TTK/MPS1 inhibitor OSU-13 targets the mitotic checkpoint and is a potential therapeutic strategy for myeloma

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SUPPLEMENTARY DATA

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SUPPLEMENTARY METHODS

Cell lines and primary cells

OPM-2, JJN3, and L363 cell lines were purchased from DSMZ (Braunschweig, Germany); MM.1S, RPMI-8226, NCI-H929, IM-9, ARH-77, KMS11, and U266 were obtained from ATCC (Manassas, VA, USA); and EJM was purchased from Creative Bioarray (Shirley, NY, USA). EJM was cultured at 37°C and 5% CO_2 in IMDM medium containing 20% fetal bovine serum (FBS; GIBCO, Life Technologies; Carlsbad, CA, USA), 100 U/mL penicillin, 0.25 μg/mL amphotericin B, and 100 μg/mL streptomycin (GIBCO, Life Technologies). Remaining cell lines were cultured in RPMI–1640 medium containing 10% FBS (GIBCO, Life Technologies) and the same antibiotics.

Viable $CD138^+$ primary human plasma cells were isolated from bone marrow of four recently diagnosed, untreated patients with MM by first performing Ficoll-Paque PLUS (GE Life Sciences; Marlborough, MA, USA) density centrifugation as previously described,[1] then either staining with Zombie-aqua dye (Life Technologies; Carlsbad, CA, USA) and CD138 PE-Cy7 (#130-119-842; Miltenyi Biotec; Bergisch Gladbach, Germany) and sorting viable CD138(+) lymphocytes on a FACS Aria II (BD; Franklin Lakes, NJ, USA) or enrichment via magnetic separation using the EasySep Human Whole Blood CD138 Selection Kit (StemCell Technologies; Vancouver, Canada).

siRNA knockdown experiments

OPM-2 and NCI-H929 cells were transfected with Alexa-fluor 647-conjugated TTK siRNA (Qiagen, Crawley, UK; SI02223207) or non-targeting scrambled siRNA (Qiagen; SI03650318)

as previously described for MM cells.[2] Briefly, 50 µl of cells (1 x 10^6) was incubated in serum-free media with 5 µM of siRNA in a 96-well plate for 10 min at 37°C, along with 0U, 10U, or 20U of streptolysin to induce reversible permeabilization and enhance siRNA uptake. The permeabilization was reversed by adding 250 µl of 10% FBS RPMI medium, and the cells were further incubated for 30 min at 37°C. Subsequently, the cells were transferred to a 6-well plate with 2.5 ml of 10% FBS RPMI medium and incubated for 48 h at 37°C. After incubation, the cells were stained with Zombie-aqua dye for viability and analyzed by flow cytometry. The uptake of siRNA was assessed by measuring Alexa-fluor 647 fluorescence.

RNA extraction and Real-time polymerase chain reaction

RNA was isolated using the Total RNA Purification Plus Kit (Norgen; Thorold, Canada), and cDNA was synthesized according to manufacturer's instructions using SuperScript VILO cDNA synthesis kit (Invitrogen; Waltham, MA, USA). Real-time PCR was performed on an ABI Prism 7900HT with Taqman primer/probe sets for human *TTK* (Hs01009870_m1) and *18S* (Hs99999901_s1) (Life Technologies). Gene expression levels were normalized to *18S* internal control, and the relative *TTK* expression was calculated using the ΔΔ Ct method as previously described.[3]

NanoBRET assay for target engagement

HEK293 cells were transiently transfected with 1 µg of TTK-NanoLuc fusion vector and 9 µg of transfection carrier DNA. The transfected cells were treated for 1 h with several concentrations of OSU-13, CFI-402257, BOS-172722, BAY 1161909, or BAY 1217389 (3-fold dilutions starting at

1 μM), and the broad-spectrum kinase inhibitor CTx-0294885 (3-fold dilutions starting at 10 μM) as a reference compound. Kinase-ligand affinity was measured by competitive displacement with 1 μM tracers (K-10 or K-5). Curve fits were performed only when % NanoBret signal at the highest concentration of compounds was less than 55%. The 600 nm/460 nm ratio was calculated and the normalized BRET response (%) was established.

Kinase panel profiling

OSU-13 was profiled against 399 kinases in assays performed by Reaction Biology Corporation (Malvern, PA, USA). OSU-13 was tested at 1 μM in duplicate, DMSO was used as negative control, and the broad-spectrum protein kinase inhibitor staurosporine was used as the main positive control, tested in 10 concentrations with 4-fold serial dilutions starting at 20 or 100 μM. LDN193189, GW5074, D4476, Ro-31-8220, SCH772984, SB202190, and BI2536 were used as alternate positive controls for specific kinases in the same concentrations as staurosporine. Reactions were carried out at 10 μM ATP, and enzyme activity (%) relative to DMSO controls were calculated.

TTK and OSU-13 Co-crystallization

The recombinant fusion protein 6xHis-tev-TTK (519-808) was produced *via* arabinose-inducible expression using *E. coli* BL21 AI cells and mixed 1:5 with 100 mM OSU-13. The mixture was then incubated on ice for 2 h. The sample was centrifuged at 17 000 × g for 10 min to sediment the residual particulate in solution. The supernatant was used for crystallization in different setups. Incubation times were 2, 3, or 8 days, with 30 or 40% glycerol or PEG 200 as cryoprotectant. The

co-crystal structure was determined using X-ray diffraction and refined to 2.37 Å resolution. Molecular replacement was performed with the CCP4 program Phaser using the publicly available TTK kinase domain structure (RCSB ID: 4HMP.pdb) stripped of all ligands and water molecules as the starting model.

Cell Viability Assay (MTS)

Cell lines (OPM-2, NCI-H929, RPMI-8266, U266, EJM, JJN3, L363, KMS11, and MM.1S) were incubated in 96-well plates (20 000 cells/well) for 72 h in medium containing either carrier alone (DMSO) or carrier containing OSU-13 at the specified concentrations. Cell viability assay was carried out in triplicate or quadruplicate wells by colorimetric [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation kit (Promega; Madison, WI, USA) as described by the manufacturer. Briefly, cells were incubated with 20 µl of the MTS reagent for 1 or 2 h at 37°C, and absorbance was read at 490 nm with the *Cytation 5* multimode reader (Biotek; Winooski, VT, USA). The mean of the replicates was used to generate the curve and calculate the IC_{50} for each experiment in Graphpad Prism 8 (Graphpad Software; San Diego, CA, USA). The final IC_{50} represents the mean ± SD from three independent MTS assay experiments.

