iScience, Volume *23*

Supplemental Information

SHP2 Inhibition Influences Therapeutic Response to Tepotinib in Tumors with MET Alterations

Linda Pudelko, Frank Jaehrling, Christof Reusch, Sanziago Vitri, Christopher Stroh, Nina Linde, Michael P. Sanderson, Doreen Musch, Catherine Jorand Lebrun, Marina Keil, Christina Esdar, Andree Blaukat, Rafael Rosell, Karl Maria Schumacher, and Niki Karachaliou

SUPPLEMENTAL INFORMATION

Supplementary Tables

Table S1: IC_{50} values for tepotinib in sensitive and resistant cells. Related to Figure 1.

Cell line	Mean tepotinib viability assay IC_{50} values (nmol/L) ± SD			
	3 days exposure		6 days exposure	
	Low passage	High passage	Low passage	High passage
EBC-1	1.2 ± 0.2	2.0 ± 0.7	1.5 ± 0.6	2.1 ± 0.5
TR1	NA	1.3 ± 0.4	NA	1.2 ± 0.2
TR2	NA	1.1 ± 0.4	NA	1.1 ± 0.2
Hs746T	0.6 ± 0.3	0.7 ± 0.1	1.3 ± 0.8	0.9 ± 0.2
TR3	NA	NA	NA	NA

Comparison of IC_{50} values for tepotinib in parental tepotinib-sensitive (EBC-1, Hs746T) and tepotinib-resistant (TR1, TR2, TR3) cell lines with low passage number (#5) and high passage number (#12) upon exposure for 3 and 6 days in 2D. TR1, tepotinib-resistant EBC-1 cell line #1; TR2, tepotinib-resistant EBC-1 cell line #2; TR3, tepotinib-resistant Hs746T #1; NA = not assessable.

Table S2: Differential phosphorylation of signaling pathway nodes in tepotinib-resistant EBC-1 and Hs746T cell lines. Related to Figure 3.

A)

	Fold change compared to parental cells		
Phosphorylated RTK	**TR1**	**TR2**	**TR3**
EGF-R	1.12	1.15	0.53
ErbB2	0.61	0.49	0.02
ErbB3	1.39	1.60	NA
ErbB4	1.07	1.34	1.47
FGF-R1	0.60	0.09	0.12
FGF-R2α	0.52	0.20	0.42
FGF-R3	0.48	0.55	0.52
FGF-R4	0.49	0.00	NA
Insulin-R	0.80	0.77	1.18
IGF-I-R	0.88	0.16	2.65
Axl	0.97	1.63	0.44
Dtk	1.54	0.35	0.01
Mer	0.73	1.07	0.00
HGF-R	1.08	1.09	0.46
MSP-R	1.23	1.80	0.07
PDGF-Rα	0.89	1.66	1.16
PDGF-Rβ	0.52	0.68	0.26
SCF-R	0.53	0.14	0.68
Flt-3	0.56	0.43	0.70
M-CSF-R	0.21	0.14	0.46
c-Ret	0.86	0.99	0.86
ROR1	0.69	0.69	1.21
ROR2	0.94	1.15	7.70
Tie-1	0.95	0.19	NA
Tie-2	0.46	0.50	NA
TrkA	1.16	1.88	NA
TrkB	0.66	0.66	NA
TrkC	0.67	0.14	0.06
VEGF-R1	0.57	0.04	NA
VEGF-R2	0.37	NA	0.49
VEGF-R3	0.79	0.49	1.91
MuSK	0.59	0.26	0.49
EphA1	0.56	0.16	0.95
EphA2	0.49	0.21	0.99
EphA3	0.61	0.07	44.97
EphA4	0.42	0.02	NA
EphA6	0.32	0.04	0.64
EphA7	0.51	0.28	0.17
EphB1	0.60	0.19	2.66
EphB2	0.62	0.53	NA
EphB4	0.56	0.16	0.19
EphB6	0.63	0.41	0.48
ALK	0.51	0.69	0.76
DDR1	0.79	2.05	0.63
DDR2	0.53	0.05	2.28
EphA5	0.57	0.27	1.48
EphA10	0.66	0.51	1.59
EphB3	0.65	0.74	1.65
Ryk	0.42	0.75	0.11

B)

