A highly potent clickable probe for cellular imaging of MDM2 and assessing dynamic responses to MDM2-p53 inhibition

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Supporting Information

Contents

Supplementary Figures S1-S4, Supplementary Table S1, Supplementary Schemes S1-2.

Experimental procedures for the synthesis of AMG232-TCO and RG7388-TCO, enzymatic assay protocol for the determination of IC_{50} values, cellular assay protocols for the determination of GI_{50} and p53 induction values, protocols for the fluorescence imaging experiments, and procedures for the protein enrichment experiments.

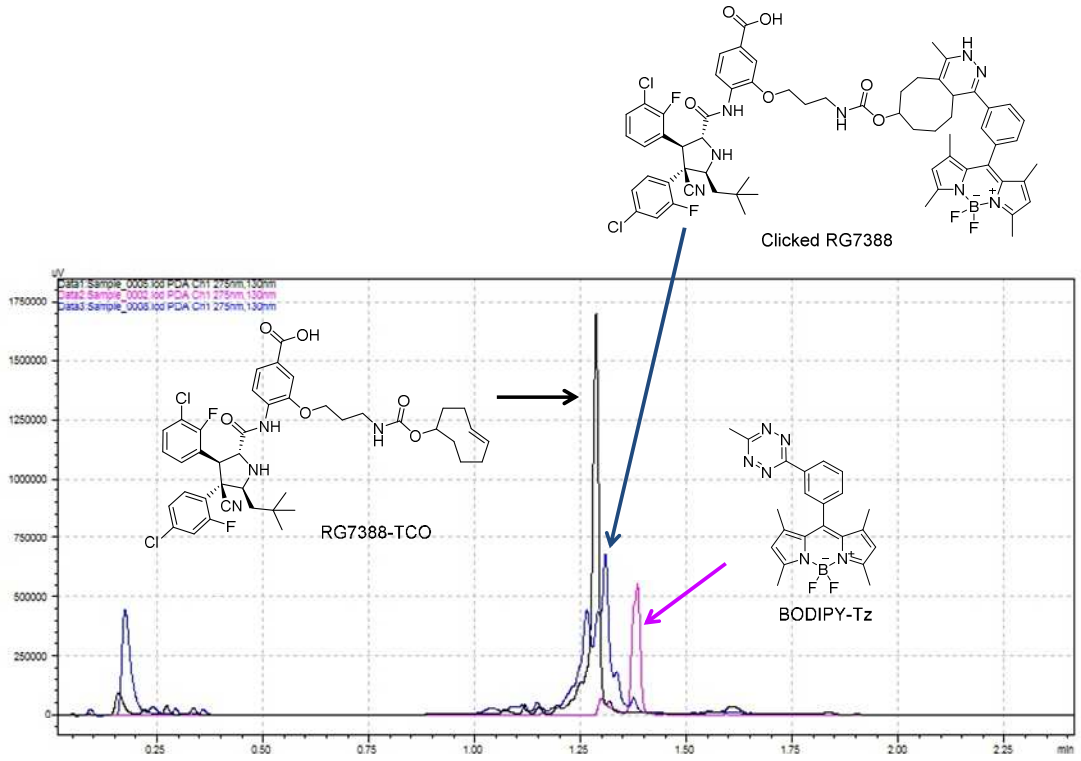
Supplementary Table S1. Physico-chemical properties of MDM2 inhibitors.

Table showing the molecular weight (MW), polar surface area (PSA) and lipophilicity (cLogP) of three MDM2 inhibitors.

	3-imidazolylindole	**AMG232**	**RG7388**
MW (g/mol)	812.2	568.6	616.5
PSA	8.33	6.49	4.97
ClogP	132.79	91.75	111.45

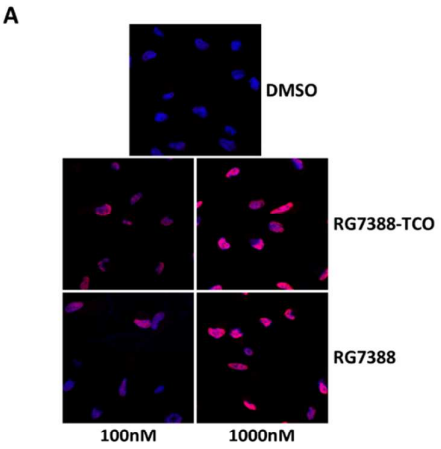
Supplementary Figure S1. LC-MS Profile of the click reaction between RG7388-TCO and BODIPY-Tz.

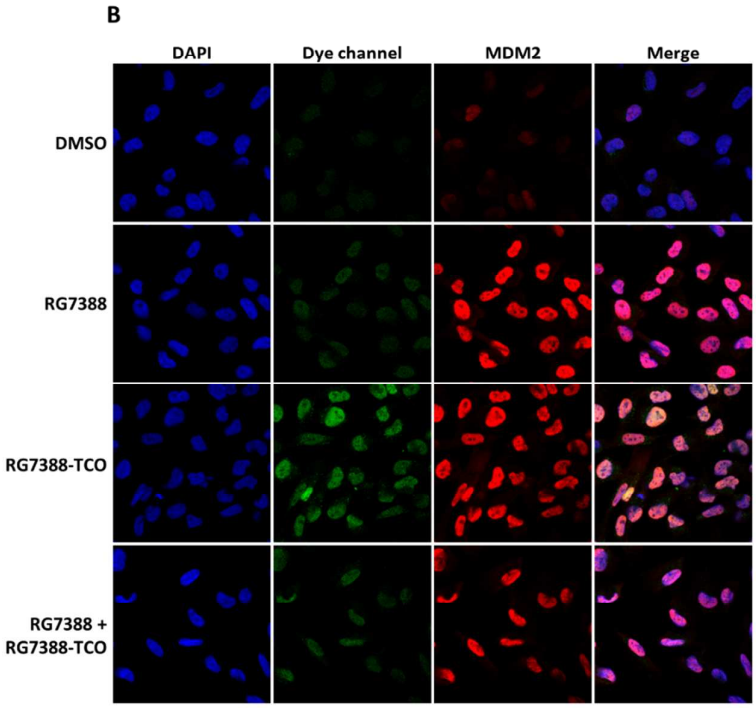
LC-MS profile of the reaction between RG7388-TCO (black) and BODIPY-Tz (in purple) forming Clicked RG7388 (blue). Multiple peaks are observed for Clicked RG7388 due to the presence of diastereoisomers in the cycloaddition product which are not expected to influence binding to MDM2.



