Oncogenic TYK2 P760L kinase is effectively targeted by combinatorial TYK2, mTOR and CDK4/6 kinase blockade

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Supplementary Methods

Cloning of *TYK2* expression cassettes

Mutations were introduced in the human *TYK2* cDNA (CCDS database: CCDS12236.1) and cloned in the MSCV-IRES-GFP plasmid (#20672, Addgene, Watertown, MA, US) by site-directed mutagenesis PCR using Phusion polymerase (Thermo Fisher Scientific, Waltham, MA, US) and listed primers (depicted in Supplementary Table S1). Mutations in TYK2 were confirmed in plasmids or gDNA by Sanger sequencing (LGC Genomics, London, UK).

Cell lines

Cells were maintained in a humidified incubator at 37°C with 5% CO_2 and were routinely tested for mycoplasma contamination by PCR with VenorGeM Classic (Minerva Biolabs, Berlin, Germany). HPC-7 cells[1] were grown in Iscove modified Dulbecco medium (IMDM) (I3390, Sigma Aldrich, St. Louis, MO, US) supplemented with 5% heat inactivated fetal calf serum (FCS) (Gibco/Thermo Fisher Scientific), 4 mM L-glutamine (Sigma Aldrich), 100 U/ml penicillin 100 µg/ml streptomycin (Sigma Aldrich), $0.75x10^{-4}$ M 1-thioglycerol (Sigma Aldrich) and 2% conditioned medium containing stem cell factor (SCF) as described previously.[2] hTERT mesenchymal stem cells (MSC)[3] (kindly provided by M. Dworzak, CCRI Vienna), Ba/F3 STAT5B^{N642H} [4], Ba/F3 TEL-JAK2[5], Ba/F3 BCR-ABL1 p210[6], Ba/F3 (ACC 300, DSMZ, Braunschweig, Germany) and 32D (ACC 411, DSMZ) cells were maintained in RPMI 1640 medium (R8758, Sigma Aldrich) complete (supplemented with 10% heat inactivated FCS, 2 mM L-glutamine and 100 U/ml penicillin 100 µg/ml streptomycin). hTERT MSC cells were supplemented with 1 µM hydrocortisone (Sigma Aldrich), parental Ba/F3 and 32D cells with 1 ng/ml IL-3 (213-13, Peprotech, Rocky Hill, NJ, US). Phoenix-ECO (CRL-3214^{TM}, ATCC, Manassas, VA, US) packaging cells were kept in Dulbecco's modified Eagle medium (DMEM) (D6546, Sigma Aldrich) complete (supplemented with 10% heat inactivated FCS, 2 mM L-glutamine and 100 U/ml penicillin 100 µg/ml streptomycin).

Generation of stable cell lines and primary cells

Bone marrow (BM) cells were isolated by flushing femur and tibia of 9-11-week-old male wildtype (WT) mice (C57BL6/N, Charles River Laboratories, Wilmington, MA, US). Red blood cell lysis was performed with Hybri Max lysis buffer (Sigma Aldrich), and cells were washed with PBS. BM cells were cultivated 1 day prior to transduction in RPMI 1640 medium complete (supplemented as described before) containing 10 ng/ml IL-3, 10 ng/ml IL-6 (216-16, Peprotech) and 2% SCF.

For production of retroviral particles Phoenix cells were transfected with the retroviral plasmid DNA using Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific) according to manufacturer's protocol and cultured for 2 days. Filtrated supernatant supplemented with 4 µg/ml (primary cells) or 8 µg/ml (cell lines) polybrene (Sigma Aldrich) was used for three consecutive rounds of spinoculations (1000 g, 90 min) (adapted from Schmoellerl et al.[7], Aigner et al.[8]). After 3 days cells were fixed with IC fixation buffer (eBio/Thermo Fisher Scientific) according to manufacturer's protocol and GFP expression was assessed by FACS Canto II (BD Biosciences, Franklin Lakes, NJ, US). Cell lines were sorted for GFP expression with FACSAria TM III (BD Biosciences).

IL-3 independent Ba/F3 and 32D outgrowth assay

5 x 10^6 cells were electroporated[4] with 25 µg plasmid DNA using GenePulser II (BioRad, Hercules, CA, US). On the next day, cells were washed three times and cultured without IL-3. Medium was exchanged 1-2 times per week. Cell growth was regularly monitored by Casy cell counter (Innovatis, Franklin, TN, US) and by measuring the GFP expression by flow cytometry.

Colony formation assay

1x10^3 sorted GFP^+ HPC-7 cells or 1x10^4 GFP^+ BM cells (for flow cytometry analysis 1x10^5 GFP^+ BM cells) were seeded in hematopoietic growth factor-free Methocult methylcellulose (M3231, Stemcell, Vancouver, Canada). HPC-7 cells were supplemented with 0.3% SCF. After 7-14 days colonies were counted, and washed cells were reseeded (three times) in new methylcellulose. Pictures were taken with CKX41 Olympus microscope (Olympus, Shinjuku, Japan) and Baumer GAPI software (Baumer, Frauenfeld, Switzerland).

Cell viability assay cell lines

Cell titer blue viability assay (Promega, Madison, WI, US) was used according to manufacturer's protocol. 1x10^4 cells/well were seeded in duplicates or triplicates into a 96-well flat bottom plate and incubated with at least five different drug / SCF concentrations for 3 days. For synergy screening two different concentrations (10 nM, 30 nM) of BMS986165 (MedChemExpress, Monmouth Junction, NJ, US) were added. Details on drugs used are depicted in Supplementary Table S2. Fluorescence was measured by EnSpire 2300 (PerkinElmer, Waltham, MA, US). Data were normalized to untreated controls / 2% SCF (100% metabolic activity) and highest drug concentration or positive controls (15% DMSO) / 0% SCF (0% metabolic activity).

IC_{50} values were calculated using a non-linear regression model in GraphPad Prism version 7.0 for Mac (GraphPad Software, San Diego, CA, US). Synergy scores were calculated with the zero interaction potency (ZIP) model using the website: synergyfinder.org.[9]

For determining the effects of the drugs on malignant transformation of Ba/F3 and 32D cells, retrovirally transduced $TYK2^{P760L}$-expressing cells were washed three times and incubated with the indicated drug concentration without IL-3 in duplicates. Cell number was determined by cell counter (BioRad, Hercules, CA, US) and medium was exchanged 1-2 times per week.

Cell viability assay patient derived xenograft (PDX) cells

PDX cells ($1.4x10^5$/well) were seeded in 96-well plates on a feeder layer of 1 day precultured hTERT immortalized MSC ($1.4x10^4$/well) in AIMV medium (12055-091, Gibco/Thermo Fisher Scientific) as similarly described.[10] At least 5 different drug concentrations (Supplementary Table S2) were added and after 3 days incubation cells were harvested and stained with LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit according to manufacturer's protocol (Thermo Fisher Scientific, L34960). The percentage of viable cells was assessed by Cytoflex S (Beckman Coulter, Brea, CA, US) with CytExpert version 2.2.0.97. Data were normalized to untreated samples.

Apoptosis and cell cycle analysis

For determining early and late apoptosis cells were stained with Annexin V APC (Biolegend, San Diego, CA, US) and 7-AAD (Miltenyi, Bergisch-Gladbach, Germany) according to manufacturer's protocol. For cell cycle analysis cells were fixed, permeabilized (both Biolegend) and stained with 1 µg/ml DAPI (Sigma Aldrich) and Ki-67 APC (Thermo Fisher Scientific, dilution 1:500) for 30 min. The cells were analyzed with Cytoflex S and CytExpert version 2.2.0.97.

Flow cytometry

PDX cells and fixed primary cells and BM colonies were first stained with fixable viability dye APC Cy7 (eBio/Thermo Fisher Scientific) at a dilution 1:1000. They were washed with PBS and surface staining was performed with antibodies according to Supplementary Table S3 at a dilution 1:100 (1:250 for PB lineage antibodies).

Analysis was performed on FACS Canto II and FlowJo version 10.

Immunoblotting

Cells were lysed as described previously.[11] Cell debris was removed by centrifugation and equal protein amounts were denatured in 1x Laemmli buffer for 10 min at 95°C.