OSU-13 effect in primary cells

Lymphocyte viability after OSU-13 treatment was assessed using cryopreserved bone marrow (BM) samples from a MM patient, PBMC from a plasma cell leukemia (PCL) patient, and PBMC from three healthy donors. Briefly, cryopreserved total ficolled lymphocytes from each patient

were thawed, counted, and cultured in alpha-MEM supplemented with antibiotic/antimycotic, 20% FBS, and 1 µl/ml of DMSO or DMSO containing 0.5 µM OSU-13. Cells were plated at a density of 1 x 10^6 cells in 1 ml per well in a 24-well plate and incubated for 24, 48, and 72 h. At the end of each time point, cells were stained with antibodies targeting CD3, CD14, CD34, CD19, CD56, and CD138, along with Zombie Aqua dye for viability assessment. Flow cytometry was performed to analyze the cells, focusing on viable, single Zombie$^-$ lymphocytes. Subsequently, lymphocyte subsets were identified based on the expression of surface markers: PC (CD138$^+$ CD56$^+$), B cells (CD138$^-$ CD19$^+$), T cells (CD56$^-$ CD3$^+$), NK cells (CD138$^-$ CD56$^+$ CD3$^-$), and NKT cells (CD138$^-$ CD3$^+$ CD56$^+$).

Cell proliferation assay

Cell proliferation was assessed by the dye dilution method using CellTrace far-red (Invitrogen) according to the manufacturer's instructions. Briefly, NCI-H929 and OPM-2 cells were stained with CellTrace far-red (1 µL/mL) for 20 min at 37ºC, protected from light. Unstained cells were kept for parallel control. The reaction was stopped by adding 5 x the original volume of RPMI + 10% FBS. Then, cells were incubated for 20 minutes to allow the CellTrace dye to undergo de-esterification. After that, cells (150 000 cells/mL) were treated with DMSO or OSU-13 (0.1, 0.65, 1.25, 2.5, and 5 µM) for 72 h. Cells were analyzed using an Attune Nxt cytometer (Invitrogen), and data analysis was performed using FlowJo software (FlowJo LLC; Ashland, OR, USA). Initially, CellTrace fluorescence intensity in viable single cells was normalized to that obtained in viable single cells from respective unstained controls. Then, relative CellTrace fluorescence intensity in OSU-13-treated cells in comparison to DMSO control was calculated and converted to proliferation (%), with the average of DMSO control data set to 100%.

Caspases 3/7 activity assay

Caspases 3/7 activity was quantified using ApoTox-Glo assay (Promega; Madison, WI, USA). NCI-H929 cells were incubated in RPMI + 10% FBS medium containing DMSO, 0.1 µM OSU-13, or 0.5 µM OSU-13 for 24, 48, and 72 h at 37°C and 5% CO_2. Then, 100 µl of Caspase-Glo® 3/7 Reagent was added, samples were incubated for 30 minutes at room temperature, and luminescence was measured.

Western blotting

OPM-2 and NCI-H929 total cell lysates were prepared in RIPA buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, 1 mM PMSF) (Cell Signaling Inc.; Danvers, MA, USA). Protein concentrations were measured using Bradford assay (Abcam; Cambridge, United Kingdom), and protein lysates were boiled for 5 min with reducing sample buffer (Thermo Fisher Scientific; Waltham, MA, USA). Then, similar amounts of each lysate, depending on the target protein, were loaded on a 4-15% mini-PROTEAN TGX precast gel and transferred onto a nitrocellulose membrane (Bio-Rad; Hercules, CA, USA) in a Trans-blot turbo transfer system (Bio-Rad). Blots were incubated at room temperature for 2 h in Intercept Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA), and incubated at 4°C overnight with the following primary antibodies: cleaved PARP1, p-H2AX (Ser139), caspase 3, cleaved caspase 3, caspase 7, cleaved caspase 7, GAPDH, and α-tubulin (Cell Signaling Inc.). All antibodies were diluted 1:1 000, except for GAPDH and α-tubulin antibodies (1:2 500 dilution), in Intercept Blocking Buffer (LI-COR Biosciences) + 0.1% Tween 20 (Bio-Rad). Then,

membranes were washed three times with Tris-buffered saline + 0.1% Tween 20 (TBST) for 15 min at room temperature, and membranes were incubated at room temperature for 1 h in anti-rabbit-IRDye 800 or anti-mouse-IRDye 680 (LI-COR Biosciences) diluted 1:10 000 in Intercept Blocking Buffer + 0.2% Tween 20. After three washes with TBST, membranes were scanned in an Odissey CLx imager (LI-COR Biosciences) and quantified in the Image Studio 5.0 software (LI-COR Biosciences).

TUNEL assay

OPM-2 and NCI-H929 cells were incubated in 24-well plates (300 000 cells/well) for 48 h in medium containing either carrier alone (DMSO) or carrier containing 2.5, 5, or 10 μM of OSU-13. Then, the TUNEL assay was performed with the TUNEL Assay kit - Edu Orange (Abcam) according to the manufacturer's instructions. Subsequentially, cells were centrifuged, resuspended in 100 μL PBS, immobilized onto a microscope slide using a Cytospin (Thermo Fisher Scientific), and mounted with Cel Prolong Glass Antifade Mountant with NucBlue (Thermo Fisher Scientific). Slides were analyzed in a spinning-disk confocal system (UltraVIEW Vox CSUX1 system; PerkinElmer, Waltham, MA, USA) with back thinned electron-multiplying charge-coupled device (EMCCD) cameras (C9100-13 or C9100-23B; Hamamatsu Photonics, Bridgewater, NJ, USA) on a Nikon Ti-E microscope (Tokyo, Japan). Images were analyzed using ImageJ (NIH; Bethesda, MD, USA), and unstained control cells were used as a blank to measure background signal. The percentage of TUNEL positive cells was calculated as TUNEL positive cells / total cells x 100.

Cell cycle analysis

For cell cycle and ploidy analysis, OPM-2 and NCI-H929 cell lines were serum-starved for 16 h and incubated at 150 000 cells/mL in medium containing DMSO or 0.5 μM OSU-13 at 37°C and 5% CO_2 for 72 h. Then, the cells were washed in PBS and resuspended in 200 μL PBS + 2% FBS. One mL of cold (−20°C) 70% (v/v) ethanol in PBS was added dropwise, and the cells were incubated on ice for 30 min. After centrifugation (2 500 rpm for 5 min) and supernatant removal, the cells were stained with 500 μL of 50 μg/mL propidium iodide (Miltenyi Biotec; Bergisch Gladbach, Germany) containing 100 μg/mL RNaseA (Thermo Fisher Scientific) for 30 min at 37°C. The DNA content was measured using an Attune Nxt cytometer (Invitrogen) with data acquisition in low speed and analyzed using FlowJo software (FlowJo LLC).