Supplementary Figure S2. Control experiments showing the equivalence of MDM2 induction due to RG7388 or RG7388-TCO through MDM2 antibody staining (A) and the co-localisation of BODIPY-TZ bound to RG7388-TCO and MDM2 antibody staining (B).

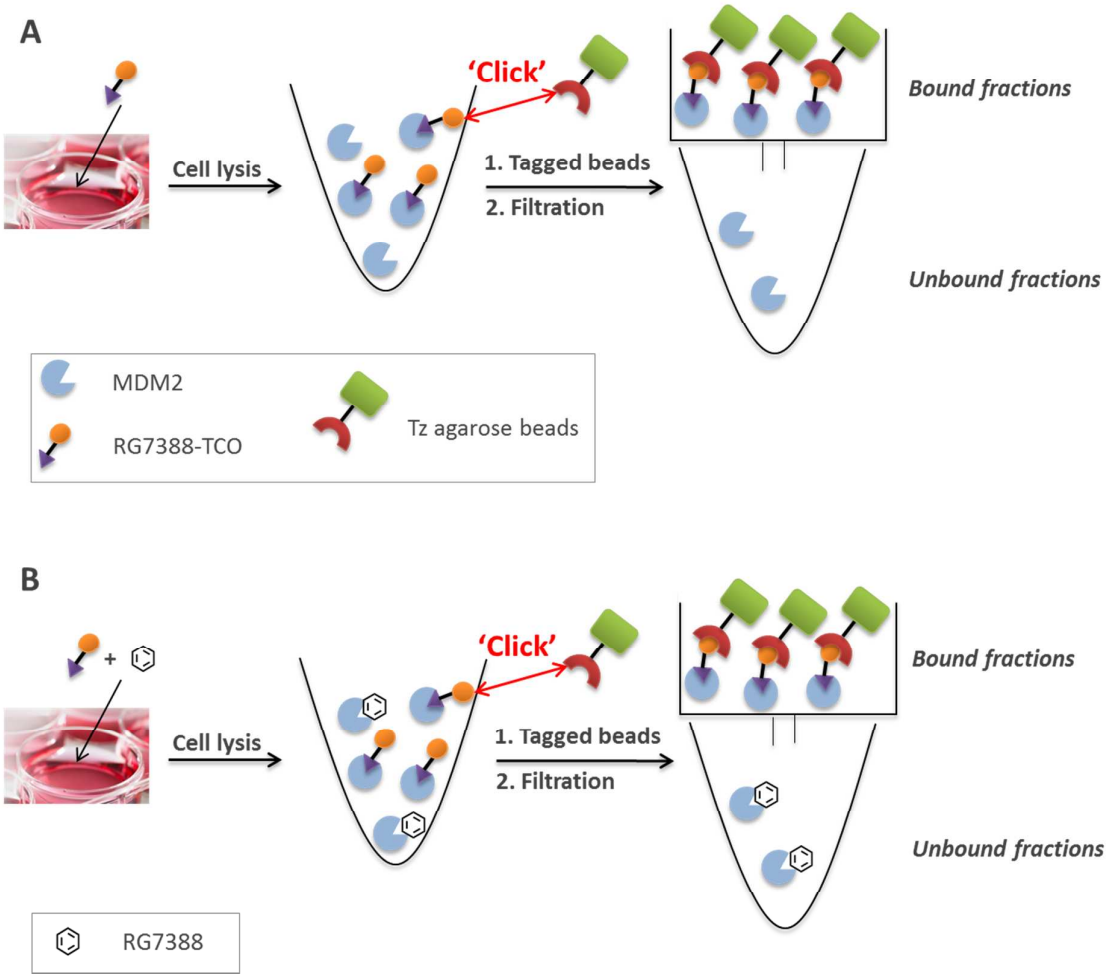
A. Dual channel (DAPI and Alexafluor 594) images showing induction of MDM2 following 4 h incubation with RG7388 or RG7388-TCO. Representative images of three independent experiments. B. SJSA-1 incubated with RG7388-TCO (100 nM, 4 h) and RG7388 (1 μM 3 h) were prepared for both MDM2 antibody and BODIPY-Tz (100 nM) staining post-fixation. The nuclei are shown in blue (DAPI channel), the fluorescence from the click reaction is shown in green (dye channel) and MDM2 protein shown in red (MDM2 channel) merged image of the three channels (merge). Data shown are representative of two experimental replicates.


A
DMSO
RG7388-TCO
RG7388
100nM
1000nM


B
DAPI
Dye channel
MDM2
Merge
DMSO
RG7388
RG7388-TCO
RG7388 +
RG7388-TCO

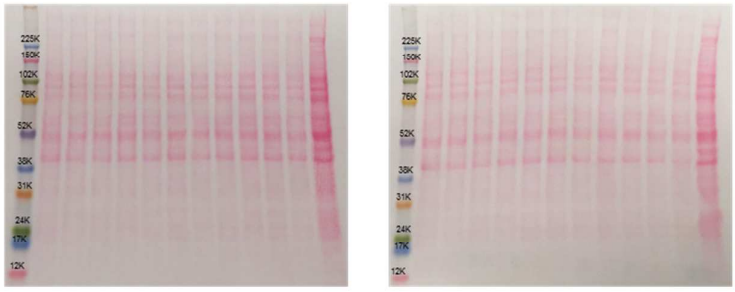
Supplementary Figure S3. Schematic representation of the pull down experiment using clickable probes.

A. SJSA-1 cells were treated with RG7388-TCO. Following cell lysis, lysates were incubated with Tz agarose beads. The proteins bound to RG7388-TCO were pulled down by filtration (bound fractions). B. Same experiment as described in A but the assay was performed in competition where SJSA-1 cells were treated simultaneously with RG7388 and RG7388-TCO.



