A potent and selective quinoxalinone-based STK33 inhibitor does not show synthetic lethality in KRAS-dependent cells

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Kinase Assays:

Kinases and Substrates
STK33 Kinase (Catalog no. 14-671-K) and Myelin Basic Protein (MBP; Catalog no. 13-104) were obtained from Millipore. ADP Glo Assay Kit (Catalog no. V9102) was obtained from Promega. PKA Kinase (Catalog no. P2912) was acquired from Invitrogen and Peptide-21 (Catalog no. 760365) was acquired from Caliper Life Sciences.

Other Reagents
3-(N-morpholino)propanesulfonic acid (MOPS, Catalog no. BDH4171) was obtained from VWR Scientific. BSA (Catalog no. ABRP) was obtained from ICP Biologicals; EDTA (Catalog no. 3624-19) and BME (Catalog no. 40409-00) were purchased from JT Baker. NaOH (Catalog no. SX0590-3) was obtained from EMD Scientific. Brij-35 (Catalog no. DG503) was acquired from G-Biosciences. Glycerol (Catalog no. 327255000) was purchased from Acros.

Cell Lines
Four cell lines were used in this study. Two KRAS dependent AML derived cell lines, NOMO-1 and SKM-1, were compared with two KRAS independent lines, THP-1 and U937, to test the hypothesis that inhibition of STK33 with the probe compounds ML280 and ML281 would lead to KRAS dependent cell death as described (18). All cell lines were the kindly provided by Gary Gilliland (Bringham and Women’s Hospital, Boston).
**STK33 Kinase ADP-Glo Assay:**

For high-throughput screening (HTS), the STK33 Kinase ADP-Glo Assay was run in white, 1536-well (Aurora Catalog no. 19210) assay-ready plates. Assay-ready plates contained predispensed compound (2.5 nl of 10 mM compound/well) in DMSO.

Briefly, 2 µl assay buffer (10 mM MOPS pH 7, 300 µM EDTA, 0.5% (V/V) Glycerol, 0.001% (V/V) Brij-35, 0.1 mg/ml BSA, 0.01% (V/V) beta mercaptoethanol) was added to all but the no-enzyme control wells using a Combi-nL Dispenser (Thermo).

Next, 2 µl of STK33/MBP (50 nM STK33 plus 0.5 mg/ml Myelin Basic Protein [MBP] in assay buffer) only was added to all other wells. The compounds were pre-incubated at room temperature with the enzyme for 15 minutes before initiation of the kinase reaction by the addition of 0.5 µl ATP/Mg (250 µM ATP, 50 mM MgAc₂ in assay buffer). The reaction was allowed to proceed for 1 hour followed by luminous readout using the ADP-Glo Assay kit per the manufacturer’s directions. Luminescence (15-second integration time) was measured using a ViewLux CCD-based imaging plate reader (Perkin-Elmer).

For dose-response experiments, the STK33 Kinase ADP-Glo Assay was run in 384-well plates (Aurora Catalog no.3052) as the 1536-well HTS assay but at 10 times the volume. STK33/MBP solution was introduced to the plates first. Using a pin tool, 25 nl of compound in DMSO stock solution was transferred into the assay plates. The plates were incubated for 15 minutes at room temperature followed by initiation of the reaction by the addition of ATP/Mg. After 1 hour, luminescence was read using the ADP-Glo Assay kit per the manufacturer’s directions with an EnVision multimode plate reader (Perkin-Elmer).

**Assay Buffer**
- 10 mM MOPS pH 7
- 300 µM EDTA
- 0.5% (V/V) Glycerol
- 0.001% (V/V) Brij-35
- 0.1 mg/ml BSA
- 0.01% (V/V) beta mercaptoethanol

**ATP/Mg**
- 250 µM ATP
- 50 mM MgAc₂ in assay buffer

**STK33/MBP**
- 50 nM STK33
- 0.5 mg/ml Myelin Basic Protein (MBP) in assay buffer

**1536-well Plate Assay**
1. Add 2.0 µl assay buffer to the last two columns of 1536-well, white Aurora high-base assay plate (containing 2.5 nl 10 mM compound or DMSO) using Combi-nL dispenser (Thermo).
2. Add 2.0 µl STK33/MBP to all but last two columns of 1536-well assay ready plates (containing 2.5 nL 10 mM compound/well).
3. Incubate 15 minutes at room temperature.
4. Add 0.5 µl ATP/Mg/well to full plate. Incubate 60 minutes followed by luminescent readout using the ADP-Glo Assay kit per the manufacturer’s directions
5. Add 2.5 µl/well ADP Glo Reagent I (Promega). Incubate 40 minutes.
6. Add 5 µl/well ADP Glo Reagent II (Promega). Incubate 30 minutes.
7. Read luminescence (15 sec integration time) on ViewLux CCD-based imaging plate reader (Perkin Elmer).

