The first-in-class ERK inhibitor ulixertinib shows promising activity in MAPK-driven pediatric low-grade glioma models

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SUPPLEMENTARY MATERIALS

Supplementary Methods

Cell culture

Cell lines were authenticated using Multiplex Cell Authentication,[1] and purity was validated using the Multiplex cell Contamination Test,[2] both performed by Multiplexion (Heidelberg, Germany).

Drug treatments *in vitro*

All three cell lines were seeded at the same time (for seeding densities, see Supplementary Table S1), left for five days to allow the repression of the SV40 Large T antigen in the DKFZ-BT66_OFF cells, and subsequently treated with the indicated drugs (see Supplementary Table S8) using a D300e Digital Dispenser (Tecan Life Sciences). 1/2 Log distribution was used to cover a range of concentrations from 10 µM to 1 nM. Each condition was done in technical duplicates for three independent biological replicates. Raw values were normalized to their corresponding death control (250 nM STS for metabolic activity; 10 µM trametinib for MAPK reporter assay) and healthy control (DMSO), and used to calculate IC_{50}. Normalized data was plotted onto a 4-parameter dose response model

High content microscopy (HCM)

For BT40 the nuclear stain Hoechst33342 was used to define whole nuclei and fragmented nuclei as readouts of total cell number and dead cells respectively. TMRE stains active mitochondria and was thus used to define viable cells, while the staining of active caspases with CellEvent Caspase3/7 detected apoptotic cells.

For DKFZ-BT66 the total cell number was determined equivalently to BT40. DRAQ7 is a nuclear stain, which cannot pass the cell membrane of living cells. Thus, dead cells with damaged membranes were determined as the ratio of DRAQ7 positive cells

to Hoechst3342 positive cells. Finally, cell size was determined as area of the live-cell stain calceinAM and expressed as change from DMSO.

All readouts were normalized to healthy control (DMSO) and death control (1000 nM staurosporine).

For Cell Prolifer pipelines details, see Supplementary Table S9.

Synergy analysis

For a given readout, when the dimensional change upon single treatment was concordant with an inhibitory effect for both combined drugs, synergy was calculated with the Loewe model. When the dimensional change upon single treatment was opposite to an inhibitory effect for one of the combined drugs, and if drug independence could be assumed, synergy was calculated with the Bliss independence model. When the dimensional change upon single treatment was absent for one of the combined drugs, synergy was calculated with the highest single agent (HSA) model. When the dimensional change upon single treatment was opposite to an inhibitory effect for both combined drugs, synergy calculation was not applied. See Supplementary Figure S21 for details on what synergy model was used in which readout/cell line.

In vitro on-target activity validation

Samples were washed twice with ice-cold PBS. For Western blot, cell were lysed in a lysis buffer containing 62.5 mM Tris/HCl (Carl Roth, cat. no. 9090.2) pH 6.8, 2% SDS (Carl Roth, cat. no. 2326.1), 10% glycerol (Sigma-Aldrich, cat. no. 33226), 1 mM dTT (AppliChem, cat. no. A1101), phosphatase inhibitor (PhosphoSTOP, Merck, cat. no. 4906837001), protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Merck, cat. no. 11697498001). For reverse-phase protein array, cell were lysed in a lysis buffer

containing 45% 2xSDS, 45%T-PER (Thermo Scientific, cat. no. 78510), 10% TCEP (Thermo Scientific, cat. no. 77720)). For immunoprecipitation, cells were lysed in a lysis buffer containing 300 mM Tris/HCl (Carl Roth, cat. no. 9090.2), 120 mM NaCl (ThermoFisher Scientific, BP358-1), 10% glycerol (Sigma-Aldrich, cat. no. 33226), 2 mM EDTA (GERBU Biotechnik GmbH, 1034), 2 mM KCl (Carl Roth, 6781.1), 1% Triton X-100 (AppliChem, A4975.0500), protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Merck, cat. no. 11697498001). Antibodies were crosslinked to Dynabeads™ using freshly dissolved 20 mM dimethyl-pimelimidate (DMP) in 0.2 M triethanolamine buffer.