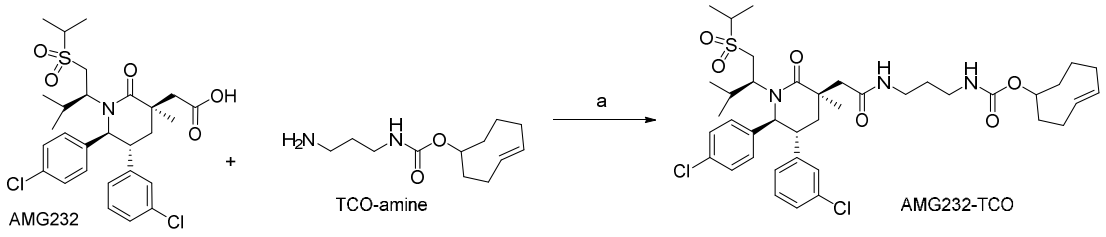
Supplementary Figure S4. Ponceau S staining of Western Blots from Figure 5.

Ponceau S staining confirming equivalent protein loading for each track. Immunoblotting showing the level of MDM2 pulled down by RG7388-TCO.



Supplementary Scheme S1. Synthesis of AMG232-TCO[a]

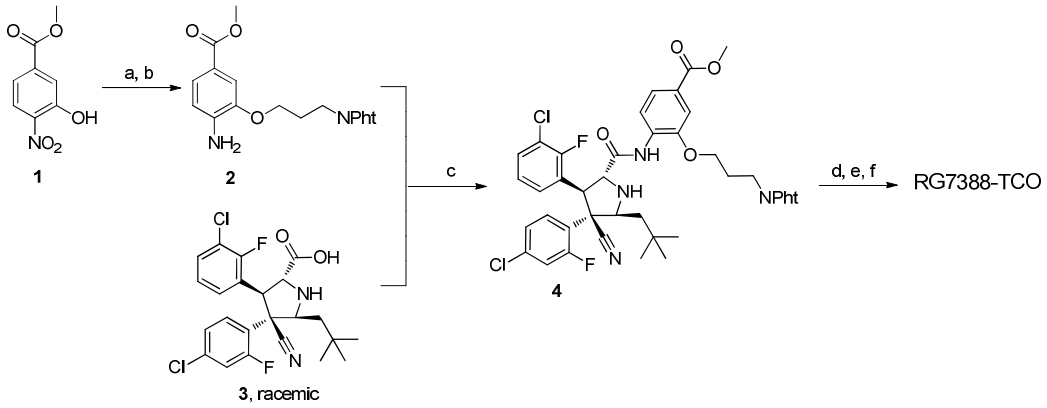
AMG232-TCO was synthesised in one step from AMG232 with the linker and TCO tag being inserted by amide coupling using the commercially available reagent TCO-amine.



[a]Reagents and conditions: (a) *N*,*N*-diisopropylethylamine, EDCI.HCl, HOAt, DCM, room temperature, 59%.

Supplementary Scheme S2. Synthesis of RG7388-TCO [a]

The synthesis of RG7388-TCO started from the alkylation of phenol **1** followed by reduction of the nitro group to give aniline **2**. Intermediate **2** was then reacted with carboxylic acid **3**[1] to give a racemic mixture which was submitted to chiral separation to afford enantiomers **4**. Hydrolysis followed by phthalimide deprotection were performed. Finally, the TCO tag was inserted using TCO-NHS ester, a commercially available reagent, to afford RG7388-TCO (Supplementary Scheme 2).



[a]Reagents and conditions: (a) *N*-(3-Bromopropyl)phtalimide, K_2CO_3, DMF, 50 ºC, 81%; (b) 10% Pd/C, H_2, MeOH, DCM, room temperature, 86%; (c) *N*,*N*-diisopropylethylamine, diphenylphosphonic chloride, DCM, room temperature, 10% (**4**); (d) 1N NaOH, THF, MeOH, 40 ºC, 100%; (e) $NH_2NH_2.H_2O$, EtOH, 60 ºC, 31%; (f) TCO-NHS ester, *N*,*N*-diisopropylethylamine, DMF, THF, room temperature, 50%.

Experimental Procedures

LC-MS method for the study of the click reaction

The compounds were solubilised in MeOH to generate a 10 mM solution. Pure and mixed samples (TCO ligand: Tz dye) were then analysed by LC-MS (liquid chromatography mass spectrometry) on a Shimadzu Nexera UPLC coupled with a Shimadzu LCMS-2020 single-quadrupole MS using a YMC-Triart C18 column (50 x 2.0 mm, 1.9 µm) at 45 °C. Gradient elution was performed from 3% acetonitrile to 99% acetonitrile in 10 mM ammonium bicarbonate pH 9.4 over 0.7 min.

MDM2 ELISA bioassay

The potency of the compounds was determined in a competition assay, in an ELISA format using chemoluminescence as the detection method. The streptavidin coated plate (Perkin Elmer, SMP103A) was incubated with the N-terminal biotinylated peptide b-IP_3 (1 µg/mL, 200 µL/well, Alta Biosciences) in buffer containing 12 mM Na_2HPO_4 and 150 mM NaCl pH 7.5 (PBS assay buffer) for 1 hour at r.t. After incubation, the plate was washed 3 times with a buffer containing 12 mM Na_2HPO_4, 150 mM NaCl pH 7.5 and 0.02% (v/v) Tween-20 (PBS-T).

MDM2 was expressed *in vitro* by mixing T7buffer (160 µL), T7 polymerase (80 µL), amino acid mixture (160 µL, Promega N2515), Recombinant RNAsin® RNAse inhibitor (80 µL, Promega L4461), DNA plasmid containing human MDM2 (1 µg/µL, 320 µL), sterile H_2O (1500 µL) and TNT® T7 coupled reticulicyte lysate system (2000 µL, Promega L4610). The mixture was heated to 30 °C for 2 hours. MDM2 expression crude solution (3 or 5 x 350 µL) was diluted in PBS assay buffer (72 mL).