Metaphase Chromosome Spread

OPM-2 cells (1 x 10^6 cells) were incubated at 400 000 cells/mL in medium containing DMSO or 1 μM OSU-13 at 37°C and 5% CO_2. After 24 h of treatment, cells were washed with PBS and incubated for 16 h in medium containing 50 ng/mL nocodazole (Sigma-Aldrich) to induce G2/M arrest. Cells were then lysed in hypotonic solution (0.056 M KCl) for 10 min at 37°C and fixed in methanol/glacial acetic acid (3:1) solution for 10 min at 4°C. One drop of suspended cells was applied to glass slides. Prolong Glass Antifade Mountant with NucBlue (Thermo Fisher Scientific) was added to stain chromosomes and mount coverslips. Slides were imaged using a Nikon DM5000 B microscope (Tokyo, Japan) equipped with fluorescence optics with a Leica X63 oil immersion objective (Wetzlar, Germany). Using ImageJ (NIH), chromosomes clearly grouped as belonging to only one cell were manually counted.

Studies in murine model

For survival studies, NCI-H929 ($1x10^7$ cells in a 0.1 mL suspension in 50% Matrigel/PBS) were subcutaneously inoculated in the right flank of 8 to 12-week-old female immunodeficient CB.17 SCID mice. Tumors were measured with a caliper in two dimensions to monitor size as mean volume approached the desired 80 to 120 mm^3 range. Fourteen days after tumor implantation (Day 1) mice were randomly assigned to daily oral treatment with vehicle control (25% PEG 400, 15% propylene glycol, and 6% Tween 80 in deionized water) or 10 mg/kg of OSU-13 (n = 10/group) for 21 days. Due to a group mean body weight loss of 10%, the OSU-13 group had a dosing holiday from Days 14-17, with treatment resumed on Day 18. Tumor volumes were monitored twice a week, and each mouse was euthanized when its tumor reached the endpoint volume of 2 000 mm^3.

For the tumor analysis, NCI-H929 cells (1×10^7 cells in a 0.2 mL suspension in 50% Matrigel/PBS) were subcutaneously injected into the right flank of female immunodeficient NOD SCID mice. Tumors were measured twice weekly. Thirteen days after tumor implantation (Day 1), when the average tumor volume reached 94 mm^3, mice were randomly assigned to daily oral treatment with vehicle control or OSU-13 (10 mg/kg on Days 1-7 and 5 mg/kg on Days 8-14, adjusted due to weight loss). The treatment period lasted until Day 14. After 24 h of the last treatment, all mice were euthanized, and the tumors were collected. Western blot analysis was performed to assess the expression levels of cleaved caspase 3, p-H2AX, and cleaved PARP1 in the tumor samples.

Mice were cared for in accordance with the American Association for Accreditation of Laboratory Animal Care (AAALAC) guidelines.

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SUPPLEMENTARY TABLES

Supplementary Table S1 Kinase profiling inhibition test of OSU-13 against 399 kinases.*

Kinase	% Enzyme Activity (relative to DMSO)		
	Replicate 1	Replicate 2	Average
TTK	-2.18	-2.90	-2.5
IRR/INSRR	2.9	2.6	2.8
LRRK2	4.7	3.7	4.2
ALK	7.2	6.9	7.1
TYK1/LTK	12.0	11.7	11.8
IR	17.2	15.9	16.5
FAK/PTK2	20.5	19.9	20.2
FER	36.1	34.9	35.5
ACK1	38.8	36.5	37.7
JNK3	40.5	40.3	40.4
PYK2	44.3	43.8	44.0
IGF1R	44.3	43.4	43.9
STK22D/TSSK1	47.7	47.6	47.6
FES/FPS	49.6	48.7	49.2
JNK1	49.8	48.7	49.2
ERN1/IRE1	61.4	50.9	56.1
MLCK/MYLK	63.8	61.2	62.5
TSSK2	64.3	61.3	62.8
MSSK1/STK23	66.5	66.4	66.5
ROS/ROS1	67.1	65.9	66.5
PDK1/PDHK1	73.7	70.3	72.0
MYO3b	74.6	74.4	74.5
DNA-PK	75.6	73.9	74.8
STK33	76.1	76.0	76.1
MYO3A	78.3	76.0	77.2
PKCmu/PRKD1	78.8	78.0	78.4
JNK2	78.9	77.4	78.1
PKCd	79.3	78.4	78.9
SRPK1	79.5	78.1	78.8
LATS1	80.8	80.7	80.8
PDK4/PDHK4	81.0	80.2	80.6
CDK19/cyclin C	81.3	79.1	80.2
AMPK(A2/B2/G2)	82.5	82.5	82.5
PAK6	83.4	80.9	82.1
BRK	83.8	83.6	83.7
NEK1	84.2	76.1	80.2

PDK3/PDHK3	84.5	80.5	82.5
PDK2/PDHK2	84.5	84.2	84.4
ALK1/ACVRL1	84.6	82.9	83.7
TRPM7/CHAK1	84.9	83.6	84.2
EPHA7	85.8	77.4	81.6
PKCnu/PRKD3	86.4	84.4	85.4
SRPK2	86.5	83.2	84.8
PAK1	86.8	85.2	86.0
YES/YES1	86.9	84.1	85.5
HIPK2	87.0	86.8	86.9
NEK8	87.2	86.4	86.8
RSK2	87.2	87.1	87.1
ERK7/MAPK15	87.2	86.9	87.1
MAST3	87.5	86.1	86.8
PKCtheta	87.7	86.4	87.1
ALK2/ACVR1	88.1	86.4	87.3
WNK1	88.1	84.8	86.5
ASK1/MAP3K5	88.5	84.2	86.3
GRK4	88.7	87.4	88.1
c-MER	88.7	86.4	87.6
BTK	88.8	87.8	88.3
p70S6Kb/RPS6KB2	88.9	88.8	88.8
CDK16/cyclin Y (PCTAIRE)	89.0	87.1	88.1
CHK1	89.1	84.3	86.7
CDK6/cyclin D3	89.1	85.6	87.3
PLK2	89.1	87.5	88.3
MTOR/FRAP1	89.3	87.8	88.6
DAPK1	89.5	86.8	88.1
NEK11	89.7	87.7	88.7
PHKg2	89.8	87.0	88.4
KSR2	89.8	89.8	89.8
ARK5/NUAK1	89.8	89.4	89.6
ULK2	89.8	87.4	88.6
YSK4/MAP3K19	90.0	89.3	89.6
Haspin	90.0	89.1	89.6
PKCeta	90.1	88.2	89.2
CAMKK1	90.2	85.5	87.9
CHK2	90.2	89.0	89.6
CDC7/DBF4	90.2	88.1	89.1
PDGFRb	90.3	89.7	90.0
DYRK1/DYRK1A	90.4	87.4	88.9
DRAK1/STK17A	90.5	88.1	89.3
PKA	90.8	89.7	90.3

