0.01% (NCI-H2122) in all wells by adding the corresponding volume of DMSO with the digital dispenser. Compound effects were assessed after 120 hours of incubation at 37°C/ 5% CO2 by adding 100 µL per well CellTiter-Glo substrate (Promega #G7573). Addition of CellTiter-Glo results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present, an indicator of metabolically active cells. The amount of ATP is proportional to the number of viable cells. After 10 minutes of incubation at room temperature, the luminescence intensity was measured by a PheraStar FSX plate reader (BMG Labtech, Germany, Lum Plus module). The viability of cells at time of compound addition (D0) was likewise assessed and subtracted from the 120 hours readout to estimate potential cell loss (toxicity). Absolute qualified AC50 were analyzed with the standard Novartis in-house assay data analysis software (Helios software application, Novartis Institutes for BioMedical Research, unpublished) using previously published methods (8,12-16). In Fig. S7C, cells were dispensed into 384-well plates (Greiner #781098) in a final volume of 25 mL per well, 500 cells per well. Cells were allowed to adhere and begin growth for twenty-four hours. One plate was measured prior to treatment (D0), and the other plate was treated with compounds or DMSO using a HP D300 digital dispenser. Cells were treated with a 1:3 serial dilution of JDQ443 to achieve final concentrations of 4.12 nM up to 1 µM, with or without 500 nM TNO155. After seventy-two hours the medium was refreshed by supplementing 25 µl per well of culture medium containing the corresponding compounds or DMSO. Seven days after treatment initiation, cell growth was determined using CellTiter-Glo as described above.

### 5.3.4 Cell proliferation measurement using the resazurin assay

Cells were seeded into 96-well plates (Costar, #3904) at a cell density of 4,000 cells/well (NCI-H358) or 1,500 cells/well (NCI-H1437) in 150 µL/well RPMI-1640 (Gibco, #61870036) containing 10% FCS (FetalClone® II, US origin, HyClone™, #SH30066.03HI) and incubated overnight at 37°C/ 5% CO2. Compounds were added to the cells by using a Tecan D300e digital dispenser (Tecan, Switzerland), to achieve final concentrations ranging from 10 µM highest and 14 nM lowest concentration for NCI-H1437 and 1 µM highest and 1.4 nM lowest concentration for NCI-H358 cells. DMSO concentration was normalized to 0.1% (NCI-H1437) or 0.01% (NCI-H358) in all wells by adding the corresponding volume of DMSO with the digital dispenser. Compound effects were assessed after 72 hours of incubation at 37°C / 5% CO2 by adding 15 µL Resazurin (Sigma #R7017-5G) per well according to the protocol of the manufacturer. Upon entering living cells, Resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. The fluorescent intensity was measured with a PheraStar plate reader (BMG Labtech, Germany), using the following filter settings (540 nm for excitation, 590 nm for emission). The viability of cells at time of compound addition (D0) was likewise assessed and subtracted from the 72 hours readout to estimate potential cell loss (toxicity). Absolut qualified AC50 were analyzed with the standard Novartis in-house assay data analysis software (Helios software application, Novartis Institutes for BioMedical Research, unpublished) using previously published methods (8,12-16).

# **6. Cysteine Profiling Proteomics**

NCI-H358 cells were seed at 1x106 cells per 15 cm dish and cultured until confluent. Cells were then treated with DMSO, JDQ443 or Sotorasib at 10 μM for 6 hours in triplicate. Cells were washed and pelleted before resuspension in 50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 0.8% NP-40, and lysis by probe sonication (amplitude 10, 1s on/ 1s off, for 30s). Lysates were cleared by centrifugation at 1000 rpm for 5 minutes at 4°C. The resulting lysate (1 mg per sample) was treated with biotin-PEG4-DADPS-C6-iodoacetamide at 100 μM for 1 hour at room temperature. Excess biotin probe was removed by cleanup through Zeba 7K MWCO columns. Lysates were denatured with 200 μL 8M urea, reduced with 10 mM DTT for 15 minutes and alkylated with 55 mM iodoacetamide for 1 hour. The denatured, alkylated proteins were digested with 20 μg LysC/trypsin (Promega) for 2 hours at 37°C. After dilution to 1M urea, biotinylated peptides were enriched by incubating with 100 μL ultralink streptavidin agarose (Thermo) for 1 hour at RT on rotator. Beads were transferred to a 1.2 μm filter plate and washed 2x with 1 mL 5% MeOH and 2x with 1 mL 50 mM HEPES pH 8. Peptides were eluted by cleaving the DADPS linker with 300 μL 10% formic acid for 1 hour at RT. The eluted peptides were collected by centrifugation and concentrated by speedvac. The resulting concentrated peptides were resuspended in 40 μL 50 mM HEPES pH 8, 60 μL acetonitrile and 50 μL TMT label in acetonitrile (Thermo). TMT labeled samples were pooled and fractionated as previously described (17). The resulting fractions were combined in 24 fractions and concentrated by speedvac.