Compound (5 µL/well, 2.5% (v/v) DMSO final concentration) and MDM2 solution (195 µL/well) were pre-incubated for 20 min at r.t. and then transferred into the streptavidin

coated plate. The reactions were allowed to proceed for 1h30 at 4 °C. The plate was washed 3 times with PBS-T buffer and incubated with primary antibody (anti-MDM2 (Ab-5) mouse (4B2C1.11), Calbiochem OP145, 0.5 µg/mL, 200 µL/well) for 1 hour at r.t. in a buffer containing 50 mM Tris-HCl, 150 mM NaCl and 0.05% (v/v) Tween-20 pH 7.5 (TBS-T buffer). The plate was washed 3 times with TBS-T buffer and incubated with secondary antibody (Goat anti-mouse IgG, HRP conjugate, Millipore, 50 ng/mL, 200 µL/well) for 45 minutes at r.t. in TBS-T buffer. The plate was washed twice with TBS-T buffer for 5 minutes and then 3 times. ECL mix (obtained from mixing ECL detection reagent 1 (20 mL) and ECL detection reagent 2 (20 mL), GE Healthcare RPN2106, 100 µL/well) was added to the plate. The plate was incubated for 1 minute at r.t. and read immediately using the TopCount NXT Microplate Scintillation and Luminescence Counter (Packard).

Cell culture

SJSA-1, SN40R2 and T778 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 2 mM glutamine and 10% (v/v) FBS (Gibco, Life Technologies) and were grown at 37 °C in an atmosphere of 5% CO_2 and air.

Proliferation assay

Inhibition of cell growth was measured using the Alamar Blue assay. The desired cells (SJSA-1 or SN40R2) were plated onto 96 well plates and allowed to recover for 16 hours prior to the addition of inhibitor compounds (in 0.1% DMSO v/v) for a further 72 hours. At the end of the incubation period 10% (v/v) Alamar Blue (Bio-Rad AbD Serotec, Oxford, UK) was added and incubated for a further 6 hours prior to determination of fluorescent product at 535 nM excitation / 590 nM emission.

p53 induction assay

Levels of p53 after MDM2 inhibition was measured using the MSD assay system following the manufacturer's protocol.[2] Briefly, SJSA-1 cells were plated onto 96-well plates (8.10^4 cells/mL, 200 µL/well) and incubated overnight at 37 °C in an atmosphere of 5% CO_2 and air prior to the addition of inhibitor compounds (in 0.1% DMSO v/v) for a further 2 hours. After incubation, the media was removed and each well was lysed with 1 x MSD lysis buffer (50 µL) supplemented with cOmplete EDTA-free protease inhibitor (Roche, 1 tablet/10 mL of lysis buffer), NaF (1 mL) and Na_3VO_4 (100 µL). The plate was incubated for 20 min at r.t. and each lysate (25 µL) was assayed as described in the literature.

MDM2 imaging

The desired cells (SJSA-1 and T778) were seeded onto methanol sterilised coverslips. Following adherence, cells were incubated as indicated with RG7388, RG7388-TCO (100-1000 nM) or DMSO vehicle. Cells were fixed with 4% w/v paraformaldehyde solution in PBS (pH 7) for 20 min then permeabilised in Triton-X (0.1% in PBS) for 10 min at room temperature. Following permeabilisation, cells were incubated with BODIPY-Tz, (100 nM, 10 mins) at r.t. Cover slips were then washed three times in PBS to remove unbound dye. Coverslips were mounted onto microscope slides using VectaShield hard-set mounting media plus DAPI and sealed. The slides were then either analysed immediately or stored at 4 °C. Images were recorded using a Zeiss LSM700 confocal microscope.

When performing MDM2 antibody immunofluorescence, samples were treated as above up to the stage of permeabilisation with Triton-X. Coverslips were then blocked in a solution of 4% w/v Goat serum/PBS at room temperature for 1 hour. Coverslips were then incubated in primary MDM2 antibody (Calbiochem OP46) diluted 1:200 in blocking solution for 16 hours at 4 °C. Coverslips were incubated with Alexa Fluor 594 secondary antibody (Invitrogen

A11032) diluted 1:200 in blocking solution for 1 hour at RT in the dark. After these steps BODIPY-Tz was applied as described above.

Corrected nuclear cell fluorescence (CNCF) was calculated with ImageJ[2] using a previously described method summarised by the equation below [3 4]. Since MDM2 staining was nuclear, three nuclei, and three background fields were selected per image to give a CNCF value per fluorescence channel. Three images were analysed per experimental condition.

$$CNCF =$$

$$Integrated\ density - (Area\ of\ selected\ cells \times Mean\ background\ fluorescence)$$

Coupling of streptavidin beads with biotin-PEG4-tetrazine[5]

The beads (400 µL of 50% slurry) were first washed twice with 200 µL of lysis buffer. The beads were then re-suspended in 200 µL of lysis buffer and 10 µL of Biotin-PEG4-tetrazine (from a 30 mM stock solution in DMSO) were added. The suspension was incubated on ice for 30 min with shaking. The suspension was then centrifuged (using centrifuge columns) at 1200 rpm for 1 min and washed twice with lysis buffer (2 x 200 µL, 1200 rpm, 1 min). The Tz-beads were re-suspended in 800 µL of lysis buffer to give a 25% slurry.

Protein enrichment experiments[5]

SJSA-1 cells were seeded in 6-well plates at 5.0×10^5 cells/mL with 2 mL/well and allowed to attach overnight. RG7388-TCO was added from a 1000x stock solution in DMSO (2 µL for a final concentration ranging from 1 nM to 10 µM) and the cells were incubated at 37 °C in an atmosphere of 5% CO_2 and air for 4 hours. The media was removed, the cells were washed with PBS (2 mL/well) and lysed with TG Lysis buffer supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche) (120 µL/well) for 20 min on ice. The lysates were centrifuged (14000 rpm, 5 min, 4 °C) and the protein concentration was determined by a Pierce™ BCA Protein Assay Kit. Samples were normalised to 1.5 mg/mL. In parallel, 60 µL