		Fold change compared to parental cells		
Kinase	**Phosphorylated site**	**TR1**	**TR2**	**TR3**
p38α	T180/Y182	1.42	1.72	0.90
Erk1/2	T202/Y204. T185/Y187	5.58	8.21	0.83
JNK1/2/3	T183/Y185. T221/Y223	1.90	3.33	0.63
GSK-3α/β	S21/S9	1.27	1.69	1.32
p53	S392	3.43	4.57	1.12
EGF-R	Y1086	1.09	1.49	0.21
MSK1/2	S376/S360	1.14	2.62	0.42
AMPKα1	T183	1.00	1.89	0.62
Akt1/2/3	S473	1.81	2.37	1.20
Akt1/2/3	T308	2.44	4.24	1.85
p53	S46	1.37	2.78	1.02
TOR	S2448	0.97	2.13	0.44
CREB	S133	1.39	2.40	0.77
HSP27	S78/S82	1.21	1.98	0.72
AMPKα2	T172	1.29	2.47	0.49
β-catenin	-	0.97	1.83	1.16
p70 S6 kinase	T389	1.32	2.37	1.62
p53	S15	4.31	13.27	2.17
c-Jun	S63	3.17	5.47	0.53
Src	Y419	1.46	2.83	0.97
Lyn	Y397	1.05	2.02	0.41
Lck	Y394	1.88	4.70	0.73
STAT2	Y689	1.44	2.65	0.78
STAT5a	Y694	0.99	1.87	0.92
p70 S6 kinase	T421/424	0.92	1.87	0.54
RSK1/2/3	S380/S386/S377	2.10	4.96	0.92
eNOS	S1177	2.22	1.34	0.48
Fyn	Y420	0.96	2.46	0.91
Yes	Y426	1.13	2.08	0.49
Fgr	Y412	1.36	2.43	0.13
STAT6	Y641	1.25	2.48	0.57
STAT5b	Y699	0.51	0.92	0.82
STAT3	Y705	1.68	2.77	0.98
p27	T198	1.73	2.27	1.06
PLC-γ1	Y783	3.18	3.30	0.99
Hck	Y411	1.30	2.46	0.40
Chk-2	T68	1.25	2.42	0.82
FAK	Y397	1.31	2.51	7.22
PDGF-Rβ	Y751	1.51	2.62	0.52
STAT5a/b	Y694/Y699	0.72	1.54	0.46
STAT3	S727	1.88	2.81	0.27
WNK1	T60	1.40	1.89	1.13
PYK2	Y402	1.88	2.87	0.83
PRAS40	T246	1.33	1.75	1.24
HSP60	-	1.76	2.50	0.45

Phospho-RTK and phospho-kinase array analysis. Fold changes for phosphorylated (**A**) RTKs or (**B**) kinases in tepotinib-resistant (TR1, TR2, TR3) cell lines compared to the matching parental cells. Values > 1 represent increased phosphorylation and values < 1 indicate decreased phosphorylation. TR1, tepotinib-resistant EBC-1 cell line #1; TR2, tepotinib-resistant EBC-1 cell line #2; TR3, tepotinib-resistant Hs746T #1; NA = not assessable, due to lack of signal in control and/or treated samples.

Table S3: IC_{50} values for tepotinib SHP2i_01, SHP2i_02 and pimasertib in MET-altered cancer cell lines. Related to Figure 5.

				Mean viability assay IC_{50} values (nmol/L ± SD)			
Cell line	**Origin**	**MET/RAS status**	**Assay**	**Tepotinib**	**SHP2i_01**	**SHP2i_02**	**Pimasertib**
EBC-1	LUSC	MET amp. RAS WT	2D	0.03 ± 0.01	474 ± 26.3	8,665 ± 2,020	25.6 ± 0.1
			3D	0.9 ± 0.1	NT	6,170 ± 1,070	NT
Hs746T	STAD	MET amp. METex14 skipping. RAS WT	2D	0.01 ± 0.006	189 ± 3.0	1,359 ± 150	107 ± 26.4
			3D	0.9 ± 0.1	NT	6,370 ± 276	NT
MKN-45	STAD	MET amp. RAS WT	2D	1.6 ± 0.05	1,392 ± 343	8,291 ± 64.3	1,867 ± 21.9
			3D	19.2 ± 5.3	NT	NA	NT
NCI-H1993	LUAD	MET amp. RAS WT	2D	0.8 ± 0.1	555 ± 163	7,358 ± 1,040	319 ± 92.8
			3D	9.4 ± 1.8	NT	NA	NT
NCI-H441	LUAD	MET amp	2D	NA	NA	NA	4.2 ± 3.6

IC_{50} values for tepotinib, SHP2i_01, SHP2i_02 and pimasertib in the indicated cell lines when cultured either in 2D or 3D. LUSC, lung squamous carcinoma; STAD, stomach adenocarcinoma; LUAD, lung adenocarcinoma; M, molar; WT, wildtype; amp, amplification; NA, not assessable (> 30 µmol/L); NT, not tested.

Table S4: Summary of synergy parameters for drug combinations in MET-altered cancer cells. Related to Figure 5.

Cell line	Drug 1	Drug 2	Synergy score	SD	CI	SD	EV	SD
EBC-1	Tepotinib	Pimasertib	0.07	0.08	0.83	0.12	-0.08	0.18
		SHP2i_01	0.68	0.94	0.70	0.33	0.13	0.60
		SHP2i_02	0.92	1.24	0.68	0.04	-0.42	0.59
Hs746T	Tepotinib	Pimasertib	1.27	0.62	0.59	0.01	-1.65	0.75
		SHP2i_01	0.51	0.56	0.62	0.14	-0.61	0.64
		SHP2i_02	0.95	0.55	0.52	0.15	-1.26	0.77
MKN-45	Tepotinib	Pimasertib	2.07	0.49	0.41	0.28	-1.67	1.42
		SHP2i_01	2.27	0.62	0.57	0.08	-1.77	1.56
		SHP2i_02	2.59	1.64	0.59	0.30	-0.99	2.61
NCI-H1993	Tepotinib	Pimasertib	1.52	1.49	0.30	0.28	-2.23	2.41
		SHP2i_01	1.60	1.51	0.52	0.30	-2.52	2.48
		SHP2i_02	1.19	0.26	0.50	0.08	-1.02	0.04

Table summarizing all synergy parameters derived using the Loewe combination method. Data are shown as mean ± SD. SD, standard deviation; CI, combination index; EV, excess volume.