Separation was performed by SDS-PAGE and proteins were transferred on a nitrocellulose membrane (Amersham, Little Chalfont, UK). Membranes were blocked with 5% BSA (Carl Roth, Karlsruhe, Germany) in TBST (Tris buffered saline Tween-20) and incubated with the following antibodies at 4°C overnight: pTYK2 (68790S), TYK2 (9312S), pSTAT1 (9167S), STAT1 (14995S), pSTAT3 (9131S), STAT3 (12640S), pSTAT5 (9356S), STAT5 (sc835), pAKT (9271S), AKT (9272S), p4EBP1 (2855T), 4EBP1 (9644T), CDK6 (sc56362) (all from Cell Signaling, Danvers, MA, US, 1:1000, except STAT5 and CDK6 from Santa Cruz Biotechnology, Dallas, TX, US, 1:500) and Actin (A2066, 1:1000, Sigma Aldrich). Primary antibodies were detected by anti-mouse HRP (7076S) and anti-rabbit HRP (7074S) secondary antibodies (all from Cell Signaling, dilution 1:2000) for 1 h at room temperature. Blots were developed with Clarity ECL Western blotting reagent (BioRad) using Chemidoc Touch Imaging System (BioRad).

Histochemistry

Harvested samples were transferred into 4% buffered formaldehyde for one day fixation at room temperature. After washing and storage in 70% ethanol the samples were dehydrated and embedded in paraffin. 2 µm sections were stained with hematoxylin and eosin (H&E). Representative pictures were taken with Olympus BX53 microscope and cellSens software (Olympus, Shinjuku, Japan).

Supplementary Table S1: Primer sequences used for site-directed mutagenesis and Sanger sequencing

Primer Sequence 5'-3'	
CAAGCTGAGTGATCTTGGCGTGGGCCTGGGC	P760L forward mutagenesis
CAGGCCCACGCCAAGATCACTCAGCTTGATG	P760L reverse mutagenesis
GAGTGATCCTGTCGTGGGCCTGGGCGCC	G761V forward mutagenesis
CCAGGCCCACGACAGGATCACTCAGCTTG	G761V reverse mutagenesis
TGCAGCTGGTCTTTGAGTACGTGCCCCTGG	M978F forward mutagenesis
GGCACGTACTCAAAGACCAGCTGCAGCGAC	M978F reverse mutagenesis
CCTAGTCACCATGACATCGC	Sequencing primer forward
GCAGGAGATTGACATTCTGC	Sequencing primer reverse
AGGAGTTTGAAGCTGCAGTG	Sequencing primer forward gDNA
TGAGCTCTGATTCTGTCCTC	Sequencing primer reverse gDNA

Supplementary Table S2: List of drugs dissolved in DMSO in the indicated concentration

Drugs	**Stock concentration and solvent**	**Source**	**Catalogue #**
Deucravacitinib	10 mM in DMSO	MedChemExpress	HY-117287
Palbociclib	8 mM in DMSO	MedChemExpress	HY-50767A
Vistusertib	10 mM in DMSO	MedChemExpress	HY-15247
Abemaciclib	10 mM in DMSO	MedChemExpress	HY-16297
Olverembatinib	10 mM in DMSO	MedChemExpress	HY-15666
Everolimus	10 mM in DMSO	Selleckchem (Houston, TX, US)	S1120
LY294002	100 mM in DMSO	Calbiochem (San Diego, CA, US)	CAS154447-36-6
Filgotinib	10 mM in DMSO	Selleckchem	S7605
Ruxolitinib	10 mM in DMSO	Eubio (San Diego, CA, US)	Tlrl-rux
Tofacitinib	10 mM in DMSO	Selleckchem	S2789

Supplementary Table S3: Antibody panels used for staining of BM cells, BM colonies and PDX cells

Antibody/Dye	**Clone**	**Source**
Panel for BM cells and BM colonies		
CD127 PB	A7R34	eBio
Ter119 PB	TER-119	eBio
B220 PB	RA3-6B2	eBio
CD11b PB	M1/70	eBio
CD3e PB	500A2	eBio
Gr-1 PB	RB6-8C5	eBio
c-Kit PE Cy5	2B8	eBio
Sca-1 PE Cy-7	D7	eBio
CD16/CD32 PE	93	eBio
Panel for PDX cells		
Human Fc block	-	BD
CD16/CD32	93	eBio
hCD10 PE	HI10a	BD

hCD45 APC	HI30	BD
mCD45 V500	30F11	BD
hCD19 PE Cy-7	HIB19	eBio

References of Supplementary Methods

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Supplementary Figure Legends

Figure S1: GFP expression of transduced bone marrow (BM) cells before and after seeding into factor-free methylcellulose. Percentage of GFP^+ cells of wildtype (WT) TYK2, $TYK2^{P760L}$, $TYK2^{P760L, M978F}$ and empty vector (EV) transduced cells before and after first seeding into methylcellulose (n=3, one-way ANOVA, arcsine square root transformed data). Cells were gated on single cells and living. *p<0.05

Figure S2: Colony formation and cell viability of transduced hematopoietic precursor cell line. (A) Representative colony pictures of transduced HPC-7 cells taken with a 10-fold magnification objective. Scale bars show 100 µm. (B) Cell viability assay of transduced HPC-7 cells in suspension (WT TYK2 in grey, mutated versions in orange (P760L) and red (G761V); kinase inactive with blue dashed line, EV with grey dashed line) supplemented with different stem cell factor (SCF) concentrations (n=2, in triplicates).

Figure S3: $TYK2^{P760L}$ induced outgrowth, signaling, tumor growth and pathohistology. (A) Table showing successful outgrowth of Ba/F3 and 32D cells expressing WT TYK2, $TYK2^{P760L}$, $TYK2^{G761V}$, kinase inactive variants harboring $TYK2^{M978F}$ or EV. (B) Western blot of $TYK2^{P760L}$-expressing Ba/F3 and 32D cells and parental cells subjected to (phospho-) TYK2-STAT1/3 analysis. Actin was used as loading control. Numbers indicate molecular weight markers in kDa. (C-G) Spleen weight, liver weight, white blood cell count, platelet count and hematocrit (HCT) of subcutaneously (*s.c).* injected mice [Ba/F3 and 32D cells: n=4, 32D $TYK2^{P760L}$ and Ba/F3 $TYK2^{P760L}$ cells: n=5, unpaired two tailed t-test with log and arcsine square root (HCT) transformed data, from two experiments]. (H) Representative H&E staining of spleen and liver of intravenously (*i.v.)* and *s.c.* injected mice. Scale bars show 50 µm. *p<0.05, **p<0.01

Figure S4: Deucravacitinib induces apoptosis and G0 cell cycle arrest. (A) Representative flow cytometry blots of Ba/F3 and Ba/F3 $TYK2^{P760L}$ cells treated with 1µM deucravacitinib for 24h, 48h and 72h. (B) Cell cycle analysis (n=3 in duplicates) and representative flow cytometry blots of Ba/F3 and Ba/F3 $TYK2^{P760L}$ cells treated with 1µM deucravacitinib for 72h.

Figure S5: Parental and transformed Ba/F3 and 32D cells and JAK inhibitors. (A) Dose-response curve of parental (dashed line, with IL-3) and control Ba/F3 cells expressing other oncogenes (shades of grey) treated with deucravacitinib for 72h (n≥3, in duplicates or triplicates, not all IC_{50} could be determined). (B-G) Dose-response curves and IC_{50} values of

parental (dashed line/white filling, with IL-3) and transformed Ba/F3 and 32D cells (shades of red) treated with JAK inhibitors for 72h (n≥3, in duplicates or triplicates, not all IC_{50} could be determined, one-way ANOVA with log transformed data). *p<0.05, **p<0.01, ***p<0.001

Figure S6: Parental and transformed Ba/F3 cells and PI3K/Akt/mTOR or CDK4/6 inhibitors. (A-C) Validation of screen result with dose-response curves and IC_{50} values of parental (black dashed line/white filling, with IL-3) and transformed Ba/F3 cells (shades of red) treated for 72h (n≥3, in duplicates, not all IC_{50} could be determined, one-way ANOVA with log transformed data). **p<0.01, ***p<0.001

Figure S7: Validation of kinase inhibitor library screen. (A-L) Dose-response curves and IC_{50} values of parental 32D cells (dashed line/white filling, with IL-3), transformed 32D cells (shades of red) and control Ba/F3 cells (shades of grey) with treatment for 72h (n≥3, in duplicates, not all IC_{50} could be determined, one-way ANOVA with log transformed data). **p<0.01, ***p<0.001, ****p<0.0001

Figure S8: Drug synergy of TYK2^{P760L}-transformed cell lines. (A-B) Representative growth curve of Ba/F3 and 32D cells expressing TYK2^{P760L} in the absence of IL-3 and parental cells in the presence of IL-3 (dashed line) with (shades of grey) and without (red) the indicated drug treatment (n=2, in duplicates). (C) Synergy scores (n≥3, in duplicates) of parental 32D cells (white filling, with IL-3) and transformed 32D cells (shades of red) calculated with the zero interaction potency (ZIP) model. Light grey shows additivity, dark grey synergy. (D-H) Dose-response curves of cells with a synergy score ≥10 (Ba/F3 TYK2^{P760L} 2 or 32D TYK2^{P760L} 3 cells) treated with different concentrations of the indicated drug and different concentrations of deucravacitinib (shades of blue) for 72h (n≥3). A representative synergy map is shown for each drug combination. Red shows synergism and green antagonism.