Zebrafish embryo toxicity assay, xenotransplantation, and treatment

Zebrafish toxicity assay

Zebrafish embryos were treated 48 hours post fertilization (hpf) in 48-well plates (cat. no. 351178, Corning) containing E3 buffer supplemented with 0.2nM 1-phenyl-2-thiourea (PTU buffer) and increasing concentrations as indicated. As determined by us and others[29,36,37], effective concentrations usually are up to 10-to 20-fold higher in the zebrafish embryo xenograft model than in cell culture studies. Thus, drugs in concentrations 10 times higher than the corresponding *in vitro* metabolic IC_{50} rounded to the closest power of ten were applied to the embryo-containing buffer solution (e.g.: IC_{50} Ulixertinib = 62nM; closest power of 10 = 100nM; 10-fold = 1 µM). During treatment (48 hpf - 120 hpf) the embryos were kept at 34°C. Imaging was performed at 72 hpf and 120 hpf using a stereo microscope (Leica). Zebrafish embryos were assessed for signs of toxicity (morphological changes, death, reaction to outside stimuli). A maximum tolerated dose (MTD) was determined as the highest tested concentration without manifestations of toxicity.

Zebrafish embryos xenografts imaging

After transplantation, zebrafish embryos were distributed in 48-well plates containing 1-phenyl-2-thiourea (PTU) buffer and kept at 34 °C. 24 h post injection (hpi), embryos were treated for 48h with drugs diluted in PTU buffer, as indicated. Imaging was done at 24 hpi and 72 hpi. Zebrafish embryos were anesthetized with tricaine (MS-222, Ethyl 3-aminobenzoate methanesulfonate, 0.02% (w/v), Sigma-Aldrich) and either transferred (one embryo/well) to Hashimoto 96-well zebrafish imaging plates (Funakoshi Co., Ltd., Tokyo, Japan) for imaging with the ImageXpress Micro Confocal High Content Microscope (Molecular Devices) at a temperature of 32-34 °C, or put into chambered coverslips (ibidi, Martinsried, Germany) for imaging with the Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany) equipped with the 10×/0.3 EC Plan-Neofluar and 20×/0.8 Plan-Apochromat objectives, Argon 514 nm laser line for DiI, standard PMT for fluorescence detection and T-PMT for transmitted light controlled by ZEN software (Zeiss). A Cy3-channel was used for the detection of RFP.

Zebrafish embryos treatment

Treatment response of tumor growth was captured by tumor volume change from baseline (day one to day three post-implantation). To determine tumor progression, we used an in-house macro for FIJI software, as described previously.[3] Best response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 adopted for zebrafish tumors:[3] progressive disease (PD), at least a 20% increase in tumor volume; partial response (PR), at least a 30% decrease in tumor volume. A PD/PR ratio was used to assess treatment's efficacy.

BT40 xenograft mouse model, treatment, and imaging

Drugs and solvents were used as indicated in Supplementary Table S10.

Pharmacokinetic study

After three weeks, mice were randomly split into two cohorts based on weight and ranked luciferase signal. The time window was covered by seven groups (four dosed mice per group) with four blood collection time points per group, due to blood volume restrictions for sampling within 24 h. Full blood samples were transferred into EDTA tubes (cat. no. 77003, KABE) and centrifuged at 3,000x g for 10 min to generate plasma samples, which were stored at -80 °C until analysis. For brain tissue determinations, tissue samples were harvested as indicated, and stored at -80 °C until analysis. Brain tissue samples were homogenized using a Bead Ruptor 4 homogenizer (Omni International Inc) in acetonitrile (ACN)/water (1/19, v/v) + 0.5% triton X and 0.1% formic acid (FA) (100 mg brain tissue/mL) in 2.0 mL tubes containing ~25 glass beads (0.75-1 mm; cat. no. A554.1,Carl Roth GmbH) for 2 × 1 min.