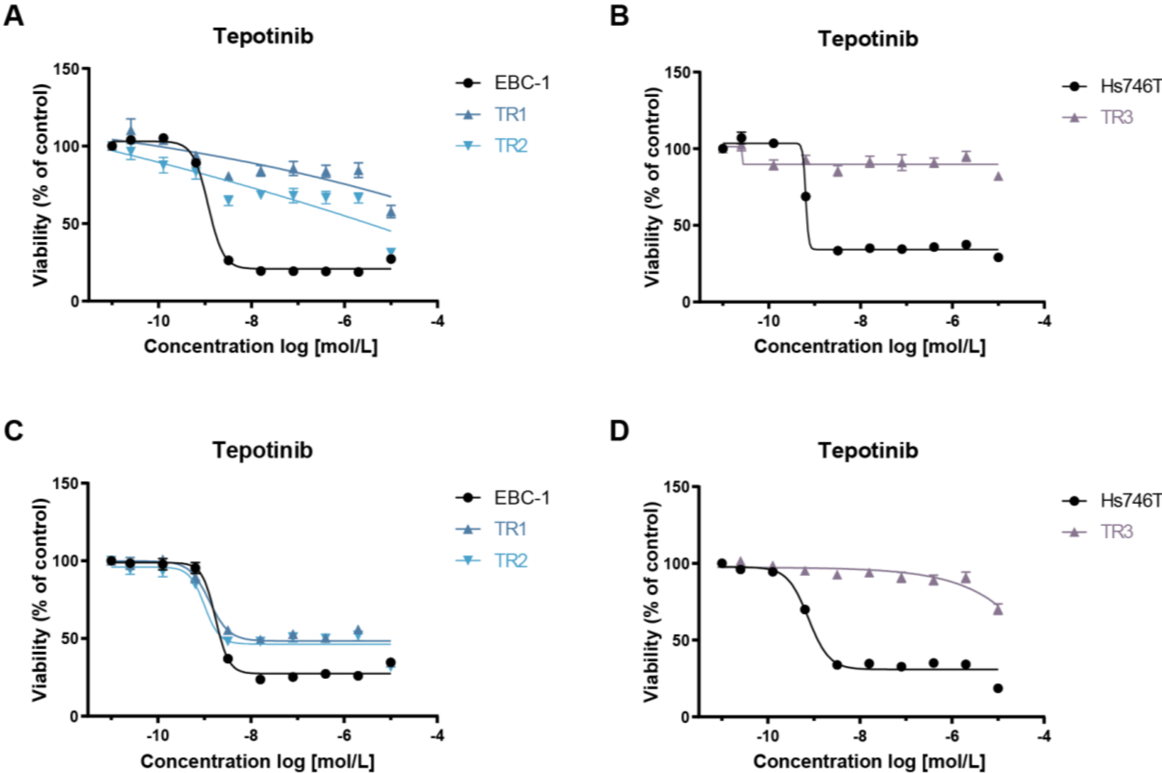
Table S5: IC_{50} values for tepotinib, SHP2i_01, SHP2i_02 and pimasertib in tepotinib-sensitive and tepotinib-resistant cell lines. Related to Figure 1 and Figure S4.

	Mean viability assay IC_{50} values (nmol/L) ± SD			
Cell line	**Tepotinib**	**SHP2i_01**	**SHP2i_02**	**Pimasertib**
EBC-1	0.5 ± 0.03	301 ± 14.1	4,000 ± 557	19.3 ± 3.0
TR1	NA	179 ± 2.1	2,751 ± 1,281	91.4 ± 1.7
TR2	NA	242 ± 2.7	5,452 ± 3,586	55.2 ± 4.5
Hs746T	0.6 ± 0.6	552	4,090 ± 195	79.3 ± 3.5
TR3	NA	NA	NA	NA

Viability assay IC_{50} values for tepotinib, SHP2i_01, SHP2i_02 and pimasertib in parental tepotinib-sensitive (EBC-1, Hs746T) and tepotinib-resistant (TR1, TR2, TR3) cell lines upon exposure for 6 days in 2D. Data are presented as mean ± SD. TR1, tepotinib-resistant EBC-1 cell line #1; TR2, tepotinib-resistant EBC-1 cell line #2; TR3, tepotinib-resistant Hs746T #1; NA = not assessable (> 30 μmol/L).