Figure S9: *TYK2^{P760L}* mutation in patient-derived xenograft (PDX) cells. (A) Presence of heterozygous mutation (2279C>T) shown in the blue bar was confirmed in both leukemias by Sanger sequencing. (B-E) Cell viability curves of hTERT mesenchymal stem cells cocultured PDX cells of leukemia 1 and leukemia 2 treated with different concentrations of the indicated drug and different concentrations of deucravacitinib (shades of blue) for 72h. A synergy map is shown for each drug combination. Synergy scores and most synergistic area score were calculated with the ZIP model (n=1). Red shows synergism and green antagonism.

Figure S1: GFP expression of transduced bone marrow (BM) cells before and after seeding into factor-free methylcellulose.

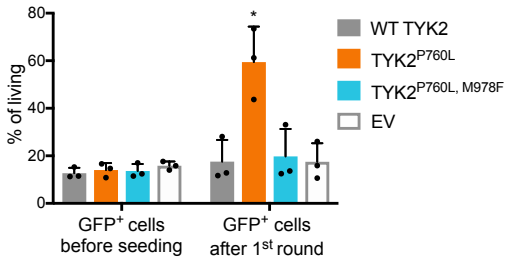
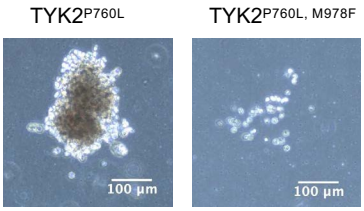


Figure S2: Colony formation and cell viability of transduced hematopoietic precursor cell lines.

A



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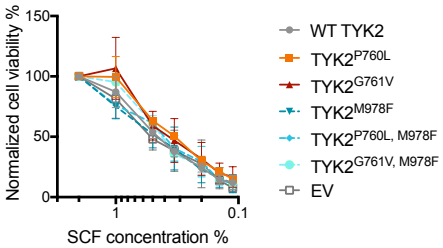


Figure S3: TYK2^{P760L} induced outgrowth, signaling, tumor growth and pathohistology.

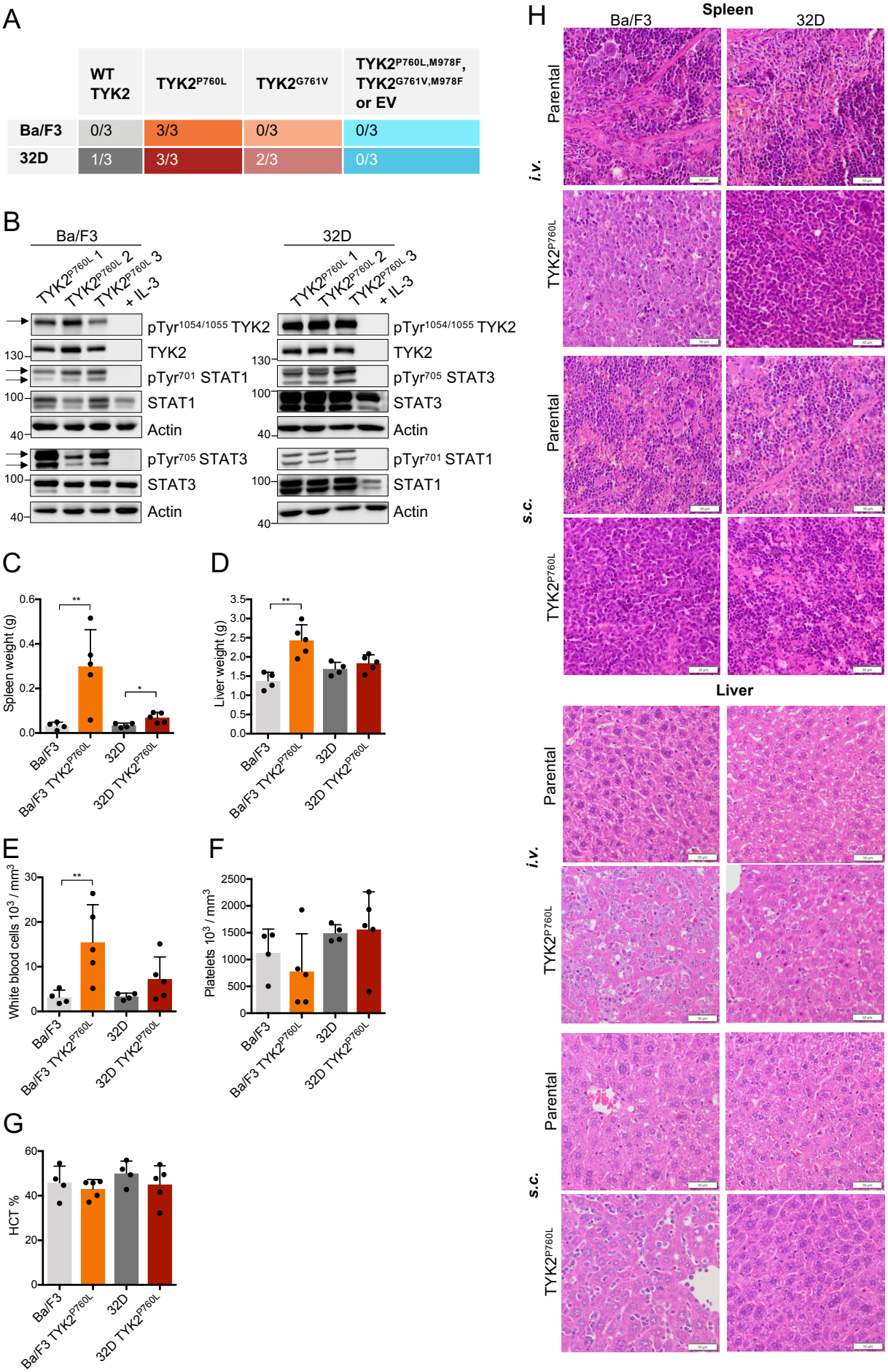
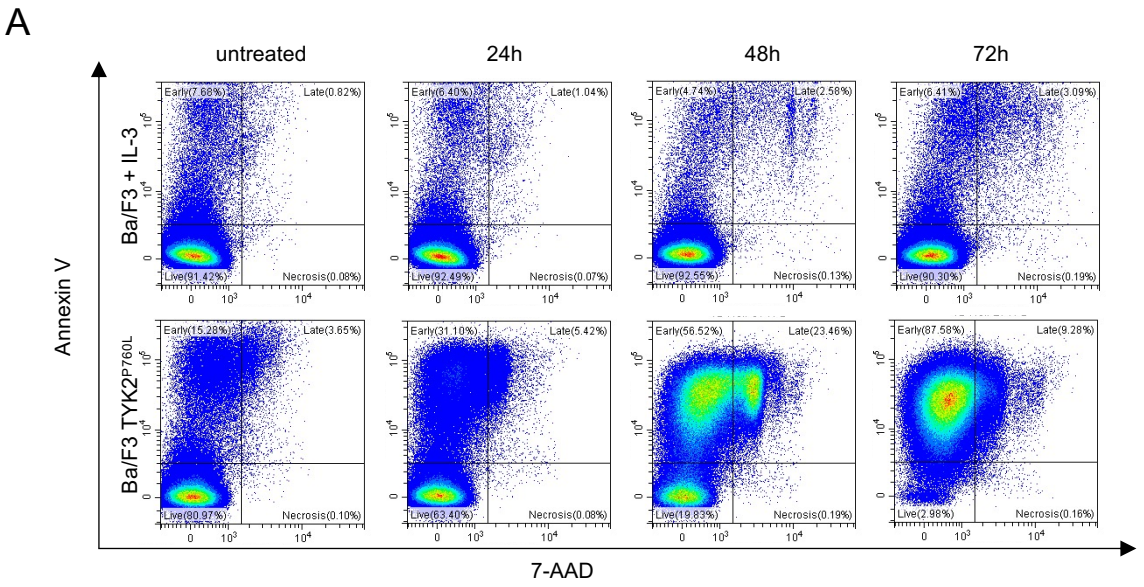
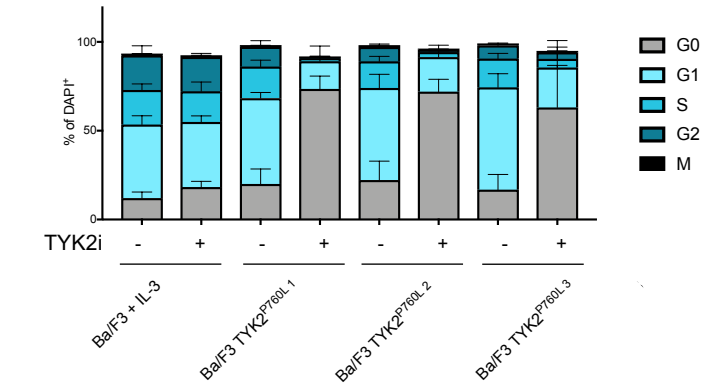


Figure S4: Deucravacitinib induces apoptosis and G0 cell cycle arrest.