Ulixertinib and navitoclax bioanalysis

UPLC-MS/MS quantification assay

Microdialysate, plasma, and brain tissue concentrations were measured using a validated ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) quantification assay following the pertinent guidelines of the US FDA and EMA.[4,5] Each of four performed validation runs included blank and internal standard controls, seven calibration samples (two-fold), and four quality control concentrations (six-fold). The assays fully complied with the applicable recommendations of the US FDA and EMA on bioanalytical method validation.

Ulixertinib quantification

Optimized MS/MS parameters for the detection of ulixertinib can be found in Supplementary Table S11. The calibrated range for ulixertinib quantification was 1 – 1,000 ng/mL (corresponding to 10 – 10,000 ng/g for brain tissue), which was additionally extended to 0.1 – 100 µg/mL, all showing linear regression coefficients > 0.99. Overall accuracies (inter- and intraday) were 87.0 to 109.3% with corresponding precision < 6.7%. A Xevo-TQ-S tandem mass spectrometer (Waters, Milford, MA, USA) coupled to an I-class UPLC (Waters) and equipped with heated electrospray ionization source was used for quantification with selected reaction monitoring using collision-induced dissociation with argon in the positive ion mode. Chromatographic separation was performed on a BEH C18 column (50 × 2.1 mm; 1.7 µm; Waters) with a gradient from 5 to 95% acetonitrile (ACN) + 0.1% FA in 1.5 min (corresponding decrease of aqueous eluent: 19/1 H2O/ACN + 0.1% FA) at a flow rate of 0.5 mL/min. Microsamples (25 µL) were spiked with internal standard (Ulixertinib-D6) and 200 µL of borate buffer (pH 9). Ulixertinib was extracted with 1 mL of tert-butyl methyl ether. After evaporation of 200 µL of the ether phase, extracts were dissolved in 400 µL ACN/H2O (1/3) + 0.1% formic acid and 5 µL were injected for analysis.

Navitoclax quantification

Optimized MS/MS parameters for the detection of navitoclax can be found in Supplementary Table S12. The calibrated range was 0.05 – 50 ng/mL (corresponding to 0.5 – 500 ng/g for tumor tissue), all showing linear regression coefficients > 0.99. Overall accuracies (inter- and intraday) were 94.7 to 110.8% with corresponding precision < 9.8 %. A Xevo-TQ-XS tandem mass spectrometer (Waters, Milford, MA, USA) coupled to an Acquity classic UPLC (Waters) and equipped with heated electrospray ionization source was used for quantification with

selected reaction monitoring using collision-induced dissociation with argon in the positive ion mode. Chromatographic separation was performed on a BEH C18 Peptide column 300 Å (50 × 2.1 mm; 1.7 µm; Waters) with a gradient from 20 to 95% ACN + 0.1% FA in 1.9 min (corresponding decrease of aqueous eluent: 19/1 H2O/ACN + 0.1% FA) at a flow rate of 0.5 mL/min. Samples (100 µL) were spiked with internal standard (venetoclax) and extracted by protein precipitation using 300 µL of ACN. After partial evaporation of the separated supernatant and addition of 50 µL of H2O + 0.1% formic acid, 20 µL were injected for UPLC-MS/MS analysis.

Sample preparation for ulixertinib on-target analysis in vivo (DUSP6 Western blot)

Tissue samples were weighed, resuspended in 1:10 w/v lysis buffer (49% SDS buffer, 50% T-PER buffer, 10mM DTT, phosphatase inhibitor (PhosphoSTOP, Merck, cat. no. 4906837001), protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Merck, cat. no. 11697498001)) and disrupted using a benchtop tissue homogenizer. The resulting lysates were centrifuged at 10,000 x g for 5 minutes and the supernatant was collected. The protein concentration of each lysate was determined using the Pierce™ BCA Protein Assay kit (ThermoFisher Scientific, cat. no. 23227) for subsequent Western blot analysis.