384-well Plate Assay

1. Add 20 µl assay buffer to the last column of the 384-well, white Aurora assay plate using Combi dispenser (Thermo).
2. Add 20 µl STK33/MBP to all but last column.
3. Using a pintool, transfer 25 nl compound in DMSO stock solution into assay plate.
4. Incubate 15 minutes at room temperature.
5. Add 5 µl ATP/Mg/well to full plate. Incubate 60 minutes.
6. Add 25 µl/well ADP Glo Reagent I (Promega). Incubate 40 minutes.
7. Add 50 µl/well ADP Glo Reagent II (Promega). Incubate 30 minutes.
8. Read luminescence (0.1 sec integration time) on Envision plate reader (Perkin Elmer).

PKA Kinase Caliper Assay:

Inhibition of PKA activity was measured using a recombinant His-tagged PKA catalytic fragment and a fluorescently-labeled peptide substrate in an end-point readout assay of compounds of interest at various concentrations.

In a 384-well polypropylene plates, purified PKA (20 µl/well) was mixed together with 15 µM ATP, 15 mM MgAc₂ in assay buffer (10 mM MOPS pH 7, 300 µM EDTA, 0.5 % (V/V) Glycerol, 0.001% (V/V) Brij-35). Using a pin tool, 25 nl of compound dilutions in DMSO were added. Next, 5 µl of 5 nM PKA and 15 µM of Peptide 21 in assay buffer were added. The plates were incubated at room temperature for 1 hour followed by the addition of 1.5 nM PKA, 3 µM Peptide 21 (Fluorescent Peptide Substrate), and 12 µM ATP (final conditions). The enzymatic reaction was allowed to proceed, and then was stopped by the addition of the 10 µl of 75 mM metal chelator EDTA.

Percent (%) conversion of substrate to product (i.e., peptide to phosphor-peptide) was quantitated using a capillary electrophoresis lab-on-a-chip (LOC) device (Caliper EZ-Reader with 12-channel Sipper chip, Caliper Life Sciences) at the conditions specified by the manufacturer for Peptide 21.
**Assay Buffer**

10 mM MOPS pH 7  
300 µM EDTA  
0.5 % (V/V) Glycerol  
0.001% (V/V) Brij-35

1. In a 384-well polypropylene plate, add 20 µl / well 15 µM ATP, 15 mM MgAc2 in assay buffer.  
2. Using a pintool, add 25 nl compound dilutions in DMSO.  
3. Add 5 µl 5 nM PKA, 15 µM Peptide 21 in assay buffer.  
4. Incubate the plates for 1 hour at room temperature followed by the addition of 1.5 nM PKA, 3 µM Peptide 21 (Fluorescent Peptide Substrate), and 12 µM ATP (final conditions).  
5. Stop the enzyme reaction by adding 10 µl 75 mM EDTA in assay buffer.  
6. Determine percent (%) conversion of peptide to phosphor-peptide using Caliper capillary electrophoresis instrument at the conditions specified by the manufacturer for Peptide 21.

**Aurora B Kinase Radiolabel Binding Assay:**

The Aurora B (AurB) kinase assay was outsourced to Millipore Corporation. The inhibition of AurB activity was measured in response to various concentrations of the compound of interest based on a filter-binding isotopic assay.