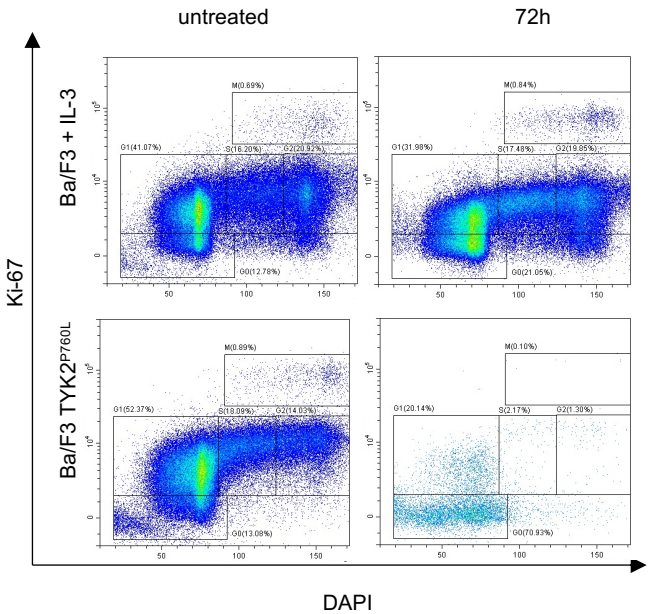


Figure S5: Parental and transformed Ba/F3 and 32D cells and JAK inhibitors.

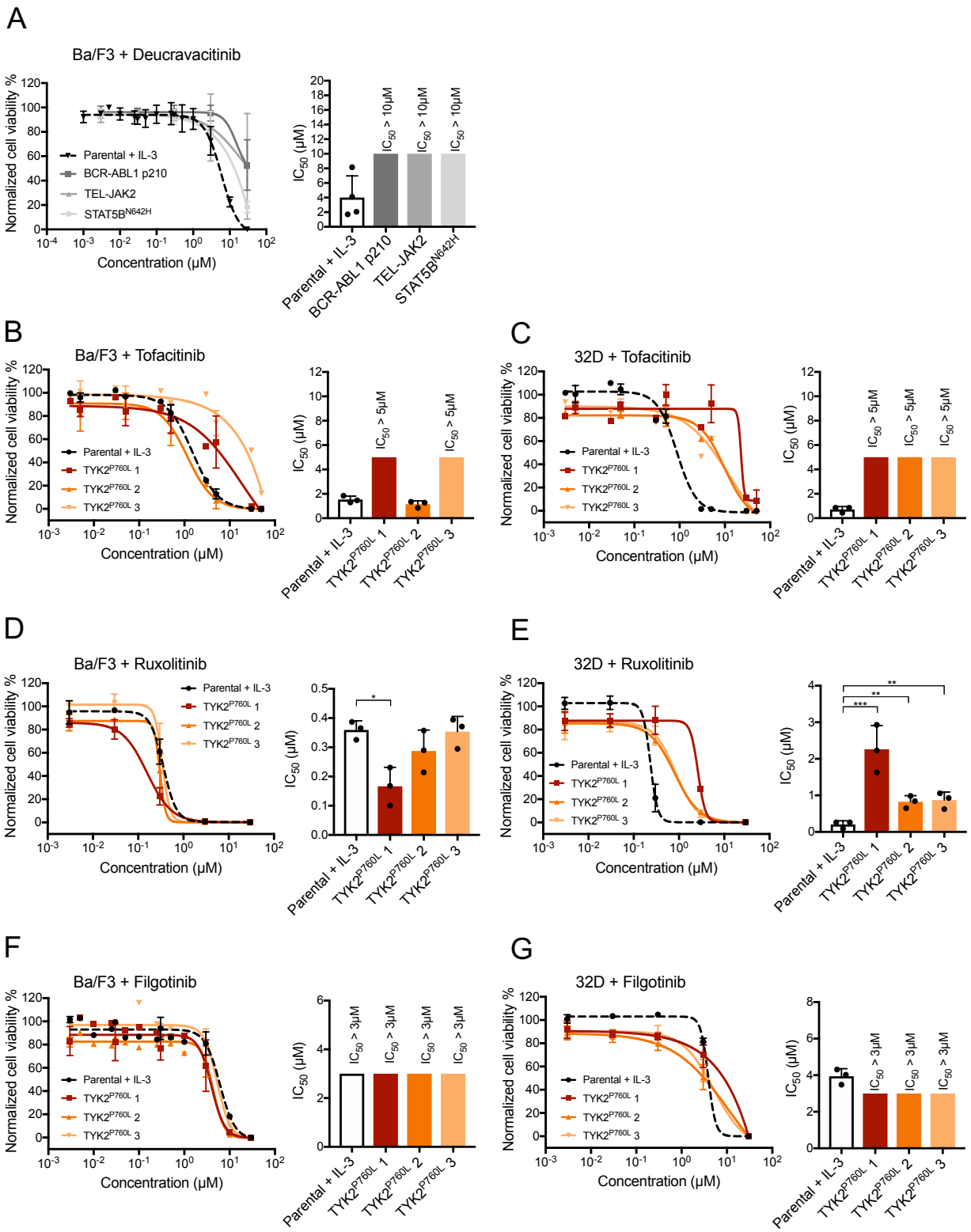


Figure S6: Parental and transformed Ba/F3 cells and PI3K/Akt/mTOR or CDK4/6 inhibitors.

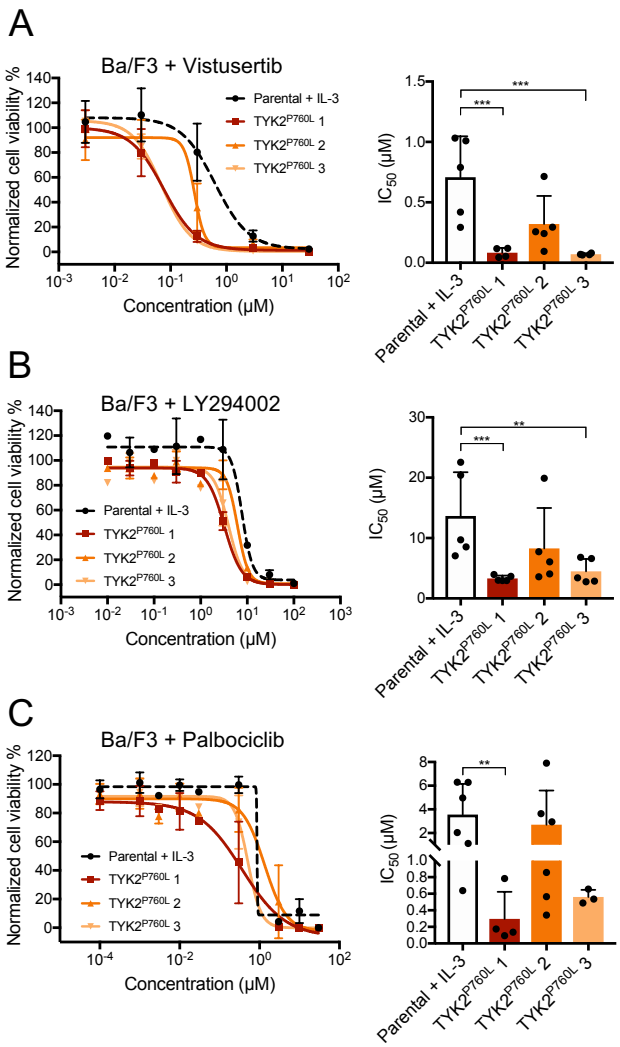


Figure S7: Validation of kinase inhibitor library screen.