***In vivo* preclinical study**

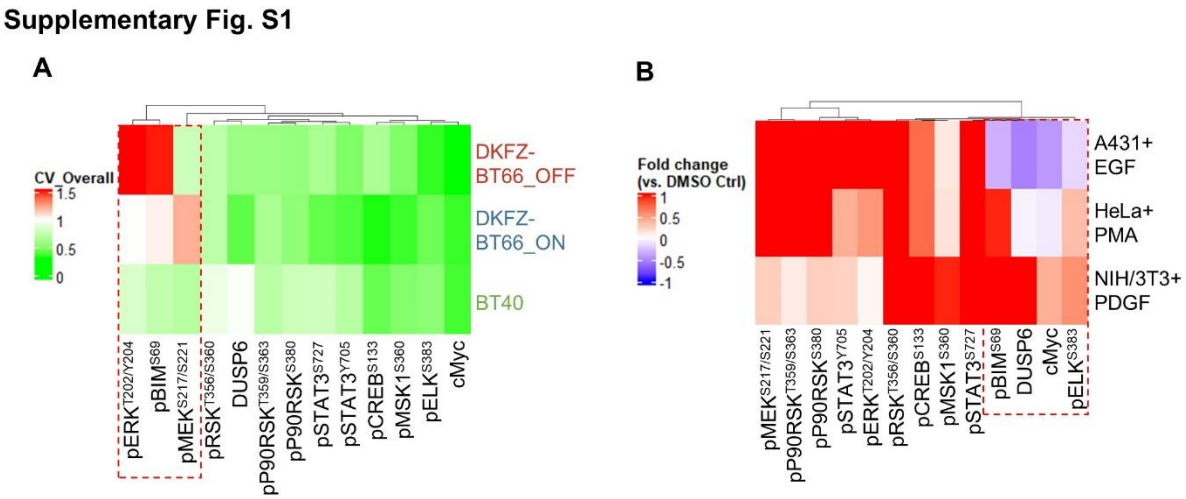
After two weeks of tumor development, animals were distributed into six treatment groups (n=8 per group) based on their ranked luciferase signal according to the following pattern: “ABCDEFFEDCBAA…”. Ulixertinib (75 mg/kg) and the vehicle were administered via oral gavage every 12 h. Navitoclax (100 mg/kg) was administered via oral gavage every 24 h. Vinblastine (0.5 mg/kg) was injected intraperitoneally

every 72 h. During the treatment period, animals were weighed every second day and tumor development was monitored twice per week by bioluminescence imaging.

Statistical analysis and graphical representations

IC50 calculation was performed using GraphPad Prism 5 software (Version 5.01, GraphPad Software Inc., San Diego, USA). RStudio (R Version 1.4.1103) was used for calculation of ANOVA followed by Tukey's honest significant test. Synergy scores were computed using the online platform SynergyFinderPlus (https://synergyfinder.org/)32 (matrix design), and the R package drugCombo 1.1.1 (ray design). Consensus rankings were generated with the R package challengeR37 using average ranks. Quantification of Western blot bands was conducted using ImageJ (version 1.53e, Wayne Rasband and co., NIH). Calibration curves for the ulixertinib bioanalysis were determined with 1/x2 weighted linear regression using peak area ratios of the analyte to IS, using the software TargetLynx (V4.2, Waters, Milford, USA). Plasma pharmacokinetics were determined with the software Kinetica (v 5.0, Thermo Fisher Scientific, Philadelphia, USA). Bioluminescence measurments were analyzed with the LivingImage software (v4.5.2, Caliper Life Sciences, Massachusetts, USA).Survival curves were generated in R using the survminer package. Significance between survival curves were calculated using the log-rank test in GraphPad Prism 8 software (Version 8.0.2.263, GraphPad Software Inc., San Diego, USA).