1. Incubate Aurora-B(h) with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 30 µM AKRRRLSSLRA, 10 mM MgAcetate, and [gamma-32P-ATP] (specific activity approx. 500 cpm/pmol, concentration as required).  
2. Initiate the reaction by adding MgATP mix.  
3. Incubate the plates for 40 minutes at room temperature.  
4. Stop the reaction by adding a 3% phosphoric acid solution.  
5. Spot 10 µl of the reaction onto a P30 filtermat and wash three times for 5 minutes in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

**Cell Based Assays:**

NOMO-1, SKM-1, THP-1, U937 were kindly provided by Gary Gilliland (Brigham and Women’s Hospital, Boston). All cell lines were maintained under the manufacturer’s recommended standard conditions. Cells were plated in 384-well plates at optimized densities and incubated overnight prior to treatment. Compounds were added to cells using a CyBio Well Vario after which cells were cultured under standard condition for 72 h. Cell viability was measured using CellTiter Glo luminescence (Promega) and readout
using an LJL Biosystems Analyst microplate reader. Raw numbers were the normalized to the median of the DMSO treated wells for each plate.

**Experimental Procedures for Analytical Assays:**

**Solubility**

Solubility was determined in phosphate buffered saline (PBS) pH 7.4 with 1% DMSO. Each compound was prepared in duplicate at 100 µM in both 100% DMSO and PBS with 1% DMSO. Compounds were allowed to equilibrate at room temperature with a 250-rpm orbital shake for 24 hours. After equilibration, samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer. The DMSO samples were used to create a two-point calibration curve to which the response in PBS was fit.

**Plasma Protein Binding**

Plasma protein binding was determined by equilibrium dialysis using the Rapid Equilibrium Dialysis (RED) device (Pierce Biotechnology, Rockford, IL) for both human and mouse plasma. Each compound was prepared in duplicate at 5 µM in plasma (0.95% acetonitrile, 0.05% DMSO) and added to one side of the membrane (200 µl) with PBS pH 7.4 added to the other side (350 µl). Compounds were incubated at 37°C for 5 hours with a 250-rpm orbital shake. After incubation, samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

**Plasma Stability**

Plasma stability was determined at 37°C at 5 hours in both human and mouse plasma. Each compound was prepared in duplicate at 5 µM in plasma diluted 50/50 (v/v) with PBS pH 7.4 (0.95% acetonitrile, 0.05% DMSO). Compounds were incubated at 37°C for 5 hours with a 250-rpm orbital shake with time points taken at 0 and 5 hours. Samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.
Experimental Procedures for the Synthesis of the Probes:

General details. All reagents and solvents were purchased from commercial vendors and used as received. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker 300 MHz or Varian UNITY INOVA 500 MHz spectrometer as indicated. Proton and carbon chemical shifts are reported in ppm ($\delta$) relative to tetramethylsilane ($\delta = 0$ for both $^1$H and $^{13}$C), CDCl$_3$ solvent ($^1$H $\delta$ 7.26, $^{13}$C $\delta$ 77.0) or DMSO-d$_6$ solvent ($^1$H $\delta$ 2.50, $^{13}$C $\delta$ 39.5). NMR data are reported as follows: chemical shifts, multiplicity (obs. = obscured, br = broad, s = singlet, d = doublet, t = triplet, m = multiplet); coupling constant(s) in Hz; integration. Unless otherwise indicated, NMR data were collected at 25°C. Flash chromatography was performed using 40-60 $\mu$m Silica Gel (60 Å mesh) on a Teledyne Isco Combiflash Rf system. Tandem Liquid Chromatography/Mass Spectrometry (LC/MS) was performed on a Waters 2795 separations module and 3100 mass detector. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F plates. Visualization was accomplished with ultraviolet (UV) light and aqueous potassium permanganate (KMnO$_4$) stain followed by heating.

Synthesis of ML281 (7r):

![Scheme S1. Synthesis of ML281](image)

5-Isopropyl-1-(thiophene-2-carbonyl)indoline-2,3-dione (S1): 5-Isopropylindoline-2,3-dione (100 mg, 0.529 mmol) was dissolved in DCM under inert atmosphere and Hunig’s base (0.184 ml, 1.057 mmol) was added. The mixture was cooled to 0°C, and thiophene-2-carbonyl chloride (0.062 ml, 0.581 mmol) was added dropwise. After 10 minutes at 0°C, the mixture was left to warm to room temperature overnight. LCMS showed selective formation of the desired product, and the mixture was extracted using DCM/aq.HCl 0.1N. Concentration of the organic phase afforded 159 mg of 6r (0.529 mmol, 100%) which was used in the next step without further purification. LRMS [M + H$^+$]: 300.34.