of Tz-beads (25% slurry) were added to centrifuge columns, the suspension was centrifuged (1200 rpm, 1 min) and the buffer was removed. The Tz-beads were then incubated with the lysates on ice for 30 min with shaking (60 µL of 25% slurry for 120 µL of lysates). The suspensions were then transferred into centrifuge columns and centrifuged (1200 rpm, 1 min). The unbound fractions were kept and the beads were washed twice with 100 µL of lysis buffer. 60 µL of elution buffer (2x LDS sample buffer) were added to each column which were incubated for 30 min at room temperature. The columns were then centrifuged (5000 rpm, 1 min) and the elution fractions were collected. Samples were separated on 4-12% NuPAGE gels (Life Technologies) and transferred onto a nitrocellulose membrane (Novex). The membrane was blocked in blocking buffer (Odyssey) at r.t. for 1 hour and subjected to immunodetection using an MDM2 primary antibody (Human/Mouse/Rat MDM2/ HDM2 antibody, R&D Systems, 1:1000) in blocking buffer, at 4°C overnight. After washing 3x with a Tris-buffered saline (TBS) with 0.1% Tween-20 solution (TBST), the membrane was incubated with fluorescently labelled secondary antibody (IRDye800CW Donkey Anti-Rabbit, 1:10 000) for 1 hour at r.t. in the dark. After washing 2x with a TBS solution, the membrane was imaged on an Li-Cor Biosciences Odyssey system in the 800 nm channel.

Competition experiments

Same procedure as described above but SJSA-1 cells were treated simultaneously with RG7388 from a 1000x stock solution in DMSO (2 µL for a final concentration ranging from 1 nM to 10 µM) and with RG7388-TCO from a 1000x stock solution in DMSO (2 µL for a 1 µM final concentration).

Synthesis of AMG232-TCO and RG7388-TCO

General information

Anhydrous solvents were purchased either from VWR or SeccoSolv and were stored under nitrogen. Other solvents were purchased from Fisher Chemicals. Commercially available reagents were used as received. TCO-Amine and TCO-NHS ester were purchased from Jena Bioscience. Petrol refers to the fraction with a boiling range between 40 and 60 ºC. All reactions were followed by TLC analysis (pre-coated TLC sheets ALUGRAM® SIL G/UV_{254}, Macherey-Nagel) or LC-MS (liquid chromatography mass spectrometry) on Agilent 1200 HPLC and 6140 MS using a YMC-Triart C18 column (50 x 2.0 mm, 1.9 µm). ^{1}H NMR spectra were recorded on a Bruker 400 UltraShield™ spectrometer. Chemical shifts are reported in parts per million (δ) referenced to the appropriate deuterated solvent employed and relative to TMS. Multiplicities are indicated by s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet). 'Flash' column chromatography was performed on pre-packed silica cartridges (Biotage SNAP cartridges, KP-Sil) on Biotage Isolera Four. Preparative SFC was performed on a Berger MultiGram II using a Phenomenex LUX Cellulose-4 column (5 µm, 250 x 21.2 mm). All reactions were carried out under nitrogen.

The purity of the final probes was determined by LC-MS and ^{1}H NMR and was always >95%.

LC-MS methodology

Eluent A: 10 mmoL ammonium bicarbonate pH 9.4

Eluent B: acetonitrile

Gradient: 3 – 99% B over 0.7 min

Flow: 0.7 mL/min

Column T: 45 ºC

Preparative SFC

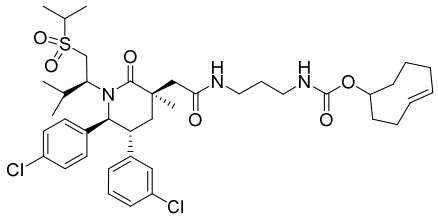
Eluent A: Methanol (NH3 added as modifier)

Eluent B: Carbon dioxide

Flow: 50 mL/min

Procedure for the synthesis of AMG232-TCO

(4E)-Cyclooct-4-en-1-yl *N*-(3-{2-[(3R,5R,6S)-5-(3-chlorophenyl)-6-(4-chloro phenyl)-3-methyl-1-[(2S)-3-methyl-1-(propane-2-sulfonyl)butan-2-yl]-2-oxopiperidin-3-yl] acetamido}propyl)carbamate (AMG232-TCO).



2-[(3R,5R,6S)-5-(3-Chlorophenyl)-6-(4-chlorophenyl)-3-methyl-1-[(2S)-3-methyl-1-(propane-2-sulfonyl)butan-2-yl]-2-oxopiperidin-3-yl]acetic acid (AMG232) (0.03 g, 0.05 mmol), TCO-amine (0.01 g, 0.05 mmol), *N*,*N*-diisopropylethylamine (0.02 mL, 0.14 mmol), EDCI.HCl (0.02 g, 0.08 mmol) and HOAt (0.01 g, 0.08 mmol) were mixed in DCM (0.5 mL) and the reaction was stirred at room temperature for 16 hours. Water (5 mL) was added and the organic phase was extracted with EtOAc (3 x 5 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography with 100% EtOAc to give (4E)-cyclooct-4-en-1-yl *N*-(3-{2-[(3R,5R,6S)-5-(3-chlorophenyl)-6-(4-chlorophenyl)-3-methyl-1-[(2S)-3-methyl-1-

(propane-2-sulfonyl)butan-2-yl]-2-oxopiperidin-3-yl]acetamido}propyl)carbamate (AMG232-TCO) (0.02 g, 0.03 mmol, 59%) as a pale yellow solid.

LCMS: Retention time 1.60 min, $[M+H]^+$ = 776

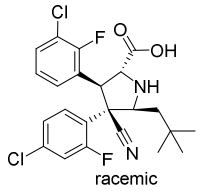
^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.29 (s, 4H), 7.16 – 7.11 (m, 1H), 7.09 (dt, *J* = 8.1, 1.6 Hz, 1H), 7.05 (t, *J* = 1.7 Hz, 1H), 6.98 (dt, *J* = 7.4, 1.6 Hz, 1H), 5.65 – 5.54 (m, 1H), 5.52 – 5.41 (m, 1H), 5.10 (d, *J* = 11.0 Hz, 1H), 4.30 (s, 1H), 4.00 (dd, *J* = 13.9, 10.5 Hz, 1H), 3.55 (ddd, *J* = 14.0, 11.2, 3.1 Hz, 1H), 3.36 – 3.15 (m, 4H), 3.16 – 3.04 (m, 3H), 2.88 (d, *J* = 13.5 Hz, 1H), 2.47 (d, *J* = 13.4 Hz, 1H), 2.38 – 2.28 (m, 3H), 2.31 – 2.22 (m, 1H), 2.22 – 2.12 (m, 1H), 2.05 (dd, *J* = 13.5, 3.1 Hz, 1H), 2.00 – 1.93 (m, 2H), 1.93 – 1.88 (m, 1H), 1.88 – 1.83 (m, 1H), 1.78 – 1.63 (m, 4H), 1.63 – 1.52 (m, 1H), 1.41 (d, *J* = 6.8 Hz, 6H), 1.34 (s, 3H), 0.67 (d, *J* = 6.6 Hz, 3H), 0.52 (d, *J* = 6.9 Hz, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 177.92, 172.82, 158.88, 145.44, 139.12, 135.89, 135.21, 133.61, 133.17, 130.78, 129.17, 128.85, 127.77, 127.16, 81.58, 70.75, 59.96, 55.52, 47.76, 45.61, 45.38, 43.17, 42.06, 39.95, 39.46, 38.90, 37.72, 34.97, 33.86, 33.30, 31.98, 30.69, 26.98, 20.94, 20.89, 15.25.