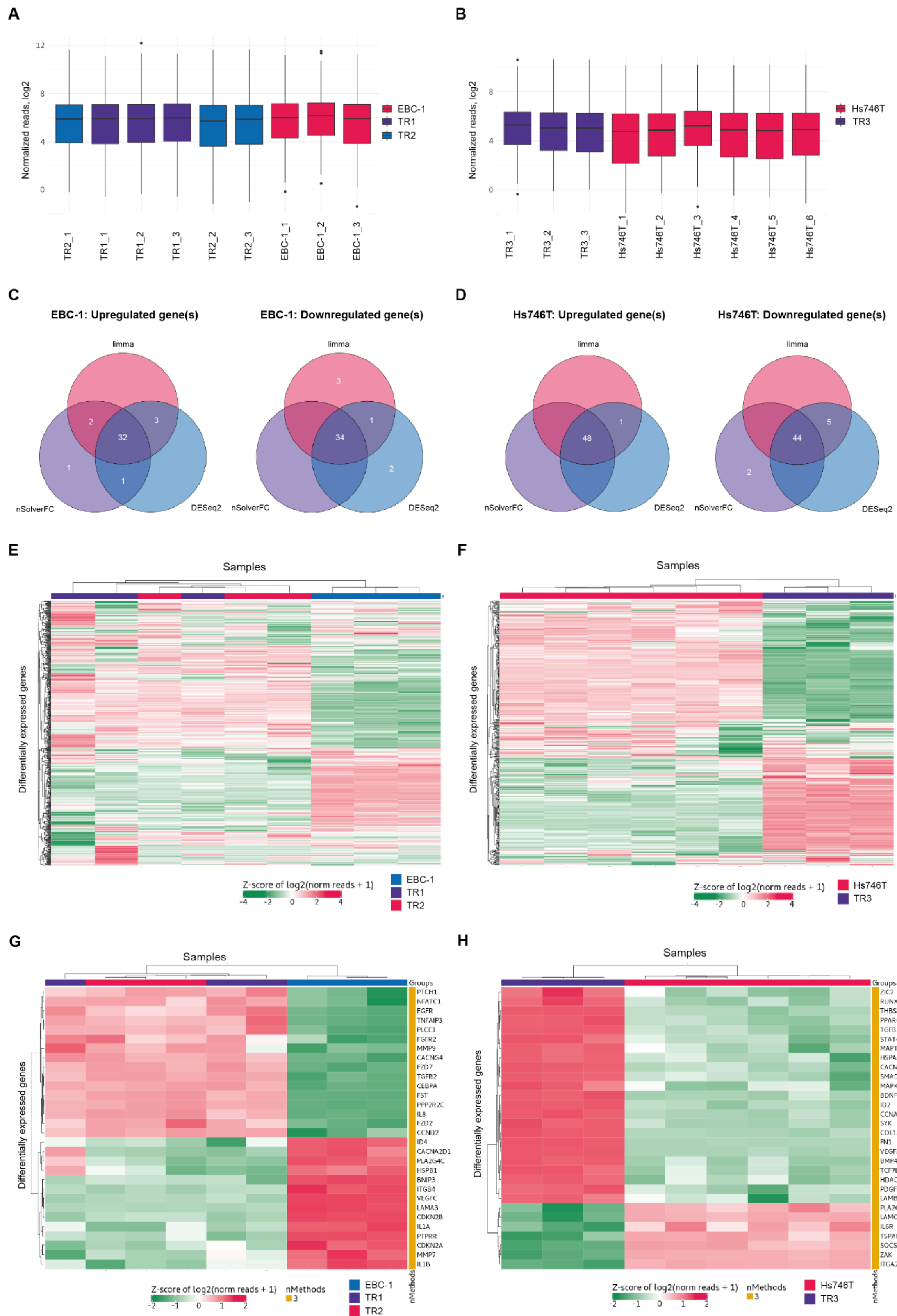
Supplementary Figures

Figure S1



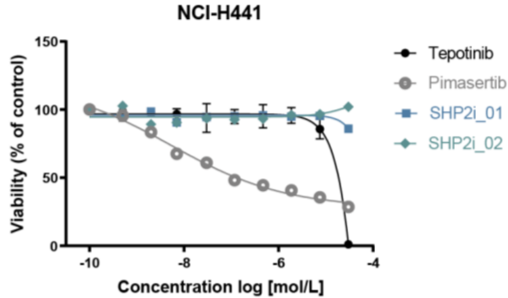
Generation of tepotinib-resistant EBC-1 and Hs746T cell lines. Related to Figure 1. Dose-response curves for tepotinib in parental tepotinib-sensitive (EBC-1, Hs746T) and tepotinib-resistant (TR1, TR2, TR3) cell lines with (**A, B**) low passage number (#5) and (**C, D**) high passage number (#12) upon exposure for 3 days in 2D. Data are shown as the mean ± SEM percentage fluorescent values relative to the corresponding DMSO control from 6 technical replicates.