N-(4-isopropyl-2-(3-oxo-3,4-dihydroquinoxalin-2-yl)phenyl)thiophene-2-carboxamide (7r): 5-Isopropyl-1-(thiophene-2-carbonyl)indoline-2,3-dione 6r (159 mg, 0.529 mmol) was dissolved in a mixture of acetic acid/water 10/1 (2 ml), heated at 80°C, and benzene-1,2-diamine (61 mg, 0.567 mmol) was added. After 5 minutes, a yellow precipitate was observed and heating was continued for 15 minutes. After cooling the mixture to room temperature, filtration followed by successive washing with acetic acid (3 ml), acetic acid/water 1/1 (5 ml), and water (10 ml) afforded 7r, 133 mg as a yellow
powder (0.341 mmol, 64%). $^1$H NMR (500 MHz, DMSO-d$_6$): δ 12.66 (s, 1H), 10.48 (s, 1H), 7.90-7.75 (m, 3H), 7.72 (d, $J = 3.2$ Hz, 1H), 7.69 (d, $J = 2.1$ Hz, 1H), 7.6-7.45 (m, 1H), 7.41 (dd, $J = 8.4$ Hz, $J = 2.1$ Hz, 1H), 7.38-7.27 (m, 2H), 7.15 (dd, $J = 4.9$ Hz, $J = 3.8$ Hz, 1H), 2.96 (sept, $J = 6.9$ Hz, 1H), 1.25 (d, $J = 6.9$ Hz, 6H); $^{13}$C NMR (125 MHz, DMSO-d$_6$): δ 159.34, 156.43, 154.62, 143.88, 139.83, 134.12, 132.05, 131.87, 131.42, 130.35, 129.01, 128.61, 128.41, 128.21, 127.90, 127.69, 124.08, 123.40, 115.22, 32.94, 23.82. LRMS [M + H]$^+$: 390.05; HRMS [M + H]$^+$: calc for C$_{22}$H$_{20}$N$_3$O$_2$S 390.1271 found 390.1277.

Experimental Procedure for the synthesis of Amgen Compound 1:

The Amgen compound 1 was synthesized in our group in order to be used as the positive control throughout the project (Scheme S2). Briefly, 1-methylpiperazine was condensed with 1-chloro-2-methoxy-4-nitrobenzene to afford the intermediate S2, which was hydrogenated to the corresponding aniline S3. In parallel, 6-chloro-1H-pyrazolo[3,4-d]pyrimidine was coupled with 4-methoxyphenylboronic acid in the presence of copper (II) acetate and pyridine to afford intermediate S4, which was characterized by X-ray crystallography (Figure S1). The crystal structure confirmed that the Cu(II) mediated N-arylation proceeded chemoselectively to deliver compound S4. Intermediate S4 was then condensed with the aniline S3 in the presence of hydrochloric acid to afford Compound 1.
1-(2-Methoxy-4-nitrophenyl)-4-methylpiperazine (S2): 1-(2-methoxy-4-nitrophenyl)-4-methylpiperazine (500 mg, 2.67 mmol) and 1-methylpiperazine (5.9 ml, 53.3 mmol) were heated at 100°C for 20 h. After cooling to room temperature, the excess methylpiperazine was evaporated and 20 ml of HCl 1M was added. The aqueous phase was washed with DCM (2 times 20 ml), basified using NaOH 2M (15 ml), and re-extracted using DCM (3 times 30 ml). The organic phase was concentrated and afforded 194 mg of intermediate S2 (0.772 mmol, 29%). LRMS [M + H]^+: 252.56.

3-Methoxy-4-(4-methylpiperazin-1-yl)aniline (S3): 1-Chloro-2-methoxy-4-nitrobenzene (194 mg, 0.772 mmol) and 10% Palladium on activated charcoal (25 mg) in ethanol (7.7 ml) were placed under hydrogen atmosphere. After 3 h at room temperature, LCMS showed complete conversion and the mixture was filtered on a pad of celite and concentrated to afford 166 mg of intermediate S3 (0.75 mmol, 97%). LRMS [M + H]^+: 222.32.