HRMS calcd for $C_{40}H_{55}Cl_2N_3O_6S$ $[M+H]^+$ 776.3261, found 776.3263.

Procedures for the synthesis of RG7388-TCO

Synthesis of carboxylic acid 3[1]



Step 1 - To a solution of 4-chloro-2-fluorophenylacetonitrile (5.0 g, 29.6 mmol) and 3-chloro-2-fluorobenzaldehyde (4.7 g, 29.6 mmol) in dry MeOH (148 mL) was slowly added MeONa in MeOH (25 w.t. %, 9.9 mL). The reaction was heated at 50 ºC for 3 hours (formation of a precipitate). The reaction was then cooled to room temperature and filtered. The precipitate was washed with water (100 mL), cold methanol (100 mL) and dried under vacuum to give (2*Z*)-3-(3-chloro-2-fluorophenyl)-2-(4-chloro-2-fluorophenyl)prop-2-enenitrile (6.3 g, 20.3 mmol, 69%) as a white solid.

LCMS: Retention time 1.61 min, $[M+H]^+ = 310$

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.99 (dd, J = 7.2, 7.2 Hz, 1H), 7.90 (s, 1H), 7.85-7.76 (m, 1H), 7.73 (dd, J = 8.4, 8.4 Hz, 1H), 7.66 (dd, J = 11.1, 2.2 Hz, 1H), 7.49 (dd, J = 8.4, 2.2 Hz, 1H), 7.43 (dd, J = 8.0, 8.0 Hz, 1H).

Step 1' - A mixture of *tert*-butyl glycinate (5.0 g, 37.9 mmol) and 3,3-dimethylbutyraldehyde (4.0 g, 39.8 mmol) in DCM (95 mL) was stirred at room temperature for 16 hours. The reaction mixture was concentrated. The residue was diluted with EtOAc (50 mL), dried over $MgSO_4$ and the solvent was removed *in vacuo* to give *tert*-butyl 2-[(*E*)-(3,3-dimethylbutylidene) amino]acetate (6.5 g, 30.5 mmol, 80%) as a pale yellow oil, which was used without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.72 (t, J = 5.5 Hz, 1H), 4.04 (s, 2H), 2.11 (d, J = 5.5 Hz, 2H), 1.40 (s, 9H), 0.96 (s, 9H).

Step 2 - Triethylamine (0.89 mL, 6.41 mmol) and silver fluoride (0.16 g, 1.25 mmol) were added to a stirred solution of *tert*-butyl 2-[(*E*)-(3,3-dimethylbutylidene)amino]acetate (0.52 g, 2.46 mmol) and (2*Z*)-3-(3-Chloro-2-fluorophenyl)-2-(4-chloro-2-fluorophenyl)prop-2-enenitrile (0.55 g, 1.78 mmol) in DCM (45 mL). The mixture was stirred at room temperature for 18 hours. More AT-1464-068 *tert*-butyl 2-[(*E*)-(3,3-dimethylbutylidene)amino]acetate (0.38 g, 1.78 mmol) and triethylamine (0.62 mL, 4.45 mmol) were added and the reaction was stirred at room temperature for 18 hours. The mixture was quenched with a saturated solution of NH_4Cl (50 mL). The organic phase was separated, washed with brine (30 mL), dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography with 1: 9 EtOAc: Petrol to give racemic *tert*-butyl (2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl) pyrrolidine-2-carboxylate (0.4 g, 0.76 mmol, 43 %) as a colourless oil.

LCMS: Retention time 1.83 min, $[M+H]^+$ = 523

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.72-7.68 (m, 1H), 7.67-7.59 (m, 1H), 7.55-7.52 (m, 1H), 7.39-7.23 (m, 3H), 4.60 (d, J = 7.2 Hz, 1H), 4.26 (dd, J = 6.8, 6.8 Hz, 1H), 4.00 (dd, J = 9.0, 9.0 Hz, 1H), 3.51-3.47 (m, 1H), 1.61-1.44 (m, 1H), 1.35 (s, 9H), 1.12-1.07 (m, 1H), 0.88-0.68 (m, 9H).

Step 3 - TFA (16.5 mL) was added to a stirred solution of racemic *tert*-butyl (2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl) pyrrolidine-2-carboxylate (3.4 g, 6.6 mmol) in DCM (47 mL). The reaction was stirred at room temperature for 18 hours. The solvent was removed *in vacuo*. The crude product was purified by flash column chromatography with 1:1 EtOAc: Petrol to give racemic

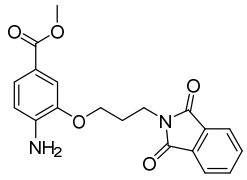
(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-carboxylic acid (3.0 g, 6.4 mmol, 98%) as a yellow foam.

LCMS: Retention time 1.21 min, $[M+H]^+ = 467$

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.74-7.69 (m, 1H), 7.68-7.62 (m, 1H), 7.56-7.51 (m, 1H), 7.36-7.28 (m, 3H), 4.70-4.66 (m, 1H), 4.57 (d, *J* = 8.5 Hz, 1H), 4.16 (d, *J* = 9.0 Hz, 1H), 1.66 (dd, *J* = 14.3, 9.0 Hz, 1H), 1.26 (d, *J* = 14.3 Hz, 1H), 0.85 (s, 9H).