Fractions were analyzed by nanoLC-MS/MS using an Easy-nLC 1000 high-performance liquid chromatography system (Thermo) interfaced with an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo). A Kasil-fritted trapping column (75 µm x 15 mm) packed with 5 μm ReproSil-Pur 120 C18-AQ, was used together with a fused silica spraying capillary pulled to a tip diameter of 8-10 μm using a P-2000 capillary puller (Sutter Instruments). The capillary tubing (75 µm I.D.) was packed with a 120 mm separation column comprised of 3 um ReproSil-Pur C18 AQ. Samples (10 µL) were injected onto the trapping column using 0.1% formic acid/2% acetonitrile in water at a flow rate of 2.5 µL/min. Trapped peptides were then introduced into the separation column and eluted at 300 nL/minute using a mobile phase A: 2% acetonitrile + 0.1% formic acid in water and a mobile phase B: 98% acetonitrile + 0.1% formic acid in water with over a gradient of 3-45% B over 90 min. MS1 scans were acquired from m/z 400-1600 at 100,000 mass resolution with a triggering intensity threshold of 400,000 ions. MS2 scans were acquired using 50,000 mass resolution, HCD with 38% collision and 0.7 Da isolation window. MS2 AGC target was 1.25e5 using a 86 ms maximum injection time. Raw files were processed using Proteome Discoverer 2.4. Data was searched against a reference human proteome including the KRASG12C mutant sequence using Mascot.

# **7. Target Occupancy (TO) Assay**

To measure target engagement of KRASG12C inhibitors *in vivo*, an LC-MS-based target occupancy assay (18) was developed using parallel reaction monitoring (PRM)*.* Protocols and reagents for the sample preparation were based on the PreOmics iST KIT (PreOmics, P.O.00027). Frozen tumor samples were pulverized using CryoPrep (Covaris, Woburn, Massachusetts, USA) and diluted in LYSE buffer (PreOmics) to a final concentration of 20 mg tissue per mL, corresponding to roughly 1 mg/ml protein. Lysis, digestion and peptide clean-up were performed with the iST kit. As internal standards, stable isotope-labeled peptides (SpikeTides L, JPT) were spiked in at the digestion step. The following peptides were used throughout the study: KRASG12C-specific peptide LVVVGACGVGK (1 fmol/µl), the KRAS-specific peptides SFEDIHHYR (6 fmol/µl) and QGVDDAFYTLVR (2 fmol/µl), the K/NRAS peptide SYGIPFIETSAK (5 fmol/µl) and the K/N/HRAS WT peptide LVVGAGGVGK (2 fmol/µl). The final eluted peptide samples were dried and stored at -20°C for analysis by LC-MS

LC-MS analysis was performed on an Exploris 480 (Thermo Scientific) with an Evosep One (Evosep) front-end. Dry peptide samples were resuspended and roughly 1 μg was loaded onto the Evosep tips according to the manufacturers’ instruction and analyzed using a 22 minutes gradient (Evosep 60 SPD method). Data was acquired using a scheduled PRM method, targeting the precursor masses of the internal standards and their endogenous counterparts. The instrument method was based on a top time method with a 3 sec cycle with an MS1 resolution of 30,000, a maximum injection time of 50 ms and a normalized AGC target of 100%. PRM (targeted MS2) scans were acquired at 60,000 resolution, a maximum injection time of 100 ms, a 0.7 m/z isolation window with a 0.25 m/z offset, a normalized AGC target of 100% and stepped normalized collision energy of 25, 27, 30. The raw data was analyzed using Skyline version 4.2 (University of Washington). Peak areas were obtained by summing at least three fragment ions for each precursor. Relative quantities of the free G12C peptide were calculated from the light/heavy (L/H) ratios and normalized to the the K/N/HRAS WT peptide LVVGAGGVGK (MIA PaCa-2 or NCI-H2122) or the KRAS-specific peptide QGVDDAFYTLVR (LU99) analogous to the described method (18). The free KRASG12C levels were then expressed as a % compared to the vehicle control within each experiment.