6-Chloro-1-(4-methoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidine (S4): 6-chloro-1H-pyrazolo[3,4-d]pyrimidine (200 mg, 1.294 mmol), 4-methoxyphenylboronic acid (295 mg, 1.941 mmol), diacetoxy copper (470 mg, 2.590 mmol), and pyridine (205 mg, 2.590 mmol) in DCM (4.0 ml) were heated in a microwave at 80°C for 15 minutes. After LCMS showed complete conversion, the mixture was filtered on a pad of celite and concentrated. Column chromatography on silica gel using a gradient of ethyl acetate in hexanes afforded 92 mg of intermediate S4 (0.353 mmol, 27%). $^1$H NMR (300 MHz, Acetone-d6): δ 9.30 (s, 1H), 8.55 (s, 1H), 8.05 (d, $J = 9.2$ Hz, 2H), 7.16 (d, $J = 9.2$ Hz, 2H), 3.90 (s, 3H); LRMS [M + H]^+: 261.22.
$N$-(3-Methoxy-4-(4-methylpiperazin-1-yl)phenyl)-1-(4-methoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-6-amine (I): 6-chloro-1-(4-methoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidine (40 mg, 0.153 mmol) and 3-methoxy-4-(4-methylpiperazin-1-yl)aniline (34 mg, 0.153 mmol) in isopropanol (0.3 ml) were sealed in a microwave tube. HCl 4.0M in dioxane (57 µl, 0.230 mmol) was added and the mixture was microwaved at 140°C for 1 h 30 minutes. The mixture was concentrated and purified on silica gel using a gradient of ethyl acetate in DCM to afford 29 mg of compound I (0.065 mmol, 43%) as a white solid. $^1$H NMR (300 MHz, CDCl$_3$): δ 8.87 (s, 1H), 8.04 (s, 2H), 8.01 (s, 1H), 7.89 (s, 1H), 7.60 (s, 1H), 6.99 (d, $J = 8.7$ Hz, 2H), 6.91 (d, $J = 8.7$ Hz, 2H), 3.86 (s, 3H), 3.79 (s, 3H), 3.12 (s, 4H), 2.71 (s, 4H), 2.41 (s, 3H); $^{13}$C NMR (300 MHz, CDCl$_3$): δ 158.06, 153.57, 152.53, 136.66, 134.99, 134.15, 132.13, 123.05, 118.39, 114.19, 111.50, 103.86, 55.57, 55.26, 50.65, 45.93. LRMS [M + H]$^+$: 446.34.
Chemical Characterization Data:

$^1$H NMR Spectrum (CDCl$_3$, 300 MHz) of Amgen’s compound 1

Chemical Formula: C$_{24}$H$_{27}$N$_7$O$_2$

Molecular Weight: 445.52

UPLC Chromatogram of Amgen compound 1
$^1$H NMR Spectrum (500 MHz, DMSO-$d_6$) of ML281 (7r)

Chemical Formula: $C_{22}H_{19}N_3O_2S$
Molecular Weight: 389.47
$^{13}$C NMR Spectrum (125 MHz, DMSO-d$_6$) of ML281 (7r)

UPLC-MS Chromatogram of ML281 (7r)
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 7a

Chemical Formula: C$_{19}$H$_{13}$N$_3$O$_2$S  
Molecular Weight: 347.39

UPLC Chromatogram of 7a
$^1$H NMR Spectrum (DMSO-$d_6$, 300 MHz) of 7b

Chemical Formula: C$_{21}$H$_{15}$N$_3$O$_2$
Molecular Weight: 341.36

UPLC Chromatogram of 7b
\(^1\)H NMR Spectrum (DMSO-\(d_6\), 300 MHz) of 7c

Chemical Formula: C\(_{19}\)H\(_{12}\)ClN\(_3\)O\(_2\)S
Molecular Weight: 381.84

UPLC Chromatogram of 7c

Peak at 7.97e-1
\(^1\)H NMR Spectrum (DMSO-\textsubscript{d6}, 300 MHz) of 7d

UPLC Chromatogram of 7d
$^1$H NMR Spectrum (DMSO-$d_6$, 300 MHz) of 7e

Chemical Formula: $C_{20}H_{14}N_4O_2$
Molecular Weight: 342.35

UPLC Chromatogram of 7e
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 7f

Chemical Formula: $C_{20}H_{14}N_4O_2$
Molecular Weight: 342.35

UPLC Chromatogram of 7f
\(^1\)H NMR Spectrum (DMSO-d\(_6\), 300 MHz) of 7g

Chemical Formula: C\(_{21}\)H\(_{14}\)FN\(_3\)O\(_2\)
Molecular Weight: 359.35

UPLC Chromatogram of 7g
$^1\text{H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 7h}$