CDK5/P25	90.8	89.4	90.1
HPK1/MAP4K1	90.9	89.0	89.9
AMPK(A2/B2/G1)	91.0	90.8	90.9
TSSK3/STK22C	91.1	88.7	89.9
HCK	91.2	90.9	91.0
NEK3	91.2	90.5	90.9
CDK4/cyclin D3	91.2	88.6	89.9
VRK2	91.4	86.9	89.1
TBK1	91.4	91.3	91.4
MELK	91.6	91.1	91.3
NIM1	91.7	85.5	88.6
AMPK(A1/B1/G2)	91.8	91.3	91.5
ERK1	91.8	91.2	91.5
PKG1b	91.9	88.4	90.2
CDK1/cyclin A	92.0	91.8	91.9
SGK1	92.1	90.5	91.3
MEKK6	92.2	91.0	91.6
MEKK2	92.2	85.5	88.8
SIK1	92.2	91.9	92.1
IKKa/CHUK	92.2	89.9	91.1
PKCiota	92.3	88.9	90.6
AMPK (A2/B1/G2)	92.5	92.3	92.4
DYRK1B	92.5	91.2	91.8
AURORA C	92.5	88.3	90.4
CLK3	92.5	92.3	92.4
CK1a1	92.6	91.4	92.0
PKCb1	92.6	91.2	91.9
MINK/MINK1	92.7	92.5	92.6
CK1g1	92.7	85.8	89.3
EIF2AK2	92.9	92.5	92.7
CDK8/cyclin C	92.9	90.8	91.8
TYRO3/SKY	92.9	90.7	91.8
JAK2	92.9	92.7	92.8
TRKA	93.0	89.5	91.3
TRKC	93.0	90.3	91.6
FRK/PTK5	93.1	91.8	92.4
OSR1/OXSR1	93.2	91.4	92.3
BMPR2	93.2	91.6	92.4
CLK1	93.2	90.7	91.9
TGFBR2	93.2	92.7	93.0
SNRK	93.3	92.2	92.7
AMPK (A1/B2/G3)	93.3	88.6	91.0
PAK3	93.3	83.7	88.5

MASTL	93.5	91.5	92.5
CAMK2a	93.5	88.2	90.9
LOK/STK10	93.7	90.9	92.3
CDK4/cyclin D1	93.7	93.3	93.5
GRK1	93.8	93.6	93.7
EPHB4	93.9	93.6	93.7
ERK5/MAPK7	93.9	92.4	93.2
CDK5/p35	93.9	93.0	93.4
BRAF	94.0	93.2	93.6
EPHA8	94.0	93.1	93.5
EPHB3	94.0	92.4	93.2
HIPK3	94.0	93.5	93.8
BMX/ETK	94.1	92.4	93.2
MAPKAPK3	94.1	91.0	92.5
NEK6	94.4	94.2	94.3
MAPKAPK5/PRAK	94.4	90.4	92.4
GLK/MAP4K3	94.4	93.8	94.1
CDK3/cyclin E	94.4	93.9	94.2
PBK/TOPK	94.6	90.6	92.6
EIF2AK1	94.6	93.9	94.3
CAMK1d	94.7	94.0	94.3
BLK	94.7	93.2	93.9
FGFR4	94.7	93.1	93.9
MYLK4	95.0	93.6	94.3
TESK2	95.0	94.9	94.9
ERK2/MAPK1	95.1	93.9	94.5
TESK1	95.1	94.8	94.9
P38g	95.1	95.0	95.0
RSK1	95.1	93.5	94.3
COT1/MAP3K8	95.1	93.4	94.3
GSK3b	95.2	95.0	95.1
EGFR	95.2	94.1	94.6
DYRK4	95.2	92.4	93.8
MARK3	95.3	93.7	94.5
ZIPK/DAPK3	95.3	89.3	92.3
KDR/VEGFR2	95.4	94.7	95.1
CLK4	95.5	94.7	95.1
AMPK(A1/B1/G1)	95.5	95.4	95.5
MSK1/RPS6KA5	95.7	94.5	95.1
AMPK(A1/B2/G1)	95.8	92.4	94.1
NEK9	95.8	94.8	95.3
PKCepsilon	95.8	94.8	95.3
ABL2/ARG	95.9	94.9	95.4

JAK1	96.0	94.0	95.0
BRSK2	96.0	93.3	94.6
LATS2	96.0	95.2	95.6
c-Src	96.0	95.5	95.7
RIPK3	96.0	95.2	95.6
MLK3/MAP3K11	96.3	95.7	96.0
PAK4	96.3	95.1	95.7
AMPK (A2/B1/G3)	96.3	91.6	94.0
CAMK1g	96.4	96.2	96.3
CDK6/cyclin D1	96.5	94.9	95.7
WNK2	96.5	92.9	94.7
CDK1/cyclin B	96.5	96.5	96.5
HGK/MAP4K4	96.7	95.8	96.2
RIPK4	96.7	96.6	96.7
IKKb/IKBKB	96.8	96.7	96.8
VRK1	96.8	96.1	96.5
LCK2/ICK	97.0	96.6	96.8
SRMS	97.0	95.7	96.3
PAK5	97.0	97.0	97.0
CAMK2b	97.0	96.7	96.9
MKK4	97.0	94.1	95.6
p70S6K/RPS6KB1	97.1	96.3	96.7
TXK	97.2	92.7	95.0
DMPK	97.3	96.9	97.1
GRK5	97.3	95.6	96.4
MLCK2/MYLK2	97.4	96.7	97.0
SIK2	97.4	96.9	97.2
LYN B	97.4	94.4	95.9
RET	97.4	95.1	96.2
PAK2	97.4	95.0	96.2
CK2a	97.4	93.0	95.2
FLT3	97.5	95.5	96.5
SLK/STK2	97.7	96.7	97.2
Aurora A	97.8	93.5	95.7
CDK2/cyclin O	97.8	97.3	97.5
DDR2	97.8	95.6	96.7
DYRK3	97.9	96.6	97.2
AMPK(A1/B1/G3)	97.9	96.5	97.2
PKCa	97.9	97.5	97.7
NEK7	97.9	97.3	97.6
PKD2/PRKD2	98.0	93.4	95.7
RAF1	98.0	97.8	97.9
STK32C/YANK3	98.0	97.3	97.7