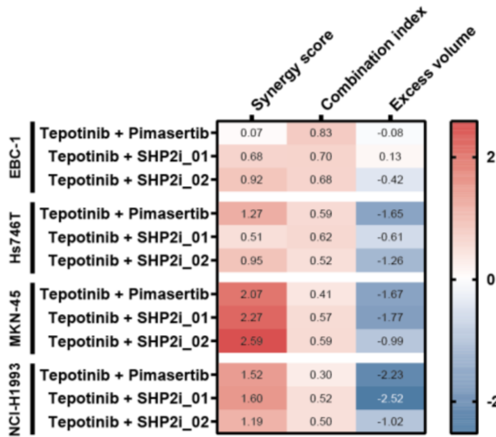
Figure S2

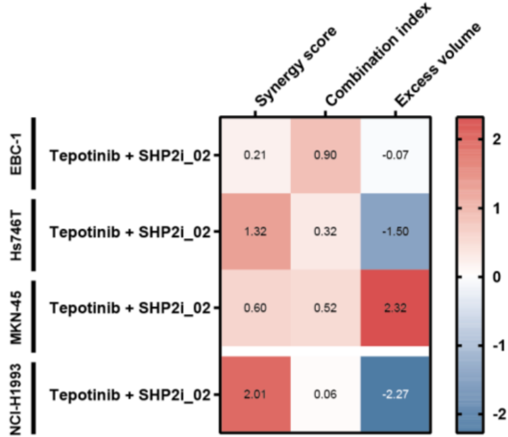


Differential expression of genes involved in RTK signaling pathways in tepotinib-resistant EBC-1 and Hs746T cell lines. Related to Figure 2. Digital gene expression quantification was performed using the NanoString nCounter® instrument with the NanoString nCounter® PanCancer Pathways Panel Assay. (**A, B**) Average gene expression across analyzed samples (n = 3 for EBC-1, TR1, TR2 and TR3; n = 6 for Hs746T), normalized and transformed to a log2 scale. (**C, D**) Venn diagrams illustrating the sum of up- and downregulated genes as derived from data analyses using three independent quantification methods: DESeq2, Limma and nSolver. (**E - H**) Relative expression data indicating the normalized and unsupervised clustering of parental and tepotinib-resistant cell lines. Horizontal rows represent individual genes, vertical columns represent the individual cell lines. The color scale at the base of each heatmap depicts the corresponding relative gene expression levels: red indicates elevated expression, green indicates reduced expression. (**E**) Heatmap clustering of differentially expressed genes on the NanoString nCounter® PanCancer Pathways Panel in the EBC-1 (blue bars), TR1 (purple bars) and TR2 (red bars) cell lines. (**F**) Heatmap clustering of differentially expressed genes on the NanoString nCounter® PanCancer Pathways Panel in the Hs746T (red bars) and TR3 (purple bars) cell lines. (**G**) Heatmap clustering of 30 genes displaying most significant differential expression in between the EBC-1 (blue bars), TR1 (purple bars) and TR2 (red bars) cell lines based on three independent quantification methods: DESeq2, Limma and nSolver. (**H**) Heatmap clustering of the 30 genes displaying the most significant differential expression between the Hs746T (red bars) and TR3 (purple bars) cell lines based on three independent quantification methods: DESeq2, Limma and nSolver. TR1, tepotinib-resistant EBC-1 cell line #1; TR2, tepotinib-resistant EBC-1 cell line #2; TR3, tepotinib-resistant Hs746T #1.

Figure S3

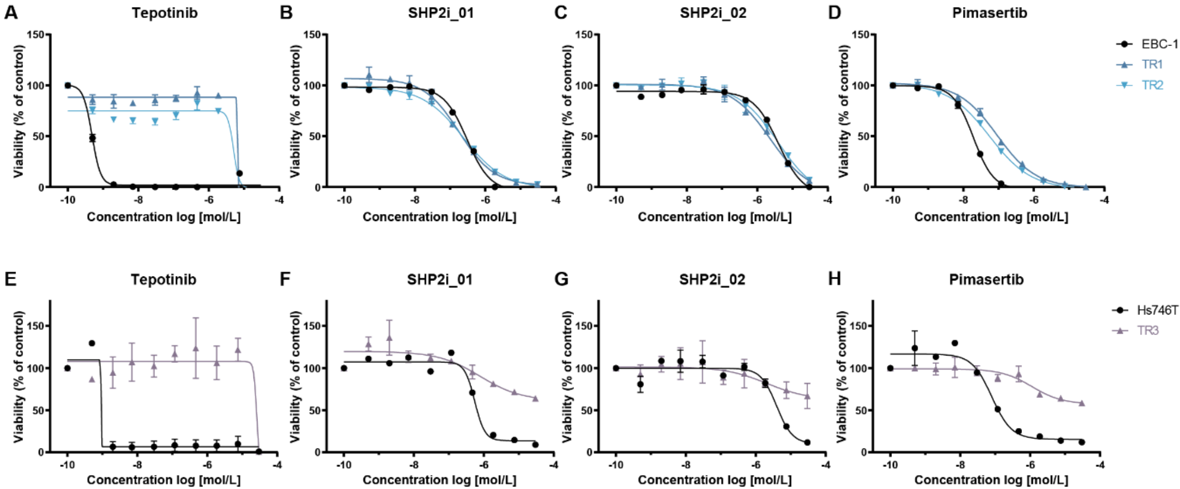






Combined inhibition of MET and SHP2 overcomes resistance to tepotinib *in vitro*. Related to Figure 5. (**A**) Dose-response curves for tepotinib, SHP2i_01, SHP2i_02 and pimasertib in the MET-amplified cell line NCI-H441 grown in 2D for 6 days. Data are shown as the mean ± SEM percentage fluorescent values relative to the DMSO control. (**B**) The indicated MET-amplified cell lines were grown in 2D and treated with a 6 x 6 combination matrix of tepotinib with either SHP2i_01, SHP2i_02 or pimasertib for 6 days. Synergy parameters including combination index (CI < 1), synergy score (SynS > 2) and excess volume (EV < 0) were analyzed using the Loewe combination method. (**C**) The indicated MET-amplified cell lines were grown in 3D and treated with a 6 x 6 combination matrix of tepotinib and SHP2i_02 titrations for 6 days. Synergy parameters were calculated as in B.