# **8. Pharmacokinetic-Target Occupancy (PK/TO) Modeling**

## 8.1 Overview

A pharmacokinetic-pharmacodynamic model was constructed to relate the PK of JDQ443 in mouse blood to the observed target occupancy in MIA PaCa-2, NCI-H2122 and LU99 CDX mice. First, a pop-PK model was fitted to oral PK data in mice with a one-compartment model with linear elimination and linear absorption. The pop-PK model in mice was estimated using Monolix2020R1 (Lixoft, Antony, France) and PK model simulations were performed using RsSimulx ([https://cran.r-project.org/package=RsSimulx](https://cran.r-project.org/package%3DRsSimulx)) in R v3.6.1. It was assumed that the target, free KRASG12C, is produced constantly and degraded at a rate proportional to its concentration. The irreversible binding of JDQ443 to the target was modeled using a Hill equation. Furthermore, it was assumed that the turnover rate of the target differs between cell lines, while the binding rate constant of JDQ443 to the target is the same across cell lines. While keeping the PK parameters fixed to the estimated population average, single-dose TO data from MiaPaCa-2, H2122 and LU99 were fitted using dMod package in R (19). Model simulations of TO were done in R via dMod and plotted in GraphPad Prism v9.2.1.  The TO model parameter estimates and their uncertainties were used to perform simulations of free KRASG12C levels in the CDX models upon repeat dosing.

## 8.2 Model assumptions

We described the oral PK of JDQ443 using a one-compartment PK model with linear elimination and linear absorption (with F of 0.43). Although KRASG12C cycles between the GTP and GDP states and is influenced by multiple feedback loops, we abstracted the complex biology with a simple turnover model. The state variable *fKRASG12C:GDP* is the percent of free KRASG12C:GDP relative to its initial concentration. We model its production with a zero-order rate constant (*kin*) and its degradation with a first-order rate constant (*kout*). Since tumor models can differ in their underlying MAPK signaling, we assume that the turnover of the target differs between tumor cell models. The free drug (*fCc*) in blood is assumed to bind to *fKRASG12C:GDP* irreversibly and prevents downstream signaling. We modeled the binding of the drug to the target using a Hill equation. The equation that describes the turnover and drug targeting is



Where n is the Hill coefficient. We assume that the initial condition of *fKRASG12C:GDP*prior to treatment of drug (denoted as *fKRASG12C:GDP*(0)) is 100%*.* Using steady state assumptions, we can derive *kout=kin/ fKRASG12C:GDP*(0)*.* The model scheme is denoted below*.*

|  |
| --- |
| ***Model scheme for the PK/TO fit to H2122 and MiaPaCa-2 xenograft models. Free drug in blood (fCc) -- calculated by accounting for the free fraction in blood (fub)-- is assumed to bind to the target (fKRAS G12C:GDP).*** C:\Users\dharmle1\AppData\Local\Microsoft\Windows\INetCache\Content.Word\ModelSupportingJDQ443.png |

## 8.3 Parameter estimation

The population-PK model was first fit to data obtained after single and repeated oral dosing in mice using Monolix2020R1 (Lixoft, Antony, France). The model was estimated with good certainty and predicted data from the microinfusion pump study well (below)

*PK parameters estimated from mice (assuming 20 g weight)*

| **Parameter** | **Population average estimate** | **Relative standard error (% RSE)** | **Inter-individual standard deviation** **(IISD)** | **IISD (% RSE)** |
| --- | --- | --- | --- | --- |
| ka (1/h) | 2.74 | 10.4 | - | - |
| V (L)Cl (L/h) | 0.190.06 | 9.044.71 | 0.410.21 | 16.5FIXED |
| Measurement error model parameter (proportional, normal error model) | 0.57 | 6.35 |  |  |

|  |
| --- |
| ***The pop-PK model estimated from oral PK data predicts data from the microinfusion pump experiment done in Lu99 xenograft model. The prediction quantiles are calculated using 200 simulations with the pop-PK model inferred in mice. A transit compartment with transit time of 2.5 h was included in the model to account for the time taken for the drug to reach the site of administration from the pump.*** Chart  Description automatically generated |

Keeping the PK parameters fixed to the estimated population average, we fitted the target occupancy (TO) model to the data from single dose PK-TO experiments in MiaPaCa-2, H2122 and LU99. We estimated a cell-line/ tumor-specific target turnover and drug-specific potency parameters (below). Notice that the confidence interval for the resynthesis rate constants of LU99 and NCI-H2122 overlap considerably. The 99% prediction intervals calculated based on the parameter uncertainty describe the data points well (below).