KSR1	98.1	97.3	97.7
WEE1	98.2	94.9	96.5
CLK2	98.2	96.7	97.4
MKK7	98.2	96.9	97.5
AXL	98.2	95.8	97.0
PKCb2	98.3	95.9	97.1
AMPK(A2/B1/G1)	98.3	98.0	98.1
PASK	98.3	95.2	96.8
EPHA5	98.4	97.5	97.9
SNARK/NUAK2	98.4	97.6	98.0
MNK1	98.4	97.7	98.1
NEK2	98.5	96.7	97.6
EPHA2	98.5	98.2	98.4
ERN2/IRE2	98.6	97.7	98.1
LCK	98.6	98.5	98.6
EPHB1	98.6	97.7	98.2
MAPKAPK2	98.7	98.4	98.5
MNK2	98.7	98.3	98.5
ARAF	98.7	98.1	98.4
EPHA6	98.7	98.4	98.5
PEAK1	98.8	97.0	97.9
PKCzeta	98.9	97.8	98.3
RSK4	99.0	98.1	98.6
CK2a2	99.0	96.9	98.0
CDK2/cyclin A	99.0	98.9	99.0
BRSK1	99.0	98.5	98.8
STK38/NDR1	99.0	98.1	98.6
EPHA4	99.1	97.7	98.4
c-Kit	99.1	98.2	98.6
TTBK2	99.1	98.0	98.5
AKT2	99.2	95.2	97.2
MST4	99.4	98.5	98.9
IRAK1	99.4	96.9	98.2
PIM1	99.4	98.5	99.0
LKB1	99.4	99.2	99.3
EIF2AK4	99.4	98.9	99.2
MEK5	99.5	98.9	99.2
CK1g2	99.5	96.2	97.9
PKAcb	99.6	99.1	99.3
TYK2	99.6	98.5	99.1
DAPK2	99.6	98.0	98.8
RIPK2	99.6	97.7	98.7
FYN	99.6	96.9	98.3

DCAMKL2	99.6	99.5	99.6
PKAcg	99.6	98.7	99.2
MLK4	99.7	98.3	99.0
ROCK1	99.7	99.2	99.4
GRK2	99.7	96.5	98.1
CAMK1b	99.9	98.5	99.2
HIPK4	100.0	98.9	99.4
RON/MST1R	100.0	96.2	98.1
PKCg	100.0	98.7	99.3
TIE2/TEK	100.0	98.9	99.5
PKN3/PRK3	100.1	99.6	99.9
PLK4/SAK	100.2	100.0	100.1
LYN	100.2	99.3	99.8
ALK3/BMPR1A	100.3	96.4	98.4
EIF2AK3	100.4	96.5	98.4
GRK3	100.4	100.1	100.3
c-MET	100.4	98.4	99.4
MUSK	100.4	97.7	99.0
GRK6	100.5	97.5	99.0
RIPK5	100.5	99.8	100.2
CDK2/Cyclin A1	100.6	99.3	100.0
TAK1	100.6	93.8	97.2
AKT1	100.7	100.5	100.6
WNK3	100.7	99.1	99.9
NEK4	100.8	99.3	100.1
TNK1	100.8	96.8	98.8
KHS/MAP4K5	100.8	100.8	100.8
CAMK1a	100.8	99.3	100.1
PIM2	100.8	99.5	100.2
PKN2/PRK2	100.9	98.4	99.6
MEKK3	100.9	97.0	98.9
PKN1/PRK1	101.0	100.4	100.7
CAMK2d	101.1	90.9	96.0
MARK2/PAR-1Ba	101.2	99.6	100.4
GRK7	101.2	101.1	101.2
CDK2/cyclin E2	101.4	99.5	100.4
FGFR2	101.4	98.9	100.1
PLK3	101.6	101.6	101.6
P38d/MAPK13	101.8	101.7	101.7
MST3/STK24	101.8	99.8	100.8
CK1G3	101.8	100.2	101.0
CSK	101.9	101.1	101.5
IRAK4	101.9	100.1	101.0

ALK4/ACVR1B	101.9	99.9	100.9
CDK14/cyclin Y (PFTK1)	101.9	101.6	101.8
CDK1/cyclin E	102.0	99.3	100.6
NEK5	102.0	98.4	100.2
TAOK3/JIK	102.0	95.9	99.0
ITK	102.1	95.8	98.9
PRKX	102.2	100.8	101.5
CDK9/cyclin T2	102.3	101.0	101.6
ALK6/BMPR1B	102.3	101.3	101.8
MARK1	102.3	99.9	101.1
CDK2/cyclin E	102.4	102.2	102.3
LIMK1	102.6	100.6	101.6
STK38L/NDR2	102.7	99.0	100.8
STK16	102.9	102.6	102.7
ZAK/MLTK	103.0	102.9	103.0
CK1a1L	103.1	99.6	101.3
CAMK4	103.1	101.1	102.1
MARK4	103.2	101.0	102.1
DDR1	103.2	101.1	102.2
FLT4/VEGFR3	103.3	101.0	102.1
SIK3	103.3	103.3	103.3
DCAMKL1	103.3	98.0	100.6
EPHB2	103.4	100.0	101.7
ERBB4/HER4	103.4	99.4	101.4
PLK1	103.4	102.3	102.9
ZAP70	103.4	98.7	101.1
ROCK2	103.4	103.3	103.4
EEF2K	103.5	102.7	103.1
DMPK2	103.6	103.4	103.5
FGFR3	103.8	100.5	102.1
EPHA3	103.8	100.9	102.3
SYK	104.1	102.7	103.4
PKG1a	104.1	103.0	103.6
MYLK3	104.1	102.0	103.0
MEKK1	104.1	103.9	104.0
MLK1/MAP3K9	104.2	99.3	101.7
CDK9/cyclin T1	104.2	102.6	103.4
CTK/MATK	104.5	99.8	102.2
P38b/MAPK11	104.8	104.7	104.7
MEK1	104.8	104.6	104.7
DYRK2	104.8	104.0	104.4
AMPK (A1/B2/G2)	104.8	99.3	102.1
MST1/STK4	104.8	103.2	104.0