Synthesis of intermediate 4

Procedure for compound 2: Methyl 4-amino-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy] benzoate.



Step 1 - *N*-(3-Bromopropyl)phtalimide (4.9 g, 18.3 mmol) was added to a stirred suspension of methyl 3-hydroxy-4-nitrobenzoate (3.0 g, 15.2 mmol) and potassium carbonate (12.0 g, 45.7 mmol) in dry DMF (59 mL). The reaction was stirred at 50 ºC for 18 hours. The reaction was then cooled to room temperature, poured into water (80 mL) and extracted with DCM (3 x 100 mL). The organic phase was then washed with brine (3 x 100 mL), dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude product was resuspended in EtOAc (50 mL) and the solid was collected by filtration to give methyl 3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy]-4-nitrobenzoate (4.7 g, 12.3 mmol, 81%) as a pale yellow solid.

LCMS: Retention time 1.40 min, $[M+NH_4^+]^+ = 402$

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.98 (d, J = 8.4 Hz, 1H), 7.88-7.79 (m, 4H), 7.72 (d, J = 1.6 Hz, 1H), 7.65 (dd, J = 8.4, 1.6 Hz, 1H), 4.29 (t, J = 5.7 Hz, 2H), 3.90 (s, 3H), 3.77 (t, J = 6.5 Hz, 2H), 2.16-2.04 (m, 2H).

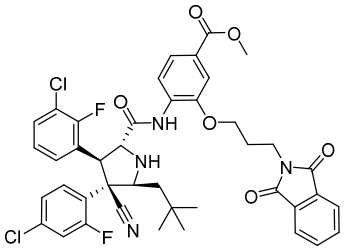
Step 2 - Methyl 3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy]-4-nitrobenzoate (2.4 g, 6.3 mmol) and 10% Pd/C (0.84 g) were mixed in MeOH (22 mL) and DCM (5.7 mL). The mixture was then shaked at room temperature under an atmosphere of hydrogen for 3 hours. The catalyst was removed by filtration on Celite and the filtrate was concentrated *in vacuo* to give methyl 4-amino-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy]benzoate (1.9 g, 5.4 mmol, 86%) as a yellow solid, which was used without further purification.

LCMS: Retention time 1.36 min, $[M+H]^+$ = 355

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.90-7.79 (m, 4H), 7.38 (dd, J = 8.2, 1.8 Hz, 1H), 7.26 (d, J = 1.8 Hz, 1H), 6.65 (d, J = 8.2 Hz, 1H), 5.56 (s, 2H), 4.02 (t, J = 6.0 Hz, 2H), 3.80 (t, J = 6.6 Hz, 2H), 3.74 (s, 3H), 2.16-2.05 (m, 2H).

Procedure for compound 4

Chiral methyl 4-[(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-amido]-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy]benzoate.



N,*N*-Diisopropylethylamine (3.3 mL, 18.9 mmol) and diphenylphosphinic chloride (2.7 g, 14.2 mmol) were added to a solution of racemic (2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-

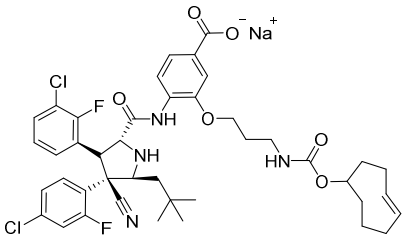
4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-carboxylic acid (2.2 g, 4.7 mmol) in DCM (44 mL). The mixture was stirred at room temperature for 20 min then methyl 4-amino-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy]benzoate (1.8 g, 5.0 mmol) was added and the reaction was then stirred at room temperature for 18 hours. The solvent was removed *in vacuo* and the crude was purified by flash column chromatography with 3: 7 EtOAc: Petrol to give a racemic mixture (1.4 g) which was purified by preparative SFC using 1:1 CO_2: MeOH (NH_3 added as modifier) to give chiral methyl 4-[(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl) pyrrolidine-2-amido]-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy]benzoate (0.36 g, 0.45 mmol, 10%) as a white solid.

LCMS: Retention time 1.75 min, $[M-H]^-$ = 801

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 10.16 (s, 1H), 8.47 (d, *J* = 8.5 Hz, 1H), 7.89 (dd, *J* = 5.5, 3.1 Hz, 2H), 7.80-7.72 (m, 2H), 7.67 (dd, *J* = 8.5, 1.8 Hz, 1H), 7.59-7.51 (m, 2H), 7.37-7.29 (m, 2H), 7.22-7.10 (m, 3H), 4.91-4.88 (m, 1H), 4.60 (t, *J* = 9.2 Hz, 1H), 4.32-4.25 (m, 1H), 4.23-4.15 (m, 1H), 4.08 (t, *J* = 10.5 Hz, 1H), 4.02-3.94 (m, 1H), 3.94-3.85 (m, 4H), 3.05 (t, *J* = 10.5 Hz, 1H), 2.37-2.20 (m, 2H), 1.62 (dd, *J* = 14.5, 9.2 Hz, 1H), 1.41 (d, *J* = 14.5 Hz, 1H), 0.91 (s, 9H).

Synthesis for RG7388-TCO

Racemic sodium 4-[(2R,3R,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-amido]-3-[3-({[(4*E*)-cyclooct-4-en-1-yloxy]carbonyl}amino)propoxy]benzoate (RG7388-TCO).



Step 1 - An aqueous solution of NaOH (1N, 2.3 mL) was added to a stirred solution of chiral methyl 4-[(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-amido]-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy] benzoate (0.46 g, 0.57 mmol) in THF (14.3 mL) and MeOH (15.5 mL). The resulting solution was stirred at 40 ºC for 18 hours. The solution was then acidified to pH 4-5 with an aqueous solution of HCl (1N). The organic layer was extracted with DCM (5 x 30 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* to give chiral 4-[(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-amido]-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy] benzoic acid (0.47 g, 0.60 mmol, 100%) as a pale orange solid, which was used without further purification.