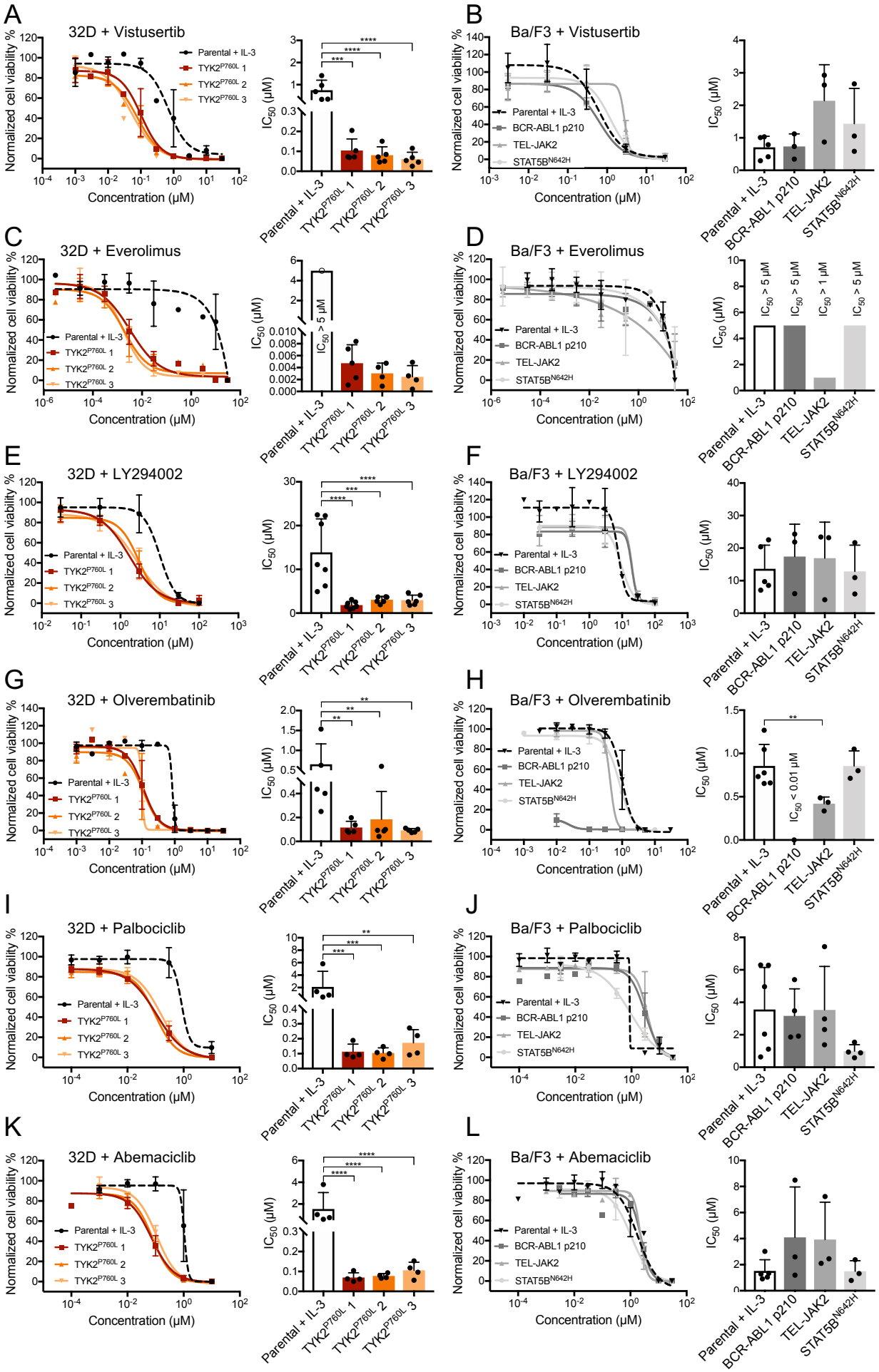


Figure S8: Drug synergy of TYK2^{P760L}-transformed cell lines.

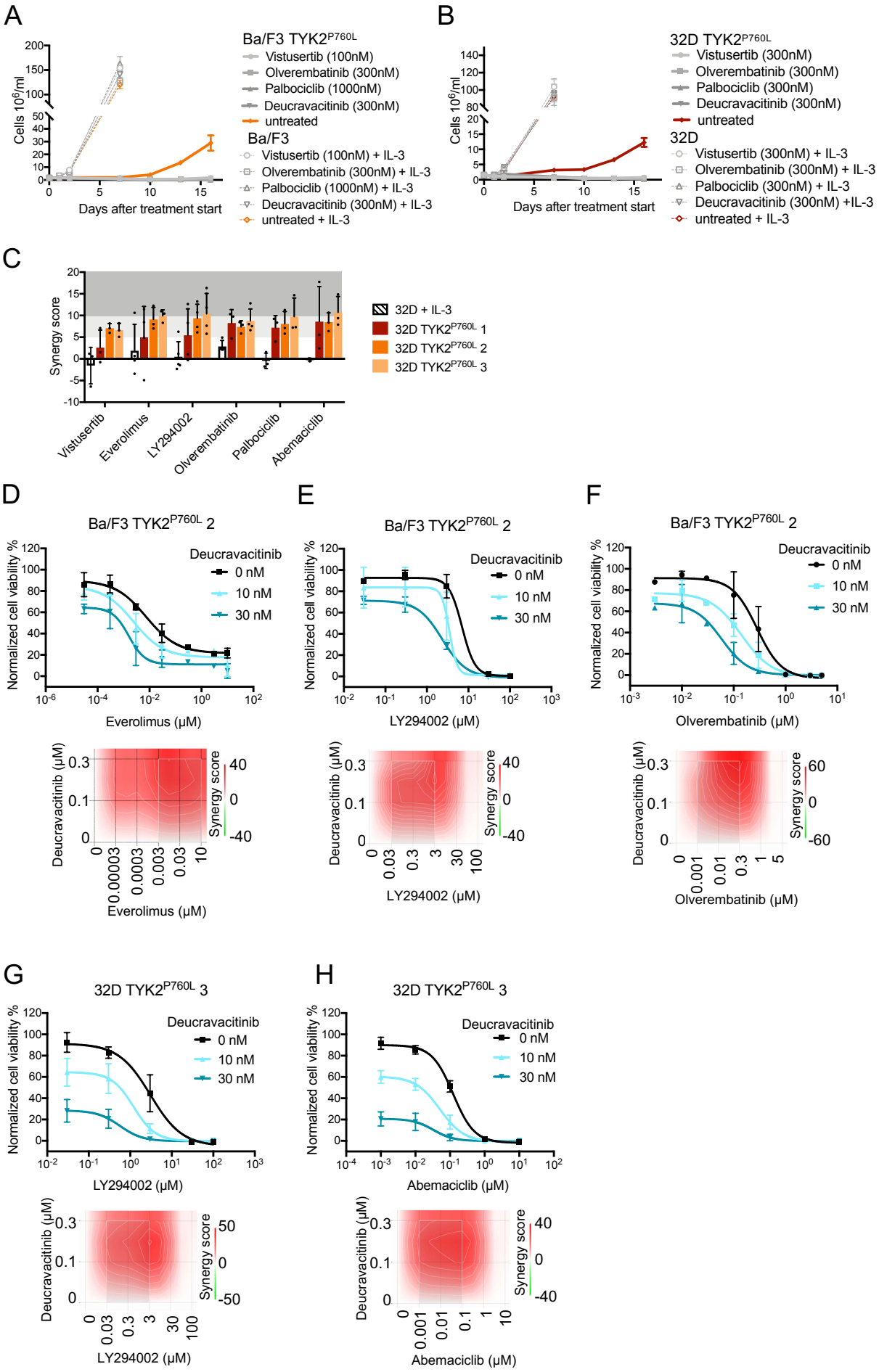
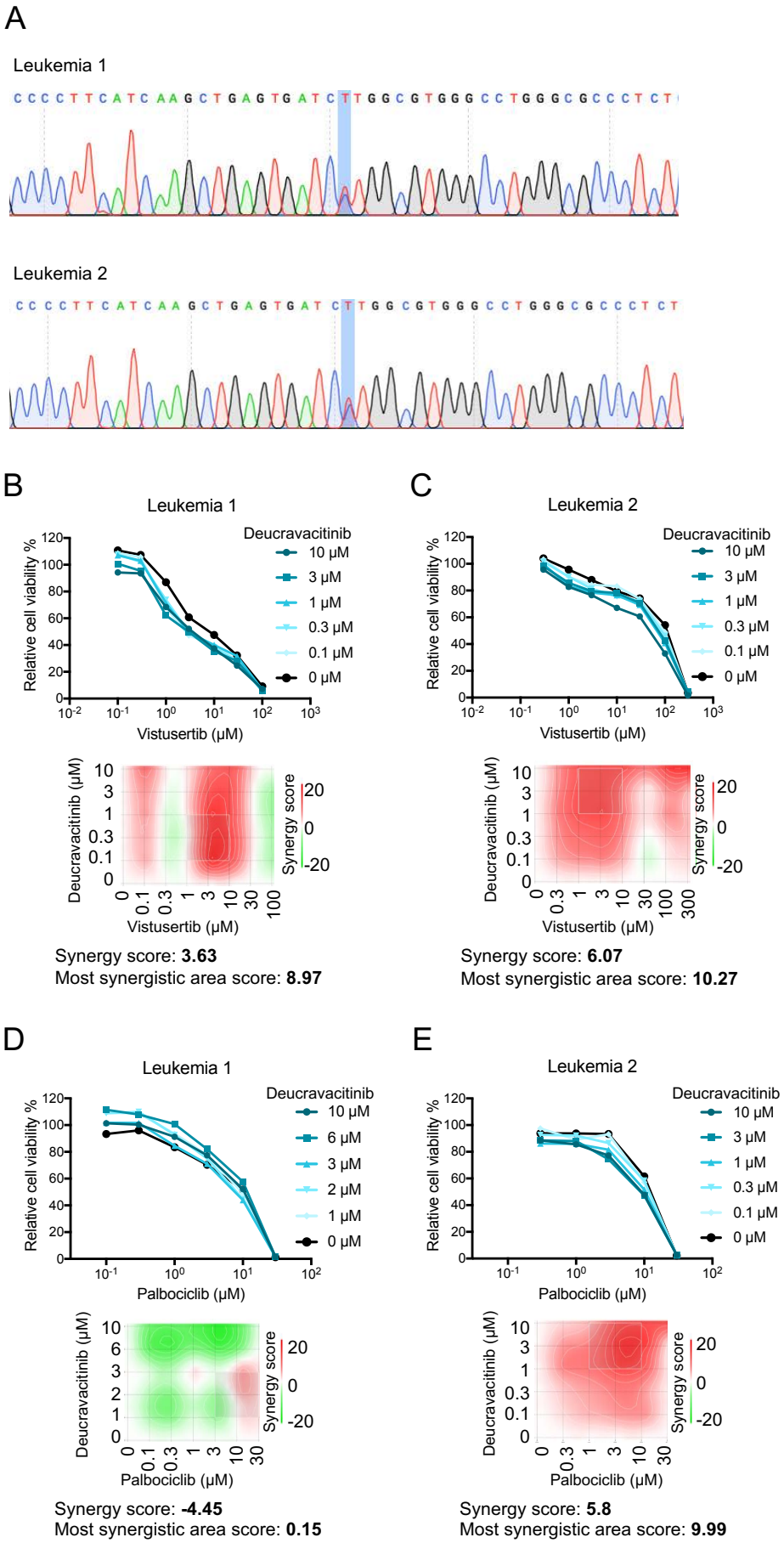