*TO model parameter estimates*

| Parameter | Estimate | 95% CI\* |
| --- | --- | --- |
|  |  | Lower limit | Upper limit |
| kinH2122 (%/h) | 3.33 | 2.71 | 4.09 |
| kinMiaPaCa2 (%/h)kinLU99(%/h) | 0.845.07 | 0.673.88 | 1.046.99 |
| kcat (1/h) | 0.68 | 0.58 | 0.82 |
| KI (µM) | 0.03 | 0.02 | 0.03 |
| fub (%), fraction unbound in bloodn | 1.98 (*in vitro* data)2 (FIXED) |  |  |

\*95% confidence intervals calculated based on profile-likelihood method in dMod (19). The residual standard deviation at any given time was modelled with the equation$ \sqrt{a^{2}+(b×fKRAS^{G12C:GDP } )^{2} }$ . a and b were estimated to be 13.3 and 0.154, respectively.

|  |
| --- |
| ***TO model fits to single-dose in vivo pharmacodynamics/ target occupancy data. Solid line and shaded region show the model fit and associated uncertainty (based on parameter confidence intervals) in the prediction, red points correspond to the data used for estimation.*** |

# **9. *DUSP6* qRT-PCR**

Frozen tumor samples were pulverized CryoPrep according to manufacturer instructions (Covaris, Woburn, Massachusetts, USA), processed using the QIAshredder kit (Qiagen, #79656) and RNA was extracted using the RNeasy mini kit (Qiagen, #74106) according to the manufacturer’s manual. RNA concentration was determined using a NanoDrop microvolume spectrophotometer and fluorometer (Thermo Fisher Scientific) at 260/280 nm. Samples were adjusted with RNase free water to 10 ng/µL final concentration. 40 ng/well of RNA were transferred into each well of a 384-well clear optical reaction plate (Applied Biosystems, #4309849). 5 µL of a 2x concentrated One Step RT qPCR Mastermix Plus (Eurogentec, #RT-QPRT-032X), 0.5 µL primer and probes 20x concentrated were added into a sterile tube, to prepare a single reaction mix. For each target gene, a separate mastermix was prepared using: DUSP6, Life Technologies, #Hs00169257\_m1; ATP5B, Life Technologies, #Hs00969569\_m1; CYC1, Life Technologies, #Hs00357717\_m1; HPRT1, Life Technologies, #Hs02800695\_m1. 0.05 µL of the Euroscript RTase and RNase inhibitor mix 200x and 0.45 µL RNase free water were added to the mastermix, and 6 µL of the mastermix were transferred to the corresponding wells of the 384 well plate. The plate was sealed with a MicroAmp clear adhesive film (Applied Biosystems, #4311971) and finally measured on the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific), using the following protocol: Stage 1, 48°C for 30 min; Stage 2: 95°C for 10 min; Stage 3: 95°C for 15 sec and 60°C for 1 min, 40 x of stage 3. The analysis of the experiment was performed using the qPCRGeneStabM in-house software, which is based on the publication by Vandesompele et al (2002) (20), by normalizing each sample on 3 housekeeping genes (ATP5B, CYC1, HPRT1). For DUSP6 assessment from cell lines (Fig. S7-B), samples were prepared as described above expect that the iTaq Universal Probes One-step kit (BioRad #172-5141) was used. In brief, a mastermix was prepared with 2x concentrated iTAq universal probes reaction mix, iScript advanced reverse transcriptase, DUSP6 gene primer/probe mix (Life Technologies #Hs00169257\_m1) and ACTB (human actin β), housekeeping gene, primer/probe mix (IDT #Hs.PT.39a.22214847). 8 µL of the mastermix were transferred to each well-containing RNA of the 384-well plate. Cycling conditions: Step1: 50°C / 10 minutes (reverse transcription); Step 2: 95°C / 3 minutes; Step 3: 95°C / 15 seconds; Step 4: 60°C / 1 minute. Steps 3 and 4 were repeated 40 cycles. The raw Ct values for the target gene DUSP6 were normalized to the respective housekeeping ACTB gene values.