Figure S4



Combined inhibition of MET and SHP2 overcomes resistance to tepotinib (A - H). Related to Figure 1. Dose-response curves for tepotinib, SHP2i_01, SHP2i_02 and pimasertib in the parental tepotinib-sensitive (EBC-1, Hs746T) and tepotinib-resistant (TR1, TR2, TR3) cell lines upon exposure for 6 days in 2D. Data are shown as the mean ± SEM percentage fluorescent values relative to the corresponding DMSO control.

TRANSPARENT METHODS

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER	ADDITIONAL INFORMATION
Chemicals			
Tepotinib	Merck KGaA, Darmstadt, Germany	NA	
Pimasertib	Merck KGaA	NA	
SHP2i_01 (RMC-4550, PTPN11 inhibitor) [1]	Synthesized at Merck KGaA	NA	(Nichols et al., 2018)
SHP2i_02 (PTPN11 inhibitor) [2]	Synthesized at Merck KGaA	NA	International Patent Application Publication No.: WO2020/033828 A1, Example #10b)
All rest of chemicals	Sigma-Aldrich, St. Louis, MO	NA	
[1] Full name: {3-[(3S,4S)-4-amino-3-methyl-2-oxa-8-azaspiro[4.5]decan-8-yl]-6-(2,3-dichlorophenyl)-5-methylpyrazin-2-yl}methanol); [2] Full name: 6-((3S,4S)-4-Amino-3-methyl-2-oxa-8-azaspiro[4.5]decan-8-yl)-3-(Ra)-(2,3-dichlorophenyl)-2,5-dimethylpyrimidin-4(3H)-one; NA, not applicable; All compounds were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mmol/L and stored at -20°C.			
Experimental Models: Cell Lines			
EBC-1	HSRRB	JCRB0920 031496	Freezing day: 2017-06-21 Internal QC: 2017-09-18
EBC-1_TR1	Established in Merck KGaA	NA	Freezing day: 2014-11-26 Internal QC: 2015-01-23
EBC-1_TR2	Established in Merck KGaA	NA	Freezing day: 2014-11-27 Internal QC: 2015-01-23
Hs746T	ATCC	ATCC® HTB-135™	Freezing day: 2017-09-12 Internal QC: 2017-10-11
Hs746T_TR3	Established in Merck KGaA	NA	Freezing day: 2014-06-24 Internal QC: 2015-01-23
MKN-45	DSMZ	ACC 409	Freezing day: 2017-02-28 Internal QC: 2017-03-24
NCI-H1993	ATCC	ATCC® CRL-5909™	Freezing day: 2007-07-27 Internal QC: *not performed*
NCI-H441	ATCC	ATCC® HTB-174™	Freezing day: 2017-04-28 Internal QC: 2017-05-23
For each cell line, the provider, freezing date of cell bank and date of finalized internal QC (STR analysis) are indicated. QC, quality control; STR, short tandem repeat; TR1, tepotinib-resistant EBC-1 cell line #1; TR2, tepotinib-resistant EBC-1 cell line #2; TR3, tepotinib-resistant Hs746T #1; HSRRB, Health Science Research Resources Bank; ATCC, American Type Culture Collection; DSMZ, "Deutsche Sammlung von Mikroorganismen und Zellkultur"; QC, quality control; NA, not applicable.			
Antibodies			
Anti-cMet/HGFR	Cell Signaling Technology, Danvers, MA	Cat# 3127	
Anti-phospho-cMet/HGFR Y1234/1235	Cell Signaling Technology	Cat# 3077	
Anti-Erk1/2	BD Biosciences, San José, CA	Cat# 610123	
Anti-phospho-Erk1/2 T202/Y204	Cell Signaling Technology	Cat# 9101	
Anti-GAPDH	Santa Cruz, Dallas, TX	Cat# sc-32233	
Anti-beta actin (clone AC-15)	Sigma-Aldrich, St. Louis, NO	Cat# A5441	
Alexa Fluor 680	Molecular Probes, Eugene, OR	Cat# A21076	
IRDye800CW	LI-COR Biosciences, Lincoln, NE	Cat# 926-32212	

EXPERIMENTAL MODEL AND SUBJECT DETAIL

Cell Lines

Human cell lines with MET amplification or METex14 skipping mutations were purchased from the American Type Culture Collection (ATCC; Hs746T, NCI-H1993, NCI-H441), the Health Science Research Resources Bank, HSRRB, Japan (EBC-1) and Deutsche Sammlung von Mikroorganismen und Zellkultur (DSMZ Germany; MKN-45) (**Supplementary Table S3**), and cultured according to provider's recommendations. The identity of each cell line was authenticated by Short Tandem Repeat (STR) analysis by the using PowerPlex^R 16 HS System (Promega, Madison, WI). All cell lines were regularly tested to confirm the absence of *Mycoplasma* via quantitative polymerase chain reaction (qPCR) using custom-made primers (TIB Molbiol, Berlin, Germany).