Chemical Formula: C$_{21}$H$_{14}$ClN$_3$O$_2$
Molecular Weight: 375.81

UPLC Chromatogram of 7h
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 7i

Chemical Formula: C$_{22}$H$_{17}$N$_3$O$_3$
Molecular Weight: 371.39

LCMS Chromatogram of 7i
\(^1\)H NMR Spectrum (DMSO-\(d_6\), 300 MHz) of 7j

Chemical Formula: \(C_{21}H_{21}N_3O_2\)

Molecular Weight: 347.41

LCMS Chromatogram of 7j
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 7k

Chemical Formula: C$_{19}$H$_{11}$Cl$_2$N$_3$O$_2$S
Molecular Weight: 416.28

UPLC Chromatogram of 7k
\(^1\)H NMR Spectrum (DMSO-d\(_6\), 300 MHz) of 7l

Chemical Formula: C\(_{19}\)H\(_{11}\)F\(_2\)N\(_3\)O\(_2\)S
Molecular Weight: 383.37

UPLC Chromatogram of 7l
$^1$H NMR Spectrum (DMSO-$d_6$, 300 MHz) of 7m

Chemical Formula: C$_{21}$H$_{17}$N$_3$O$_2$S
Molecular Weight: 375.44

UPLC Chromatogram of 7m
\(^1\)H NMR Spectrum (DMSO-\(d_6\), 300 MHz) of 7n

Chemical Formula: \(C_{19}H_{12}ClN_3O_2S\)
Molecular Weight: 381.84

UPLC Chromatogram of 7n
Chemical Formula: C$_{19}$H$_{12}$FN$_3$O$_2$S  
Molecular Weight: 365.38
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 7p

Chemical Formula: $C_{20}H_{15}N_3O_3S$
Molecular Weight: 377.42

UPLC Chromatogram of 7p
\textsuperscript{1}H NMR Spectrum (DMSO-\textit{d}_6, 300 MHz) of 7q

Chemical Formula: $C_{20}H_{12}F_3N_3O_3S$
Molecular Weight: 431.39

UPLC Chromatogram of 7q
$^1$H NMR Spectrum (DMSO-$d_6$, 300 MHz) of 7s

Chemical Formula: C$_{19}$H$_{12}$ClN$_3$O$_2$S
Molecular Weight: 381.84

UPLC Chromatogram of 7s
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 7t

Chemical Structure:

Chemical Formula: C$_{10}$H$_{12}$F$_3$N$_3$O$_2$S
Molecular Weight: 365.38

UPLC Chromatogram of 7t
\textbf{\textsuperscript{1}H NMR Spectrum (CDCl\textsubscript{3}, 300 MHz) of 8a}

Chemical Formula: C\textsubscript{19}H\textsubscript{15}N\textsubscript{3}OS
Molecular Weight: 333.41

\textbf{UPLC Chromatogram of 8a}

3: UV Detector: 210

7.218e-1

Range: 7.217e-1
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 8b

Chemical Formula: $C_{21}H_{17}N_3O$
Molecular Weight: 327.38

UPLC Chromatogram of 8b
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 8c

Chemical Formula: C$_{20}$H$_{16}$N$_4$O
Molecular Weight: 328.37

UPLC Chromatogram of 8c
\(^1\)H NMR Spectrum (DMSO-d\(_6\), 300 MHz) of 8d

Chemical Formula: C\(_{21}\)H\(_{16}\)FN\(_3\)O
Molecular Weight: 345.37

UPLC Chromatogram of 8d
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 8e

Chemical Formula: C$_{18}$H$_{17}$N$_3$O  
Molecular Weight: 291.35

UPLC Chromatogram of 8e
$^1$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 9a