CDK18/cyclin Y (PCTK3)	104.9	102.3	103.6
HIPK1	105.0	102.2	103.6
PDGFRa	105.2	103.9	104.6
P38a/MAPK14	105.3	102.1	103.7
CDK9/cyclin K	105.3	103.8	104.5
GSK3a	105.3	105.1	105.2
PIM3	105.4	99.9	102.6
TTBK1	105.4	103.3	104.3
SGK2	105.4	101.8	103.6
TEC	105.4	100.8	103.1
CAMKK2	106.0	103.4	104.7
CAMK2g	106.1	105.8	106.0
MSK2/RPS6KA4	106.1	105.6	105.9
TAOK1	106.2	104.0	105.1
PKMYT1	106.4	104.2	105.3
CDK3/cyclin E2	106.5	104.1	105.3
MLK2/MAP3K10	106.7	103.4	105.1
FLT1/VEGFR1	106.9	106.8	106.8
SGK3/SGKL	107.2	105.3	106.2
CK1d	107.5	104.0	105.7
EPHA1	107.9	105.5	106.7
NLK	107.9	103.1	105.5
MRCKa/CDC42BPA	107.9	105.8	106.9
LIMK2	108.0	106.4	107.2
STK39/STLK3	108.0	105.4	106.7
CDK17/cyclin Y (PCTK2)	108.1	108.1	108.1
RSK3	108.6	103.0	105.8
ABL1	108.7	107.5	108.1
SSTK/TSSK6	108.7	105.2	106.9
MST2/STK3	108.8	104.4	106.6
SBK1	108.9	103.9	106.4
CK1epsilon	109.0	106.7	107.8
PDK1/PDPK1	109.3	108.9	109.1
TNIK	109.5	99.3	104.4
ULK3	109.5	108.6	109.0
AKT3	109.9	108.4	109.2
MRCKb/CDC42BPB	110.0	108.0	109.0
ULK1	110.2	109.3	109.7
STK25/YSK1	110.8	109.8	110.3
AMPK(A2/B2/G3)	110.8	107.8	109.3
MEK2	111.1	109.8	110.5
AURORA B	111.2	99.1	105.1
STK32B/YANK2	111.3	110.1	110.7

FGFR1	111.3	109.8	110.5
PKG2/PRKG2	111.5	104.3	107.9
FGR	111.8	111.7	111.7
TAOK2/TAO1	111.8	106.8	109.3
DLK/MAP3K12	112.8	111.2	112.0
ALK5/TGFBR1	112.8	111.4	112.1
GCK/MAP4K2	113.0	102.6	107.8
IKKe/IKBKE	113.7	111.0	112.3
CDK7/cyclin H	113.7	111.2	112.4
MKK6	114.2	109.3	111.8
FMS	115.7	112.5	114.1
JAK3	116.8	114.5	115.6
TLK2	117.5	111.0	114.3
TRKB	118.3	115.2	116.8
ERBB2/HER2	120.2	120.2	120.2
MAK	120.3	119.6	119.9
PHKg1	121.7	112.1	116.9
TLK1	122.2	105.6	113.9
MEK3	122.5	118.8	120.6

* OSU-13 was tested in duplicate at a concentration of 1 μM. Values represent the percentage of enzyme activity relative to DMSO control after inhibition with OSU-13 at 10 μM ATP. Kinases that were inhibited >80% are highlighted in gray.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1 *TTK* expression does not correlate with some high-risk related genetic alterations present in MM. A-D MMRF CoMMpass database analysis of the *TTK* expression in MM patients with or without del(17p), t(4;14), t(8;14), and t(11;14). No statistically significant difference was observed in any of the analyses.

Supplementary Figure S2 *TTK* knockdown leads to decreased viability of MM cell lines. OPM-2 and NCI-H929 cells were transfected with Alexa-fluor 647-conjugated TTK siRNA or non-targeting scrambled siRNA (5 μM) and incubated for 48 h at 37°C. Streptolysin SLO (0U,

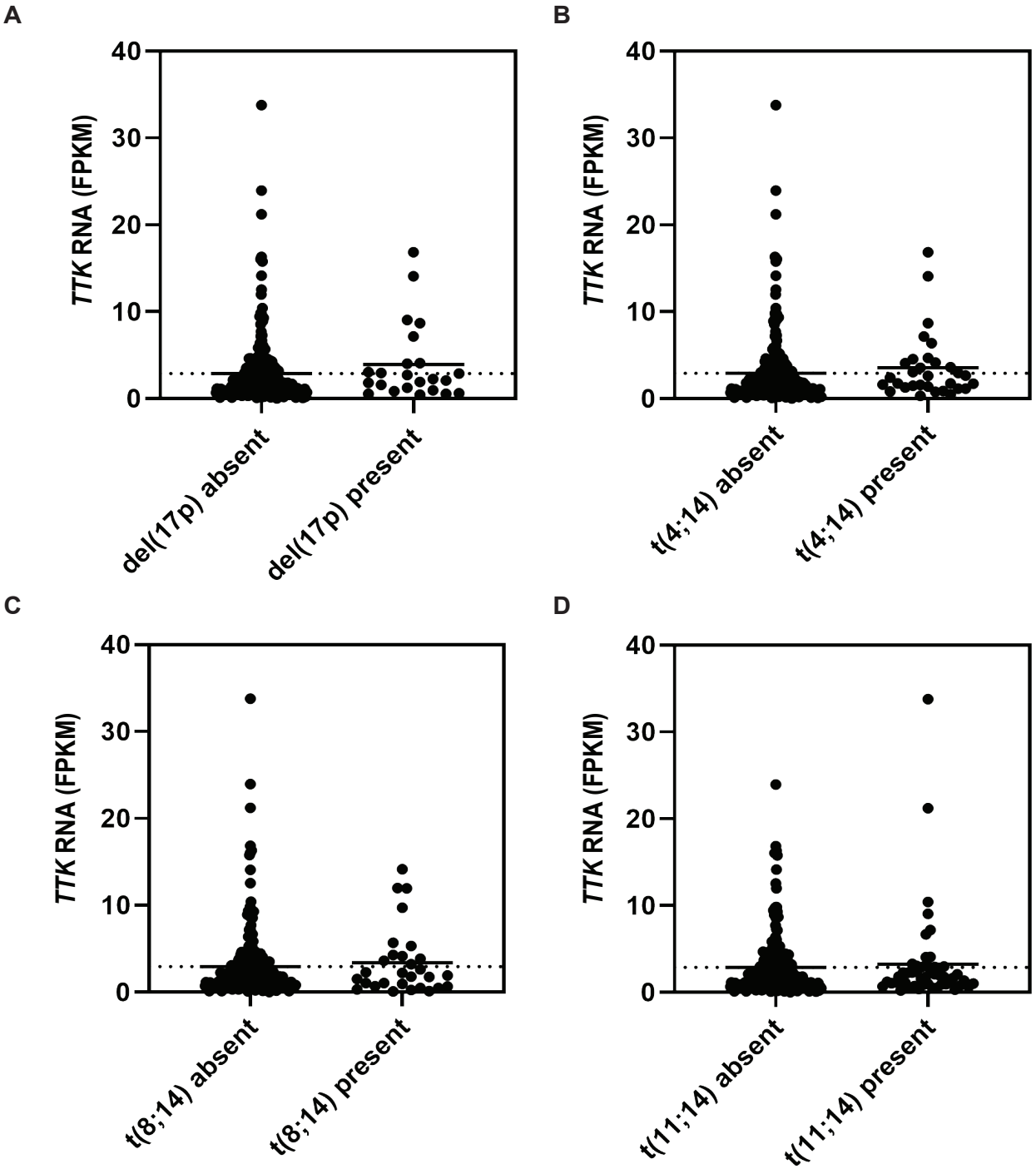
10U, or 20U) was used to enhance siRNA uptake. After incubation, flow cytometry analysis revealed the percentage of siRNA^{+} cells (AF 647^{+}) that were dead (Zombie^{+}). Data represent the mean of three independent experiments ± SD. *P*-value < 0.05 (*), *P*-value < 0.001 (***).