Figure S9: *TYK2*^{P760L} mutation in patient-derived xenograft (PDX) cells.



Supplementary Table S4: Results of the drug screen ranked with decreasing difference of the area under the curve (delta AUC) between parental Ba/F3 and Ba/F3 TYK2^{P760L} percentage of control (POC) curves.

All information regarding synonyms, primary target and reported IC_{50}/Kd/Ki was retrieved from MedChemExpress (except: Selleckchem: Berzosertib, Selinexor; Cayman: CAY10626, PI3K inhibitor 2, Sphingosine Kinase Inhibitor 2).

Inhibitors targeting the PI3K/AKT/mTOR pathway or CDK4/6 are shown in bold.

Inhibitor	**Synonym**	**delta AUC**	**Primary target**	**Reported IC_{50} / Kd / Ki**
Olverembatinib Dimesylate	**HQP1351 Dimesylate, GZD824 Dimesylate**	**153.02**	**pan BCR-ABL[3]**	**IC_{50}: 0.68 nM (Bcr-Abl^{T315I}), 0.27 nM (Bcr-Abl^{E255K}), 0.71 nM (Bcr-Abl^{G250E}), 0.15 nM (Bcr-Abl^{Q252H}), 0.35 nM (Bcr-Abl^{H396P}), 0.29 nM (Bcr-Abl^{M351T}), 0.35 nM (Bcr-Abl^{Y253F}), Bcr-Abl^{F317L}**
Olverembatinib	**HQP1351, GZD824**	**132.21**	**pan BCR-ABL[3]**	**IC_{50}: 0.68 nM (Bcr-Abl^{T315I}), 0.27 nM (Bcr-Abl^{E255K}), 0.71 nM (Bcr-Abl^{G250E}), 0.15 nM (Bcr-Abl^{Q252H}), 0.35 nM (Bcr-Abl^{H396P}), 0.29 nM (Bcr-Abl^{M351T}), 0.35 nM (Bcr-Abl^{Y253F}), Bcr-Abl^{F317L}**
Pimasertib[2]	AS-703026	112.48	MEK1/2	IC_{50}s ranging from 0.005 to 2 μM
Vistusertib	**AZD2014**	**111.25**	**mTOR (mTORC1 mTORC2)**	**IC_{50}: 2.81 nM, mTORC1 target S6: 210nM, mTORC2 target AKT: 78nM, PI3Ka: 3766 nM**
Barasertib[2]	AZD1152-HQPA	109.90	Aurora B	IC_{50}: 0.37 nM
AZD1208		107.76	PIM kinases	n.a.
Cucurbitacin B		107.31	cancer cell progression	n.a.

SNS-314 Mesylate[2]		103.83	Aurora A, B, C	$IC_{50:}$ of 9 nM (Aurora A), 31 nM (Aurora B), 6 nM (Aurora C)
Sapanisertib	**INK128**	**93.74**	**mTOR 1/2**	**IC_{50}: 1 nM (mTOR), 219 nM (PI3Kα), 221 nM (PI3Kγ), 230 nM (PI3Kδ), 5.293 μM (PI3Kβ)**
GNF-7		89.56	BCR ABL	IC_{50}: 133 nM ($Bcr\text{-}Abl^{WT}$), 61 nM ($Bcr\text{-}Abl^{T315I}$), 25 nM (ACK1), 8 nM (GCK)
Paclitaxel[2]		88.42	stabilizes tubulin polymerization	n.a.
Ponatinib	AP24534	86.31	multiple kinases	IC_{50}: 1.5 nM (VEGFR2), 1.1 nM (PDGFRα), 2.2 nM (FGFR1), 12.5 nM (c-Kit)
BX912		84.89	PDK1	IC_{50}: 26 nM
Palbociclib Isethionate	**PD0332991**	**84.14**	**CDK4/6**	**IC_{50}: 9 nM (Cdk4/cyclin D3), 11 nM (Cdk4/cyclin D1), 16 nM (Cdk6/cyclin D2), 2000 nM (DYRK1A), 8000 nM (MAPK)**
Rapamycin[1]	**Sirolimus**	**83.49**	**mTOR**	**IC_{50}: 0.1 nM**
Berzosertib	VE822	83.30	ATM ATR	IC_{50}: 19 nM
Omipalisib	**GSK2126458**	**81.96**	**PI3K**	**Ki: 0.019 nM (p110α), 0.008 nM (p110α-E545K), 0.008 nM (p110α-E542K), 0.009 nM (p110α-H1047R), 0.13 nM (p110β), 0.024 nM (p110δ), 0.06 nM (p110γ), 0.18 nM (mTORC1), 0.3 nM (mTORC2)**
ENMD-2076[1]		81.93	multiple kinases	IC_{50}: 14 nM (Aurora A), 58.2 nM (KDR), 15.9 nM (Flt-4), 92.7 nM (FGFR1), 70.8 nM (FGFR2), 56.4 nM (PDGFRα), 1.86 nM (Flt3)
Trametinib[2]	GSK1120212	81.58	MEK 1/2	IC_{50}: 2 nM
Mirdametinib[2]	PD0325901	81.23	MEK	Ki: 1 nM (MEK1), 1 nM (MEK2); IC_{50}: 0.33 nM (MEK)
Pluripotin	SC-1	80.35	ERK1, RasGAP	Kd: 98 nM (ERK1), 212 nM (RasGAP); IC_{50}: 0.5 μM (RSK1), 2.5 μM (RSK2), 3.3 μM (RSK3), 10 μM (RSK4)