Chemical Formula: C$_{18}$H$_{13}$N$_3$O$_3$S$_2$
Molecular Weight: 383.44

UPLC Chromatogram of 9a
$^{1}$H NMR Spectrum (DMSO-d$_6$, 300 MHz) of 9b

Chemical Formula: C$_{20}$H$_{15}$N$_3$O$_3$S
Molecular Weight: 377.42

UPLC Chromatogram of 9b
Kinase profiling of ML281:

| Kinase                              | % Activity |
|-------------------------------------|------------|
| Abl(h)                              | 115        |
| ALK(h)                              | 92         |
| AMPKα1(h)                           | 113        |
| ASK1(h)                             | 107        |
| Aurora-A(h)                         | 83         |
| Aurora-B(h)                         | 99         |
| CaMKI(h)                            | 104        |
| CaMKIβ(h)                           | 108        |
| CaMKIγ(h)                           | 100        |
| CaMKIδ(h)                           | 102        |
| CaMKIV(h)                           | 116        |
| CDK1/cyclinB(h)                     | 107        |
| CDK2/cyclinA(h)                     | 96         |
| CDK2/cyclinE(h)                     | 98         |
| CDK3/cyclinE(h)                     | 109        |
| CDK5/p25(h)                         | 95         |
| CDK5/p35(h)                         | 103        |
| CDK6/cyclinD3(h)                    | 114        |
| CDK7/cyclinH/MAT1(h)                | 85         |
| CDK9/cyclin T1(h)                   | 101        |
| CHK1(h)                             | 101        |
| CK1γ1(h)                            | 102        |
| CK2α2(h)                            | 121        |
| c-RAF(h)                            | 96         |
| DRAK1(h)                            | 91         |
| eEF-2K(h)                           | 113        |
| EGFR(h)                             | 93         |
| EphA5(h)                            | 112        |
| EphB4(h)                            | 101        |
| Flt3(h)                             | 51         |
| Fyn(h)                              | 103        |
| GSK3β(h)                            | 128        |
| IGF-1R(h)                           | 101        |
| IKKα(h)                             | 104        |
| IR(h)                               | 91         |
| IRAK4(h)                            | 101        |
| JAK2(h)                             | 123        |
| KDR(h)                              | 25         |
| LKB1(h)                             | 76         |
| LOK(h)                              | 103        |
| Lyn(h)                              | 83         |
| MAPK1(h)                            | 92         |
| MAPK2(h)                            | 110        |
| MAPKAP-K2(h)                        | 103        |
| MEK1(h)                             | 112        |
| MKK7β(h)                            | 82         |
| MLK1(h)                             | 92         |
| MnK2(h)                             | 97         |
| MSK2(h)                             | 96         |
| MST1(h)                             | 118        |
| Protein (h)       | Value |
|------------------|-------|
| mTOR(h)          | 85    |
| NEK2(h)          | 93    |
| p70S6K(h)        | 93    |
| PAK2(h)          | 101   |
| PDGFRβ(h)        | 105   |
| PhKγ2(h)         | 96    |
| Pim-1(h)         | 99    |
| PKA(h)           | 108   |
| PKBα(h)          | 120   |
| PKBβ(h)          | 102   |
| PKCa(h)          | 100   |
| PKCβI(h)         | 109   |
| PKCβII(h)        | 95    |
| PKCγ(h)          | 106   |
| PKCδ(h)          | 104   |
| PKCε(h)          | 109   |
| PKCζ(h)          | 108   |
| PKC(1)           | 94    |
| PKCμ(h)          | 97    |
| PKCθ(h)          | 83    |
| PKCζ(h)          | 97    |
| PGIα(h)          | 123   |
| PGIβ(h)          | 95    |
| Ptk3(h)          | 97    |
| PRAK(h)          | 106   |
| ROCK-I(h)        | 122   |
| ROCK-II(h)       | 119   |
| Rse(h)           | 103   |
| Rsk1(h)          | 96    |
| SAPK2a(h)        | 102   |
| SRPK1(h)         | 100   |
| STK33(h)         | 39    |
| TAK1(h)          | 106   |

**Profiling of ML281 in 17 KRAS-dependent (red) and KRAS-independent (blue) cell-lines:**

Cell viability measured by CellTiter Glo 72hrs after treatment. All values are normalized to the median of the DMSO values for each cell line (Positive Control: Staurosporine).

Cell lines used:
**KRAS mut:** MDA-MB-231, KOPN-8, NOMO-1, SKM-1, NB4, RPMI-8226, Karpas-620
**KRAS WT:** PL-21, EOL-1, GDM-1, EJM, U937, MM1S, MOLM-16, Jurkat, THP-1 and OCI-AML3