# **10. Resistance Analysis Methods**

## 10.1 Cell lines and KRASG12C Inhibitors

The Ba/F3 cell line is a murine pro-B-cell line and is cultured in RPMI 1640 (Bioconcept, #1-41F01-I) supplemented with 10% FBS (BioConcept, #2-01F30-I), 2 mM Sodium pyurvate (BioConcept, # 5-60F00-H), 2 mM stable Glutamine (BioConcept, # 5-10K50-H), 10 mM HEPES (BioConcept, # 5-31F00-H) and at 37°C with 5% CO2, except as otherwise indicated. The parental Ba/F3 cells were cultured in the presence of 5 ng/ml of recombinant murine IL-3 (Life Technologies, #PMC0035). Ba/F3 cells are normally dependent on IL-3 to survive and proliferate, however, by expressing oncogenes they are able to switch their dependency from IL-3 to the expressed oncogene (21)

## 10.2 Individual plasmid mutagenesis and generation of Ba/F3 stable cell lines

QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent; # 210519) was used to generate the resistant mutations on the pSG5\_Flag-(codon optimized) KRASG12C\_puro plasmid template and sequences were confirmed by sanger sequencing.

|  |  |
| --- | --- |
| **Primer**  | **Primer sequence** |
| H95R\_forward | 5'-gtcatttgaagatatccaccgttatcgcgagcagattaaga-3' |
| H95R\_reverse | 5'-tcttaatctgctcgcgataacggtggatatcttcaaatgac-3' |
| H95Q\_forward | 5'-tcatttgaagatatccaccagtatcgcgagcagattaagag-3' |
| H95Q\_reverse | 5'-ctcttaatctgctcgcgatactggtggatatcttcaaatga-3' |
| H95D\_forward | 5'-agtcatttgaagatatccacgattatcgcgagcagattaag-3' |
| H95D\_reverse | 5'-cttaatctgctcgcgataatcgtggatatcttcaaatgact-3' |
| MR68S\_forward | 5'-gaagagtactccgcaatgagcgatcaatacatgaggacg-3' |
| R68S\_reverse | 5'-cgtcctcatgtattgatcgctcattgcggagtactcttc-3' |
| Y96C\_forward | 5'-cgaagtcatttgaagatatccaccattgtcgcgagcagatta-3' |
| Y96C\_reverse | 5'-taatctgctcgcgacaatggtggatatcttcaaatgacttcg-3' |
| Y96D\_forward | 5'-cgaagtcatttgaagatatccaccatgatcgcgagcagatt-3 |
| Y96D\_reverse | 5'-aatctgctcgcgatcatggtggatatcttcaaatgacttcg-3' |

The mutant plasmids were transfected into the Ba/F3 WT cells by electroporation with the NEON transfection kit (Invitrogen, #MPK10025). Therefore, two million Ba/F3 cells have been electroporated with 10 μg pf plasmids with the NEON System (Invitrogen, #MPK5000), using following conditions Voltage (V) 1635, Width (ms) 20, Pulses 1. After 72 hours of electroporation, puromycin selection was performed at 1 μg / ml to generate stable cell lines.

## 10.3 IL-3 withdrawal

Ba/F3 cells are normally dependent on IL-3 to survive and proliferate, however, by expressing oncogenes they are able to switch their dependency from IL-3 to the expressed oncogene.

To assess if the KRASG12C single and double mutants are able to sustain the proliferation of Ba/F3 cells, the engineered Ba/F3 cells expressing the mutant constructs were cultured in absence of IL-3. Cell number and viability was measured every three days and after seven days the IL-3 withdrawal was completed. The expression of the mutants after the IL-3 withdrawal were confirmed by Western Blot (data not shown, an upwards shift was observed for KRASG12C/R68S).

## 10.4 Drug response curves for KRASG12C inhibitors and validation of resistance mutations

1000 Ba/F3 cells/well were seeded at in 96-well plates (Greiner Bio-One, #655098). Treatment was performed on the same day with a Tecan D300e drug dispenser. Viability was detected on the same day of treatment for the start plate (Day 0) and three days post-treatment (Day 3) using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, #G7573) on a Tecan infinity M200 Pro reader (Integration Time 1000ms).

To determine the growth, the three days post-treatment (Day 3) readout was normalized to start plate (Day 0). The percentage viability was then calculated by normalizing treated wells to DMSO treated control samples. XLfit was used to make the fitted curve with a Sigmoidal Dose-Response Model (four-parameter curve).