Fedratinib	SAR302503, TG101348	80.17	JAK2	IC_{50}: 3 nM (JAK2), 3 nM (JAK2(V617F)), 15 nM (Flt3), 48 nM (Ret)
MK-2206 dihydrochloride		**79.39**	**AKT**	**IC_{50}: 8 nM (Akt1), 12 nM (Akt2), 65 nM (Akt3)**
Palbociclib hydrochloride	**PD-0332991**	**76.77**	**CDK4/6**	**IC_{50}: 9 nM (Cdk4/cyclin D3), 11 nM (Cdk4/cyclin D1), 16 nM (Cdk6/cyclin D2), 2000 nM (DYRK1A), 8000 nM (MAPK)**
BI 2536		75.54	PLK1, BRD4	IC_{50}: 0.83 nM (PLK1), 3.5 nM (Plk2/Snk), 9 nM (Plk3/Fnk), 25 nM (BRD4)
PF-477736		74.70	Chk1/2	Ki: 0.49 nM (Chk1), 47 nM (Chk2), 8 nM (VEGFR2), 9.9 μM (CDK1); IC_{50}: 10 nM (Fms), 14 nM (Yes), 23 nM (Aurora-A), 23 nM (FGFR3), 25 nM (Flt3), 39 nM (Ret)
Ruboxistaurin hydrochloride	LY333531	74.32	PKCβ	IC_{50}: 4.7 nM (PKCβI), 5.9 nM (PKCβII), 52 nM (PKCη), 250 nM (PKCδ), 300 nM (PKCγ), 360 nM (PKCα), 600 nM (PKCε)
Palbociclib	**PD-0332991**	**72.54**	**CDK4/6**	**IC_{50}: 9 nM (Cdk4/cyclin D3), 11 nM (Cdk4/cyclin D1), 16 nM (Cdk6/cyclin D2), 2000 nM (DYRK1A), 8000 nM (MAPK)**
Dimethylenastron[1]		72.15	Eg5	IC_{50}: 200 nM
Pimasertib (different company) [1]	AS-703026	71.49	MEK1/2	IC_{50}s ranging from 0.005 to 2 μM
CAY10626[1]		**71.12**	**PI3Ka/mTOR**	**IC_{50}: 0.9 nM (PI3Ka), 0.6 nM (mTOR)**
Sphingosine Kinase Inhibitor 2		70.74	SPHK2	IC_{50}: 500 nM
OTSSP167[2]		69.66	MELK	IC_{50}: 0.41 nM

Cobimetinib[1]	GDC-0973, RG7420	69.50	MEK1	IC_{50}: 4.2 nM
Tunicamycin		69.27	N-linked glycosylation, GlcNAc phosphotransferase	n.a.
SB743921[2]		69.03	Eg5	Ki: 0.1 nM
HTH-01-015		67.93	NUAK1	IC_{50}: 100 nM
PI3-Kinase Inhibitor 2		**66.24**	**PI3K**	**IC_{50}: 2 nM (p110α), 16 nM (p110β), 660 nM (p110γ), 220 nM (PI3K C2β), 49 nM (mTOR)**
PP121		**65.38**	**multiple kinases**	**IC_{50}: 10 nM (mTOR), 2 nM (PDGFR), 12 nM (VEGFR2), 14 nM (Src), 60 nM (DNK-PK)**
Abemaciclib mesylate[1]	**LY2835219 mesylate**	**63.57**	**CDK4/6**	**IC_{50}: 2 nM (Cdk4/cyclin D1), 10 nM (CDK6/cyclinD1), 57 nM (CDK9/cyclinT1), 287 nM (CDK5/p35), 355 nM (Cdk5/p25), 504 nM (CDK2/cyclinE), 1627 nM (CDK1/cyclinB1), 3910 nM (CDK7/Mat1/cyclinH1), 50 nM (PIM1), 3400 nM (PIM2), 31 nM (HIOK2), 61 nM (DYRK2), 117 nM (CK2), 192 nM (GSK3b), 389 nM (JNK3), 403 nM (FLT3 (D835Y)), 659 nM DRAK1, 3960 nM (FLT3)**
UNC0064-12[1]	VEGFR-2-IN-5	62.75	VEGFR2	n.a.
Sotrastaurin	AEB071	62.27	pan PKC	Ki: 0.22 nM (PKCθ), 0.64 nM (PKCβI), 0.95 nM (PKCα), 1.8 nM (PKCη), 2.1 nM (PKCδ), 3.2 nM (PKCε)

Rabusertib[(1)]	LY2603618	61.83	Chk1	IC_{50}: 7 nM (Chk1), 12000 nM (Chk2), 893 nM (PDK1), 1550 nM (CAMK2), 2128 nM (VEGFR3), 2200 nM (MET), 4930 nM (JNK1), 5700 nM (RSK2), 12000 nM (NTRK1)
Ceralasertib[(1)]	AZD6738	61.62	ATR	IC_{50}: 1 nM (ATR), 6.8 μM (PI3Kδ), 10.8 μM (DYRK)
Torkinib[(1)]	**PP242**	**61.47**	**mTOR**	**IC_{50}: 8 nM (mTOR), 30 nM (mTORC1), 58 nM (mTORC2), 100 nM (p110δ), 410 nM (PDGFR), 410 nM (DNA-PK), 1.3 μM (p110γ), 2 μM (p110α), 2.2 μM (p110β), 1.2 μM (Hck), 1.4 μM (Scr), 1.5 μM (VEGFR2), 3.6 μM (Abl), 3.4 μM (EphB4), 4.4 μM (EGFR), 5.1 μM (Scr(T338I))**
XMD8-92[(1)]		60.63	BMK1, BRD4	Kd: 80 nM (BMK1), 190 nM (BRD4)
Alisertib	MLN8237	60.16	Aurora A	IC_{50}: 1.2 nM (Aurora A), 396.5 nM (Aurora B)
Selinexor	KPT-330, (E)-RN	60.14	CRM1	n.a.
KN-93[(1)]		59.58	CaMK	Ki: 370 nM (CaMK)
Fangchinoline		59.24	HIV, FAK, apoptosis, autophagy	n.a.
SGI-1776 free base		58.53	Pim kinases	Ki: 7 nM (Pim-1), 363 nM (Pim-2), 69 nM (Pim-3)
R112[(1)]		58.49	Syk kinase	Ki: 96 nM; IC_{50}: 226 nM
MK8745[(1)]		58.23	Aurora A	IC_{50}: 0.6 nM
Tivozanib[(1)]	AV-951, KRN951	57.66	VEGFR1/2/3	IC_{50}: 30 nM (VEGFR1), 6.5 nM (VEGFR2), 15 nM (VEGFR3)
Vandetanib[(1)]	ZD6474	57.60	VEGFR2/KDR	IC_{50}: 40 nM (VEGFR2), 110 nM (VEGFR3), 500 nM (EGFR/HER1)