Supplementary Figure S3 OSU-13 has minimal effect in non-plasma cell PBMC. A Primary cells derived from the bone marrow (BM) of a MM patient and the PBMC of a plasma cell leukemia (PCL) patient were cultured *in vitro* at 37ºC in the presence of DMSO with or without 0.5 μM of OSU-13 for 72 h. The graph shows the comparative viability (Zombie^{-}) of B cells (CD138^{-} CD19^{+}), T cells (CD56^{-} CD3^{+}), NK cells (CD138^{-} CD56^{+} CD3^{-}), and NKT cells (CD138^{-} CD3^{+} CD56^{+}) treated with OSU-13 for 72h compared to DMSO. **B** A similar experiment was conducted as described in **A** using PBMC from three healthy donors. Graph shows the comparative viability (Zombie^{-}) of the cell populations from each donor treated with OSU-13 for 72h compared to DMSO.

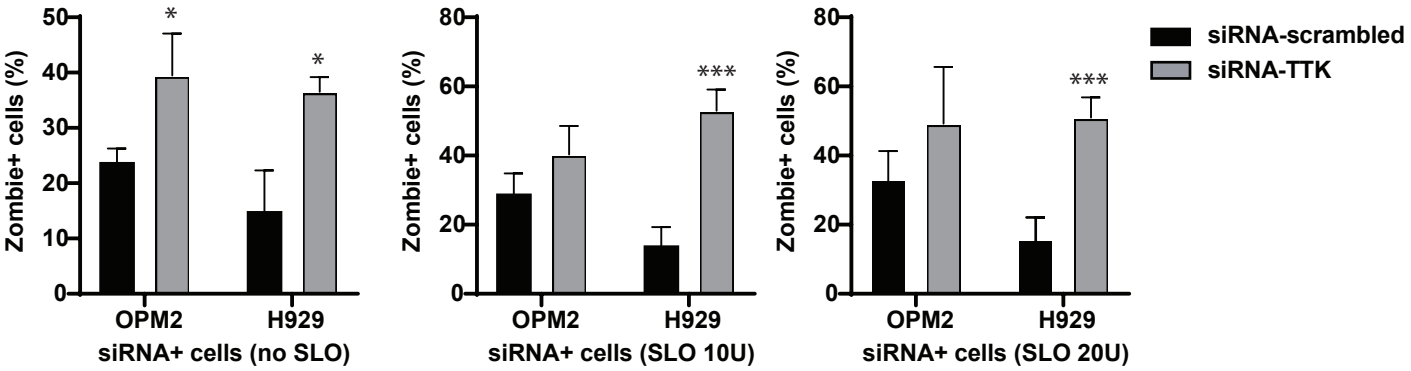
Supplementary Figure S4 Necroptosis and autophagy are not involved in OSU-13-induced cell death. A Effect of necrostatin-1s in the cell viability of OSU-13-treated cells. OPM-2 cells were pre-treated for 1 h with 100 μM necrostatin-1s or DMSO and then incubated for 72 h in 1 μM OSU-13. Cell viability was assessed with Zombie-aqua dye staining by flow cytometry. Data represent the mean of three independent experiments ± SD. **B** Western blot analysis of LC3B in lysates from OPM-2 and NCI-H929 cells incubated in DMSO or 0.5 μM OSU-13 for 72 h. GAPDH was used as loading control. Images depict a representative experiment from two independent experiments.

Supplementary Figure S5 OSU-13 causes cell cycle and chromosome segregation abnormalities in NCI-H929 cells. A DNA content analysis of NCI-H929 cells treated with DMSO (red) or 1 μM OSU-13 (blue) for 72 h. After treatment, DNA was stained with the intercalating agent propidium iodide (PI) and analyzed by flow cytometry. 2N and 4N populations are indicated. Data are representative of three independent experiments. **B** Graphical representation of the cell cycle analysis from data depicted in panel **A**. Analysis was performed by the Cell Cycle tool in FlowJo_V10 software using the Watson model. Results are mean ± SD of three independent experiments. *P*-value < 0.05 (*), *P*-value < 0.01 (**). **C** Chromosome spread analysis of NCI-H929 cells treated with DMSO or 1 μM OSU-13 for 24 h. Slides were mounted in Prolong Glass Antifade Mountant with NucBlue to stain the chromosomes. On the left, representative fluorescence microscopy images from the metaphase spreads. Scale bars, 10 μm. On the right, graph showing the distribution of the cell population according to the number of chromosomes, manually quantified in ImageJ. Results show the mean of three independent experiments ± SD. *P*-value < 0.05 (*).