## 10.5 Western blot

After treatment with the different compounds at the indicated concentrations and for the indicated time, the cells were collected, pelleted and snap frozen at - 80°C. Sixty μL of lysis buffer (50 mM Tris HCl, 120 mM NaCl, 25 mM NaF, 40 mM β-glycerol phosphate disodium salt pentahydrate, 1% NP40, 1 μM microcystin, 0.1 mM Na3VO3, 0.1 mM PMSF, 1 mM DTT and 1 mM benzamidine, supplemented with 1 protease inhibitor cocktail tablet (Roche) for 10 mL of buffer) was added to each sample. The samples were then vortexed, incubated on ice for 10 min, vortexed again and centrifuged at 14000 rpm at 4°C for 10 min. Protein concentration was determined with the BCA Protein Assay kit (Pierce, #23225). After normalization to the same total volume with lysis buffer, NuPAGE™ LDS Sample buffer 4X (Invitrogen, #NP0007) and NuPAGE™ Sample reducing agent 10X (Invitrogen, #NP0009) was added. The samples were heated at 70°C for 10 minutes before loading on a NuPAGE™ Novex™ 4 – 12% Bis-Tris Midi Protein Gel, 26 - wells (Invitrogen, #WG1403A). Gels were run for 45 minutes at 200 V (PowerPac HC, Biorad) in NuPAGE MES SDS running buffer (Invitrogen, #NP0002). The proteins were transferred for 7 minutes at 135 mA per gel on a Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs membrane (Biorad, #1704159) using the Trans-Blot® Turbo™ system (Biorad) before staining the membrane with Ponceau red (Sigma, # P7170). The membranes were blocked with TBST with 5% of milk at RT. Anti-RAS (Abcam, #108602) and anti-phospho-ERK 1/2 p44/42 MAPK (Cell Signaling, 4370) antibodies were incubated overnight at 4°C, the anti-vinculin (Sigma, V9131) and anti-α-tubulin (Sigma, T6199) antibodies were incubated for 1 hour at RT. Membranes were washed 3X for 5 minutes with TBST and the anti-rabbit (Cell Signaling, 7074) and anti-mouse (Cell Signaling, #7076) secondary antibodies were incubated for 1 hour at RT. All antibodies were diluted in TBST to 1/1000, except for anti-vinculin and anti-α-Tubulin (1/3000). Revelation was performed with WesternBright ECL (Advansta, # K-12045-D20) for Ras and vinculin and with SuperSignal West Femto maximum sensitivity substrate (Thermo Fischer, #34096), on a Fusion FX (Vilber Lourmat) using the FusionCapt Advance FX7 software. For Fig. S7A, Cells were lysed using MPER lysis buffer (Pierce #78501) supplemented with protease and phosphatase inhibitor cocktail tablets (Roche #04 693 124 001, Roche #04 906 837 001) on ice for 30 minutes. Lysates were clarified by centrifugation at 12000x g for 15 minutes and protein concentration was determined using the DC protein assay reagents (BioRad #500-0116) and a BSA standard. For immunoblotting, 25 µg of protein lysates were loaded into 4-12% NuPAGE Bis-Tris gels (Novex #WG1402BX10) and blotted onto PVDF membranes. Primary antibodies were added overnight at 40°C with rocking. The next day, filters were washed with 1xPBS + 0.1% Tween-20 and secondary antibodies were added for 30 minutes at room temperature with shaking. After secondary incubation, the filters were washed and SuperSignal detection system (Thermo Scientific #34076) added prior to image acquisition with Fusion FX reader (Vilber).

Primary antibodies: mouse monoclonal antibody anti-KRAS (clone 3B10-2F2), Novus Biologicals #H00003845-M01; polyclonal antibody anti-phospho-p44/42 MAPK (Erk1/2), Cell Signaling Technologies #9101; polyclonal antibody anti-p44/42 MAPK (Erk1/2), Cell Signaling Technologies #9102; polyclonal antibody anti-pRSK3 (Thr356/Ser360), Cell Signaling Technologies #9348; rabbit monoclonal antibody anti-RSK1/RSK2/RSK3 (clone 5G10), Cell Signaling Technologies #9355.

Secondary antibodies: goat anti-rabbit-HRP, DAKO #P0448; sheep anti-mouse-HRP, Amersham GE Healthcare #NA931.

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