LCMS: Retention time 1.17 min, $[M+NH_4^+]^+ = 807$

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.94-12.72 (br s, 1H), 8.45-8.36 (m, 2H), 7.82-7.76 (m, 1H), 7.73 (dd, J = 7.1, 7.1 Hz, 1H), 7.63-7.47 (m, 6H), 7.45-7.38 (m, 2H), 7.38-7.30 (m, 2H), 4.66 (s, 2H), 4.33-4.23 (m, 2H), 4.09-4.05 (m, 2H), 3.48-3.34 (m, 2H), 2.11-1.97 (m, 2H), 1.66 (dd, J = 14.3, 9.4 Hz, 1H), 1.33-1.22 (m, 1H), 0.95 (s, 9H).

Step 2 - Hydrazine hydrate (0.24 mL) was added to a stirred solution of chiral 4-[(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl) pyrrolidine-2-amido]-3-[3-(1,3-dioxo-2,3-dihydro-1*H*-isoindol-2-yl)propoxy] benzoic acid (0.47 g, 0.60 mmol) in EtOH (4.0 mL) at room temperature. The

reaction was stirred at 60 ºC for 2 days. The solvent was removed *in vacuo*. The crude product was resuspended in EtOH (10 mL) and the solvent was removed *in vacuo* (repeated 3 times). The crude was then partitionned between brine (10 mL) and DCM (10 mL) and the organic phase was collected. At that point, a precipitate was observed in the organic phase and was collected by filtration to give chiral sodium 3-(3-aminopropoxy)-4-[(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-amido]benzoate (0.12 g, 0.18 mmol, 31 %) as a beige solid.

LCMS: Retention time 1.32 min, $[M+H]^+ = 659$

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.09 (br s, 1H), 8.25 (d, *J* = 8.4 Hz, 1H), 7.73 (dd, J = 7.0, 7.0 Hz, 1H), 7.66-7.44 (m, 4H), 7.44-7.26 (m, 3H), 4.66-4.58 (m, 2H), 4.34-4.20 (m, 2H), 4.03-3.92 (m, 2H), 2.94 (t, *J* = 7.1 Hz, 2H), 2.10-2.00 (m, 2H), 1.64 (dd, *J* = 14.5, 9.3 Hz, 1H), 1.33-1.21 (m, 1H), 0.95 (s, 9H).

Step 3 - TCO-NHS ester (0.02 g, 0.08 mmol) was added to a stirred solution of chiral sodium 3-(3-aminopropoxy)-4-[(2R,3S,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-amido]benzoate (0.05 g, 0.08 mmol) and N,N-diisopropylethylamine (0.03 mL, 0.19 mmol) in DMF: THF (1: 1, 1.9 mL). The reaction was stirred at room temperature for 30 min. The solvent was removed *in vacuo* and the crude was partitioned between water (5 mL) and DCM (5 mL). The pH was adjusted to 4 with a 1M solution of HCl. The aqueous phase was extracted with DCM (3 x 10 mL). The organic phases were combined, dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography using 1: 9 MeOH: DCM to give racemic sodium 4-[(2R,3R,4R,5S)-3-(3-chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)pyrrolidine-2-amido]-3-[3-({[(4*E*)-cyclooct-4-en-1-yloxy] carbonyl} amino)propoxy]benzoate (0.03 g, 0.04 mmol, 50 %) as a white solid.

LCMS: Retention time 1.34 min, $[M+H]^+$ = 811

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.14 (s, 1H), 8.30 (d, *J* = 8.3 Hz, 1H), 7.80-7.66 (m, 1H), 7.57 (s, 1H), 7.57 (dd, *J* = 12.6, 2.1 Hz, 1H), 7.52 (td, *J* = 7.6, 7.0, 1.5 Hz, 2H), 7.40 (t, *J* = 8.4 Hz, 1H), 7.37-7.30 (m, 2H), 7.20-7.03 (m, 1H), 5.68-5.49 (m, 1H), 5.49-5.35 (m, 1H), 4.64 (s, 2H), 4.32-4.15 (m, 3H), 4.09 (q, *J* = 8.6, 8.0 Hz, 1H), 3.98 (t, *J* = 10.6 Hz, 1H), 3.25-3.05 (m, 2H), 2.37-2.16 (m, 3H), 1.97-1.87 (m, 5H), 1.87-1.79 (m, 1H), 1.71-1.59 (m, 2H), 1.59-1.46 (m, 2H), 1.27 (d, *J* = 14.1 Hz, 1H), 0.93 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 170.83, 159.40, 155.74, 155.47, 146.51, 134.74, 134.60, 132.36, 130.87, 129.80, 129.32, 128.42, 125.61, 125.19, 122.01, 119.43, 119.09, 117.48, 117.40, 117.26, 111.98, 78.90, 66.05, 64.32, 63.11, 62.92, 49.50, 44.13, 40.51, 38.03, 36.79, 33.55, 31.95, 30.36, 29.71, 29.49, 29.01.

HRMS calcd for $C_{42}H_{46}Cl_2F_2N_4O_6$ $[M+H]^+$ 811.2835, found 811.2844.

References

(1) Ding, Q., Zhang, Z., Liu, J.-J., Jiang, N., Zhang, J., Ross, T. M., Chu, X.-J., Bartkovitz, D., Podlaski, F., Janson, C., et al. (2013) Discovery of RG7388, a Potent and Selective p53–MDM2 Inhibitor in Clinical Development. *Journal of Medicinal Chemistry 56*, 5979-5983.

(2) Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods 9*, 671-5.

(3) McCloy, R. A., Rogers, S., Caldon, C. E., Lorca, T., Castro, A., and Burgess, A. (2014) Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. *Cell cycle (Georgetown, Tex.) 13*, 1400-12.

(4) Potapova, T. A., Sivakumar, S., Flynn, J. N., Li, R., and Gorbsky, G. J. (2011) Mitotic progression becomes irreversible in prometaphase and collapses when Wee1 and Cdc25 are inhibited. *Molecular biology of the cell 22*, 1191-206.

(5) Rutkowska, A., Thomson, D. W., Vappiani, J., Werner, T., Mueller, K. M., Dittus, L., Krause, J., Muelbaier, M., Bergamini, G., and Bantscheff, M. (2016) A Modular Probe Strategy for Drug Localization, Target Identification and Target Occupancy Measurement on Single Cell Level. *ACS Chemical Biology 11*, 2541-2550.