Tepotinib-resistant cell lines derived from EBC-1 and Hs746T were generated by continuous exposure to sequentially increasing concentrations of tepotinib for 12 months to a final concentration of 5 µmol/L and 6 µmol/L, respectively. The EBC-1 and Hs746T cell lines were sourced from the providers listed above. The identify of each cell line was most recently confirmed by STR analysis on the dates indicated summarized in the **Key Resources Table**. EBC-1 cells were grown in culture flasks in the presence of tepotinib at concentrations between of 20 - 100 nmol/L for 5 days. Dead cells were then removed, and the remaining viable cells cultured further in medium without tepotinib until colonies were visible. A portion of the viable cells were then passaged to new flasks and treated with tepotinib again at concentrations between 20 - 100 nmol/L. Additionally, selected original flasks of cells of another part were cultured further in the presence of tepotinib between 20 - 500 nmol/L.

Tepotinib resistant cells were then cultured further and medium and tepotinib was replenished on a weekly basis. Cells were passaged when the culture flasks reached near confluence. Final selection rounds were performed with incremental tepotinib concentration increases up to 5000 nmol/L. Resistant cells were then maintained in tepotinib at 5000 nmol/L for 9 weeks. In total, 11 - 12 months of exposure to tepotinib was performed to generate the TR1 and TR2 cell lines, which were then cryopreserved at Merck KGaA, Darmstadt, Germany. STR analysis was performed to confirm the identity of the cells and the cultures were routinely tested to confirm the absence of *Mycoplasma*.

The tepotinib resistant cell line TR3 was derived from the parental Hs746T cell line using a comparable protocol to that described above for EBC-1.

Mice

Xenograft tumors were established by subcutaneous injection of 5 million cells suspended in 100 µL PBS into the right flanks of immunodeficient CD-1 nude mice. Tepotinib was formulated in a buffer containing 100 mmol/L Na-citrate (Merck, Darmstadt, Germany), 0.5% Methocel K4M (Colorcon, Kent, UK) and 0.25% Tween20 (Sigma-Aldrich, St. Louis, MO) at pH 3. SHP1i_02 was formulated in a buffer containing 50 mmol/L Na-citrate buffer, 0.5% Methocel K4M and 0.25% Tween-20 at pH3. Solutions were administered once daily by oral gavage.

METHOD DETAILS

Cell Viability Assays

To determine sensitivity of MET-altered cell lines to compounds in 2D culture, cells were seeded into clear 96-well plates at seeding densities ranging between 2000 to 3000 cells per well and cultured overnight. The cells were then incubated with serial dilutions (linear or cross-titration) of compounds with a constant DMSO concentration (≤ 0.5%). Compounds were dispensed using a Tecan D300e Digital Dispenser (Tecan, Männedorf, Switzerland). Following exposure for 3 or 6 days, viability was assessed using the Resazurin assay (R&D Systems, Minneapolis, MN). According to signal strength, cells were incubated with Resazurin for at least 1 h at recommended culture conditions prior to measurement of fluorescence (excitation: 531 nm, emission: 590 nm) using an Envision 2104 Multilabel Reader (Molecular Devices, San José, CA). For linear serial dilutions for determination of IC_{50} values, blank correction was performed in Microsoft Excel and dose-response curves were generated using GraphPad Prism (version 8.2.0). For cross-titration experiments of compound combinations, data was analyzed according to the plate layout by Loewe combination method using GeneData Screener® Software (Version 16.0.5).

Sphere Formation Assays

To assess the sensitivity of cell lines to compounds in 3D culture, cells were seeded into black-walled/clear round-bottom ultra-low attachment 96-well plates (Corning, NY, #4520) at seeding densities of 2000 cells per well and cultured overnight. Cells were exposed to compounds as described above. Following 6 days of culture, viability was assessed using the CellTiter-Glo 3D reagent (Promega, Madison, WI). Luminescence was measured using an Envision 2104 Multilabel Reader. IC_{50} values and synergy/antagonism was determined as described above.

Long-term Proliferation Assays

Cells were seeded into clear 12-well plates (Corning, NY) at a density of 1.0×10^5 cells per well and cultured for 5 days. Baseline confluency was determined using the IncuCyte S3 System (Essen BioScience, Ann Arbor, MI). Cells were exposed in weekly cycles (depicted in **Fig. 5A**) to tepotinib and SHP2i_02 as monotherapies or in combination. Following 24 h, tepotinib was removed from all wells and exposure to SHP2i_02 was renewed for a further 6 days, prior to re-initiation of the treatment cycle. Compounds were added to each well manually and DMSO concentrations were kept ≤ 0.1%. Confluency was measured using the IncuCyte S3 System as described above.

Gene Expression Profiling

For molecular characterization, RNA from parental tepotinib-sensitive (EBC-1, Hs746T) and tepotinib-resistant (TR1 - TR3) cell lines was isolated using RNAqueous®-4PCR Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. All RNA samples were quantified using a Qubit® 3.0 Fluorometer (Life Technologies, Carlsbad, CA) and stored at −80 °C prior to use. Digital gene expression quantification was performed from 50 ng of RNA using the NanoString nCounter® applying the

NanoString nCounter® PanCancer Pathways Panel Assay (NanoString Technologies, Seattle, WA) with a CodeSet containing 770 genes from 13 cancer-associated canonical pathways including MAPK, STAT, PI3K, RAS, Cell Cycle, Apoptosis, Hedgehog, Wnt, DNA Damage Control, Transcriptional Regulation, Chromatin Modification, and TGF-β, and 40 reference genes. Gene expression profiling was performed according to the manufacturer's instruction.