LY-2874455[1]		57.31	pan FGFR	IC_{50}: 2.8 nM (FGFR1), 2.6 nM (FGFR2), 6.4 nM (FGFR3), 6 nM (FGFR4)
Pelitinib[1]	EKB-569	57.08	EGFR	IC_{50}: 38.5 nM
Zotiraciclib	TG02, SB1317	56.61	CDK2, JAK2, FLT3	IC_{50}: 13 nM (CDK2), 73 nM (JAK2), 56 nM (FLT3)
AG-490[2]	Tyrphostin B42	56.27	EGFR, STAT3, JAK2/3	n.a.
JNJ-10198409		54.75	PDGFR	IC_{50}: 4.2 nM (PDGFRβ), 45 nM (PDGFRα)
SB 202190[1]		54.72	p38	IC_{50}: 50 nM (p38α), 100 nM (p38β2)
Entrectinib[1]		54.02	pan-Trk, ROS1, ALK	IC_{50}: 1 nM (TrkA), 1 nM (TrkB), 1 nM (TrkC), 1 nM (ROS1),1 nM (ALK)
Midostaurin[1]	PKC-412	53.45	multiple kinases	IC_{50}: 22 nM (cPKC-α), 24 nM (cPKC-γ), 30 nM (cPKC-β1), 31 nM (cPKC-β2), 33 nM (nPKC-δ), 160 nM (nPKC-η), 1250 nM (nPKC-ε), 465000 nM (aPKC-ζ), 38 nM (PPK), 86 nM (KDR), 95 nM (c-Syk), 570 nM (cdk1/cycB), 570 nM (Protein kinase A), 790 nM (c-Fgr), 800 nM (c-Src), 912 nM (Flt-1), 1100 nM (EGF-R), 1900 nM (Myosin-light chain kinase), 3900 nM (Flk-1), 4300 nM (c-Lyn), 5000 nM (P70S6 kinase), 8000 nM (CSK)
PIK-93[1]		**52.10**	**PI4K, PI3K**	**IC_{50}: 19 nM (PI4KIIIβ), 1.1 μM (PI4KIIIα), 16 nM (p110γ), 39 nM (p110α), 120 nM (p110δ), 590 nM (p110β), 140 nM (PI3KC2β), 16 μM (PI3KC2α), 320 nM (hsVPS34), 64 nM (DNA-PK), 490 nM (ATM), 1.38 μM (mTORC1), 17 μM (ATR)**
CDK4-IN-1[1]		**51.65**	**CDK4**	n.a.

Mollugin[2]		51.50	TNF-α-induced NF-κB activation	n.a.
CGK 733[1]		51.26	ATM/ATR	n.a.
Ispinesib	SB715992	51.05	KSP	Ki app: 1.7 nM
Abemaciclib[1]	**LY2835219**	**50.89**	**CDK4/6**	**IC_{50}: 2 nM (Cdk4/cyclin D1), 10 nM (CDK6/cyclinD1), 57 nM (CDK9/cyclinT1), 287 nM (CDK5/p35), 355 nM (Cdk5/p25), 504 nM (CDK2/cyclinE), 1627 nM (CDK1/cyclinB1), 3910 nM (CDK7/Mat1/cyclinH1), 50 nM (PIM1), 3400 nM (PIM2), 31 nM (HIOK2), 61 nM (DYRK2), 117 nM (CK2), 192 nM (GSK3b), 389 nM (JNK3), 403 nM (FLT3 (D835Y)), 659 nM DRAK1, 3960 nM (FLT3)**
Bosutinib[1]		50.60	Src, Abl	IC_{50}: 1.2 nM (Src), 1 nM (Abl)
PRT062607 hydrochloride	P505-15, BIIB057	50.55	Syk	IC_{50}: 1 nM (Syk), 81 nM (Fgr), 88 nM (MLK1), 123 nM (Yes)[1]
AZD2858[1]		50.32	GSK3	IC_{50}: 0.9 nM (GSK-3α), 5 nM (GSK-3β), 356 nM (CDK5/p25), 366 nM (Haspin), 387 nM (CDK5/p35), 491 nM (DYRK2), 810 nM (CDK2/cyclin A), 1246 nM (CDK1/cyclin B), 1269 nM (PIM3), 1381 nM (TLK2), 2462 nM (PKD2), 3310 nM (CDK2/cyclin E), 4966 nM (Aurora-A)
GSK2334470[1]		49.13	PDK1	IC_{50}: 10 nM
Apitolisib[1]	**GDC-0980, RG7422**	**49.03**	**PI3K, mTOR**	**IC_{50}: 5 nM (PI3Kα), 7 nM (PI3Kδ), 14 nM (PI3Kγ), 27 nM (PI3Kβ); Ki: 17 nM (mTOR)**
PI3K-IN-2[1]		**48.72**	**PI3K**	**IC_{50}: 13 nM (PI3Kα), 7.1 nM (PI3Kβ), 190 nM (PI3Kδ), 8.6 nM (PI3Kγ)**

Amuvatinib[1]	MP470	48.41	multiple kinases	IC_{50}: 40 nM (PDGFRαV561D), 81 nM (PDGFRαD842V), 10 nM (c-KitD816H), 34 nM (c-KitV560G), 127 nM (c-KitV654A), 950 nM (c-KitD816V)
MLN0905[1]		48.37	PLK1	IC_{50}: 2 nM
PIK75[1]		**48.29**	**DNA-PK, PI3K**	**IC_{50}: 2 nM (DNA-PK), 5.8 nM (p110α), 76 nM (p110γ), 510 nM (p110δ), 1.3 μM (p110β), 2.6 μM (hsVPS34), 1 μM (PI3KC2β), 10 μM (PI3KC2α), 1 μM (mTORC1), 10 μM (mTORC2), 2.3 μM (ATM), 21 μM (ATR), 50 μM (PI4KIIIβ)**
AT 7519 hydrochloride salt[1]		**47.53**	**CDK**	**IC_{50}: 10 nM (CDK9/Cyclin T), 13 nM (CDK5/p35), 47 nM (cdk2/cyclin A), 100 nM (Cdk4/cyclin D1), 170 nM (cdk6/cyclin D3), 210 nM (Cdk1/cyclin B), 2400 nM (CDK7/Cyclin H/MAT1), 89 nM (GSK3β)**
NU 6102[1]		47.41	CDK1, CDK2	IC_{50}: 9.5 nM (Cdk1/cyclin B), 5.4 nM (CDK2/cyclin A3), 1.6 μM (CDK4), 0.9 μM (DYRK1A), 0.8 μM (PDK1)
Dequalinium chloride[1]		47.41	apamin-sensitive K+ channels	n.a.
CL-387785[1]	EKI-785	47.27	EGFR	IC_{50}: 370 pM
Volasertib[1]	BI 6727	47.14	PLK1	IC_{50}: 0.87 nM (PLK1), 5 nM (PLK2), 56 nM (PLK3)
LDN-214117[1]		47.02	ALK2	IC50: 22 nM (ALK2), 100 nM (BMP6)
LY294002[1]		**46.95**	**PI3K**	**IC_{50}: 0.5 μM (p110α), 0.57 μM (p110δ), 0.97 μM (p110β), 98 nM (human CK2), 3.869 μM (human CK2α2), 1.4 μM (DNA-PK)**
CNX-774[1]		46.71	BTK	IC_{50}: 1 nM
XMD17-109[1]		46.08	ERK5	IC_{50}: 162 nM (ERK5), 339 nM (LRRK2[G2019S])
Brigatinib[1]	AP-26113	45.82	ALK	IC_{50}: 0.6 nM

AZD4547[(1)]		45.63	FGFR	IC_{50}: 0.2 nM (FGFR1), 2.5 nM (FGFR2), 1.8 nM (FGFR3), 165 nM (FGFR4)
Dasatinib monohydrate[(1)]	BMS-354825	45.01	Src, Bcr-Abl	IC_{50}: 1.0 nM (Bcr-Abl), 0.5 nM (Src), 0.4 nM (lck), 0.5 nM (yes), 5.0 nM (c-kit), 28 nM (PDGFRβ), 100 nM (p38), 180 nM (Her1), 710 nM (Her2), 880 nM (FGFR-1), 1700 nM (MEK)

n.a. = not available

[(1)] Inhibitors excluded by the software (criteria: delta AUC >50 and AUC Ba/F3 Standard Deviation (StdDev) Ba/F3 > AUC Ba/F3 $TYK2^{P760L}$ + StdDev Ba/F3 $TYK2^{P760L}$)

[(2)] Manually excluded inhibitors

[(3)] Effects of olverembatinib have been reported in Ph^- leukemia cells via inhibition of the SRC and PI3K pathway (Ye et al. 2017); it also inhibits several other kinases (Liu et al. 2019, Wang et al. 2020)

Liu X, Wang G, Yan X, et al. Preclinical development of HQP1351, a multikinase inhibitor targeting a broad spectrum of mutant KIT kinases, for the treatment of imatinib-resistant gastrointestinal stromal tumors. *Cell Biosci*. 2019;9(1):1-12.

Wang Y, Zhang L, Tang X, et al. GZD824 as a FLT3, FGFR1 and PDGFRα inhibitor against Leukemia in vitro and in vivo. *Transl Oncol.* 2020;13(4):100766.

Ye W, Jiang Z, Lu X, et al. GZD824 suppresses the growth of human B cell precursor acute lymphoblastic leukemia cells by inhibiting the SRC kinase and PI3K/AKT pathways. *Oncotarget*. 2017;8(50):87002-87015.