Supplementary Figure S1

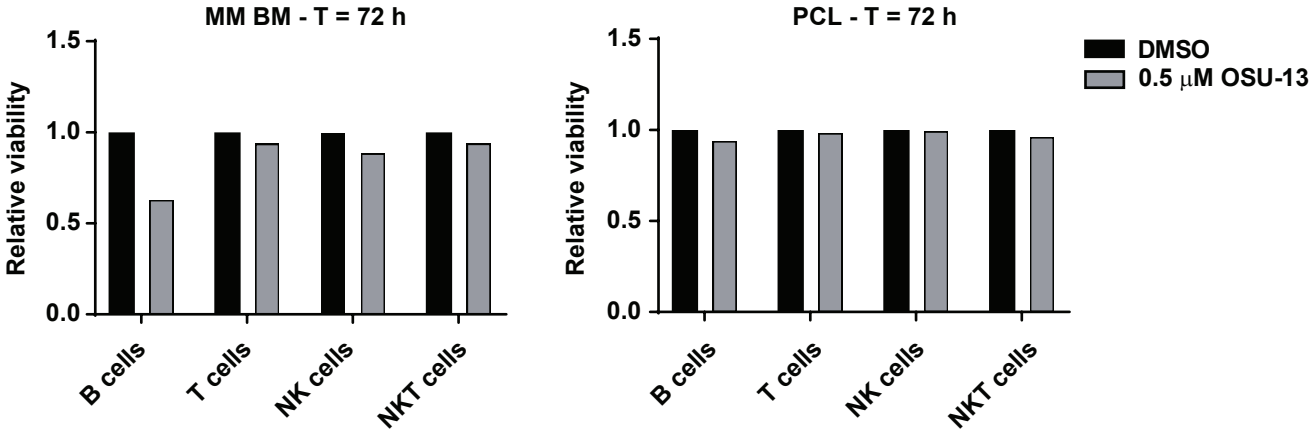


Supplementary Figure S2



Supplementary Figure S3

A



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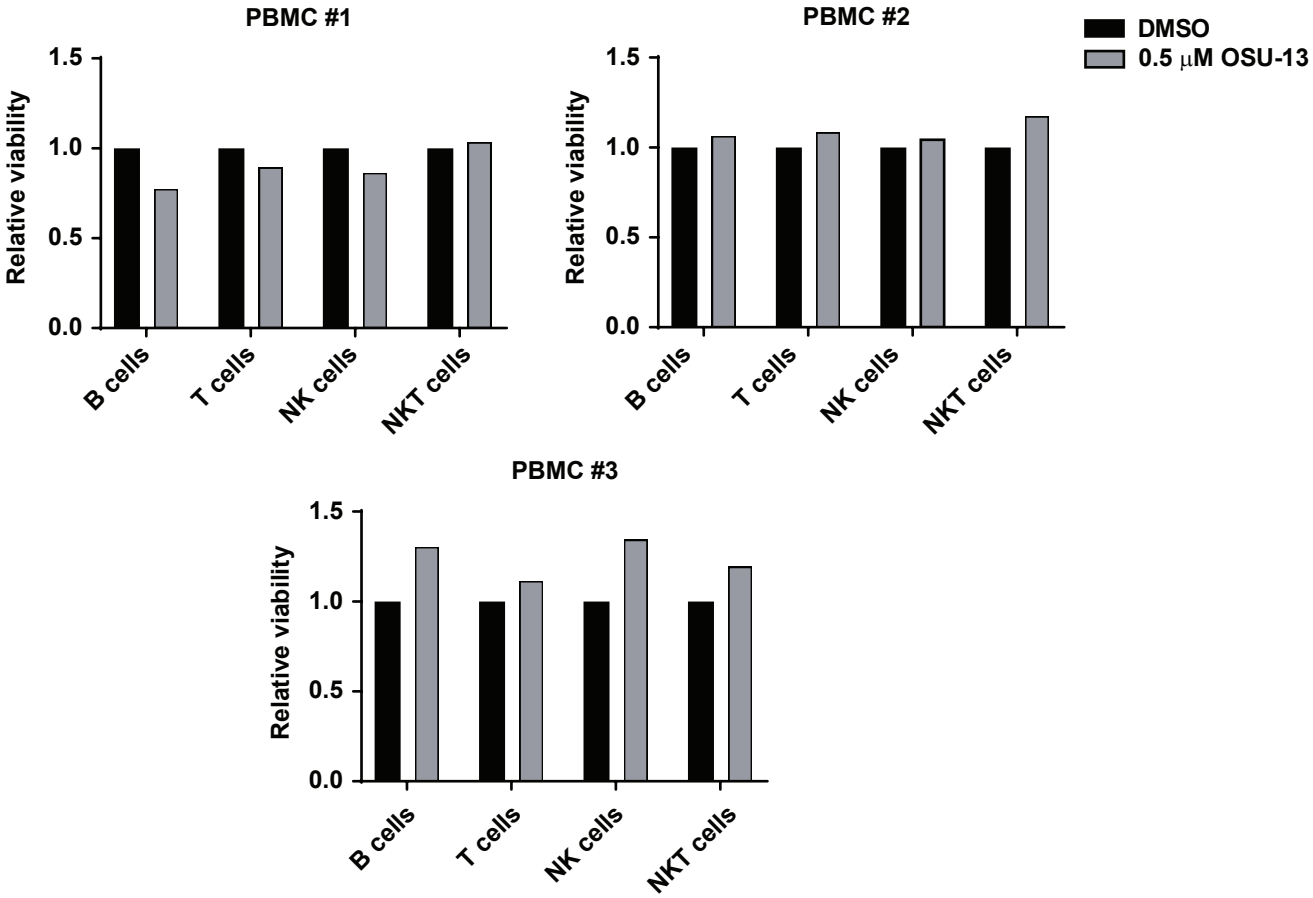
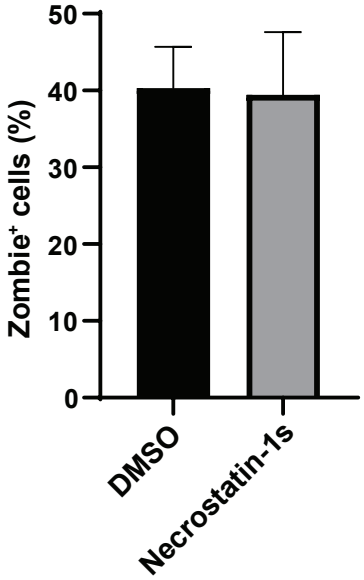


Supplementary Figure S4

A



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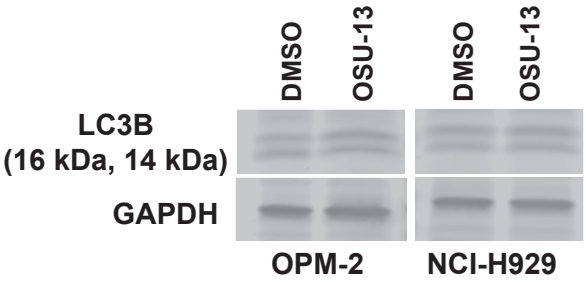

Supplementary Figure S5