Data analysis and processing was conducted by combining three independent quantification methods: DESeg2, Limma and nSolver. The corresponding R packages DESeg2 and Limma were used for analysis. Background correction was performed by subtracting the "mean + 2x standard deviation" values of the negative controls from the raw counts. Adjusted raw counts were then normalized to the geometric mean of 6 positive controls in each sample. The resulting data was normalized again using the geometric mean of 40 reference genes prior to further normalization to the 40 reference genes. The cut-off for data exclusion was set at a minimum of 30 counts of mRNA template. The Bonferroni-Hochberg method was used for multiple testing correction. An adjusted *P* value 0.05 was applied as a significance cut-off for each method. The selected log2FC threshold for data presentation of differentially regulated genes was set to 1.

Phospho-Protein Array Analysis

Changes in phosphorylation levels of selected target molecules involved in signal transduction pathways in human cancer cell lines were determined using commercially available phospho-protein arrays (R&D Systems, Minneapolis, MN). The Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems, #ARY001B) was used for the simultaneous detection of the phosphorylation status of 49 RTKs. The Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, #ARY003B) was used for the simultaneous detection of the phosphorylation status of 43 human kinases and 2 related proteins. Both phospho-protein arrays were used as recommended by the manufacturer. Cells of interest were washed with PBS and harvested using Lysis buffer 17 or Lysis buffer 6 (both from R&D Systems). 300 µg and 500 µg of total cellular protein were used for the phospho-RTK array and phospho-kinase array, respectively.

For data analysis, pixel densities on developed X-ray films were detected using the Versadoc Imaging System (Bio-Rad, Hercules, CA; Model 5000) and quantified using ImageJ software (version 1.49v). Prior to quantification, images were transferred to an 8-bit gray scale and inverted. Pixel densities within a defined area surrounding a pair of duplicate dots were then determined. The pixel density of the PBS-negative control served as a background value and was subtracted from all values. The average pixel density of the reference spots was used for normalization prior to comparative analysis. Data was visualized using GraphPad Prism software (version 8.2.0).

Colony Formation Assay

Cells were seeded into clear 6-well plates at a seeding density of 3.5 x 10^4 cells per well and compound treatment initiated on the following day. Cells were then rinsed with phosphate-buffered saline (PBS) and then fixed and stained using 0.5% crystal violet dissolved in 20% methanol for 15 min at room temperature (RT). Following incubation, the plates were rinsed thoroughly under running water and dried

at RT. Cell surface coverage was measured using the IncuCyte S3 System and analyzed using the IncuCyte S3 software.

Western Blot Analysis

Cells were seeded in 6-well plates prior to compound treatments. After drug exposure, cells were harvested for Western Blot Analysis. Cell lysis was performed using RIPA lysis buffer: 10 mmol/L Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 7.4, 150 mmol/L sodium chloride (NaCl), 1% (v/v) Nonidet P40 (NP40), 1% (v/v) Triton X-100, 0.4% (w/v) deoxycholic acid sodium salt, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.3% (w/v) sodium dodecyl sulfate (SDS). Following the determination of protein concentrations using the BCA Protein Assay Kit (Pierce, Appleton, WI), cell lysates were mixed with 4x NuPAGE™ LDS Sample Buffer (Invitrogen) and 10x NuPAGE™ Reducing Agent (Invitrogen) and heated at 95°C for 10 mins. Samples were loaded into NuPAGE™ 4 - 12% Bis-Tris Midi Protein gels (Invitrogen) and electrophoresed in NuPAGE™ MOPS SDS Running Buffer (Invitrogen). Separated proteins were then transferred onto Immun-Blot ® Low Fluorescence PVDF membranes (Bio-Rad) using the Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked using the Odyssey® Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h on a rocking platform. Primary antibodies were diluted as recommended by the provider and incubated with membranes overnight at 4°C on a rocking platform. Following washing steps, membranes were then incubated with secondary antibodies for 1.5 h at RT at dilutions recommended by the provider. Signals were detected using the Odyssey® Imager (LI-COR Biosciences, Model 9120).

QUANTIFICATION AND STATISTICAL ANALYSIS

Image data was processed with ImageJ or Adobe Illustrator without image modification. Experiments have been performed in duplicates. The results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using the two-tailed Student's t-test with the following categorization of P values: * $P < 0.05$; **: $P < 0.001$; ***: $P < 0.0001$.

REFERENCES

Nichols, R.J., Haderk, F., Stahlhut, C., Schulze, C.J., Hemmati, G., Wildes, D., Tzitzilonis, C., Mordec, K., Marquez, A., Romero, J., *et al.* (2018). RAS nucleotide cycling underlies the SHP2 phosphatase dependence of mutant BRAF-, NF1- and RAS-driven cancers. Nat Cell Biol *20*, 1064-1073.