Supplementary Figures

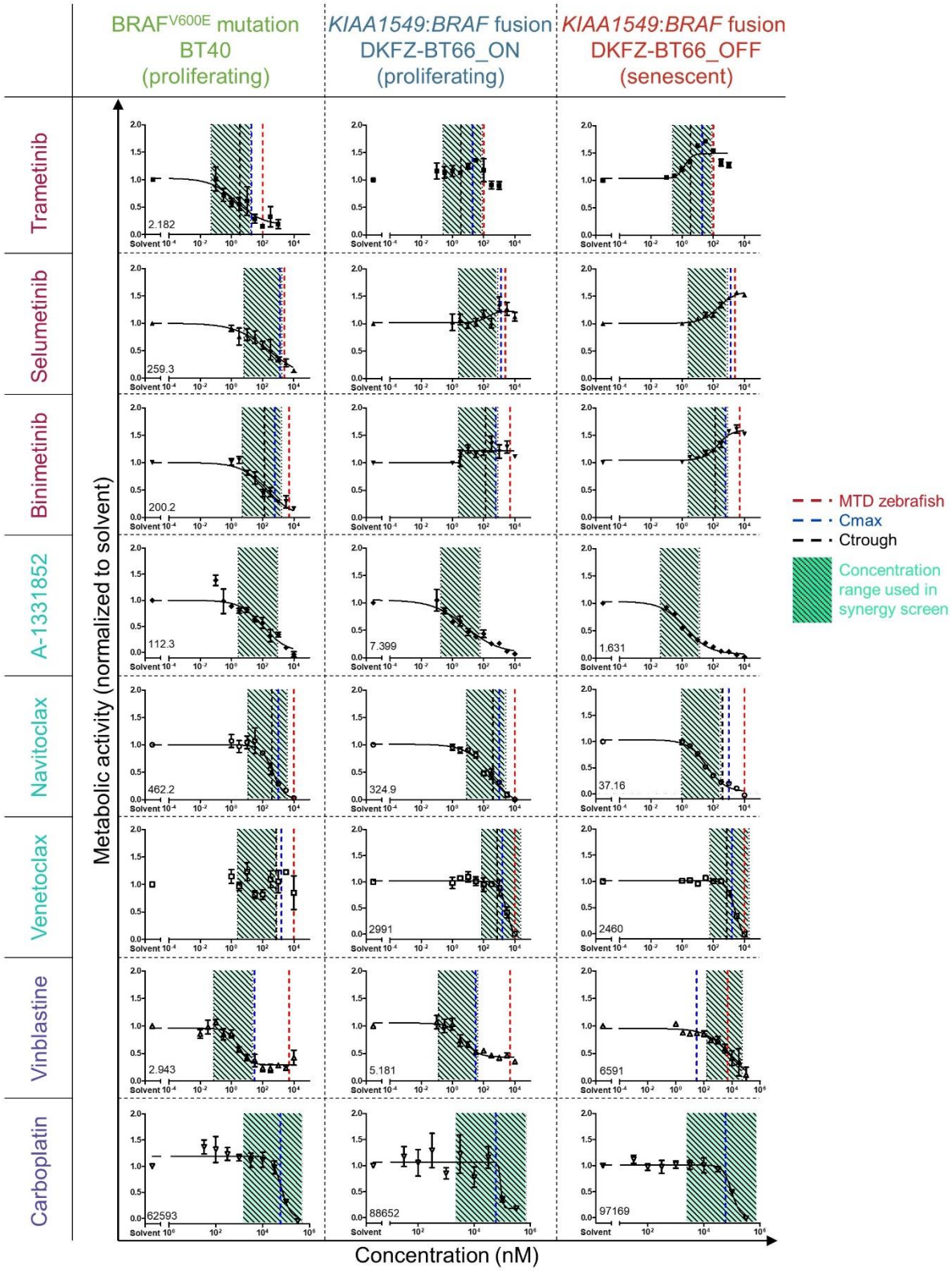


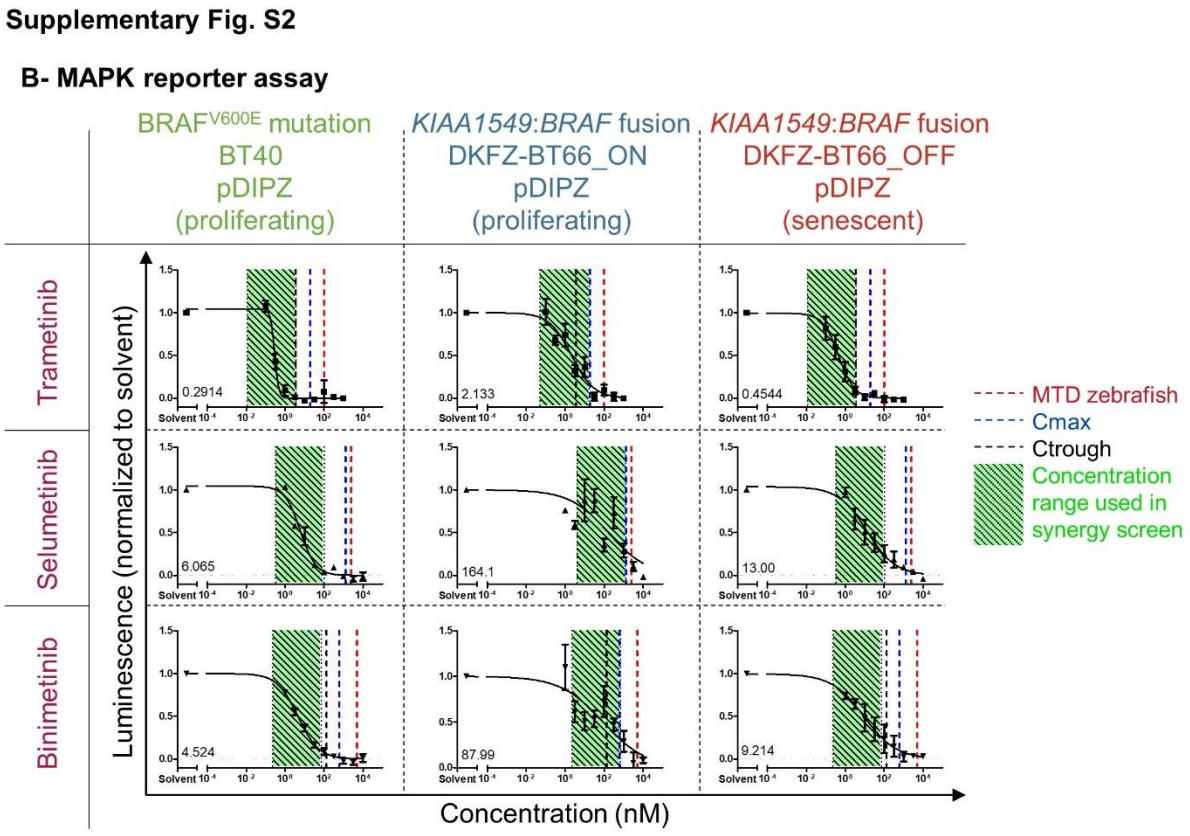
Supplementary Fig. S1: Reverse-phase protein array (RPPA) quality control

A, Heatmap depicting the coefficient of variation (CV) obtained in the reverse-phase protein array (RPPA) data across biological replicates. Green indicates low variance (CV < 1), red indicates high variance (CV > 1). Red dotted square indicates markers that didn't pass the threshold and were excluded. B, Heatmap depicting phosphoprotein fold change in the indicated control cell lines upon treatment with a MAPK stimulator relative to solvent control. Red indicates an increased fold change, while blue indicates a decreased fold change. Red dotted rectangle indicates markers that showed discrepancy and were excluded. Depicted are data from three biological replicates.

Supplementary Fig. S2

A- Metabolic activity

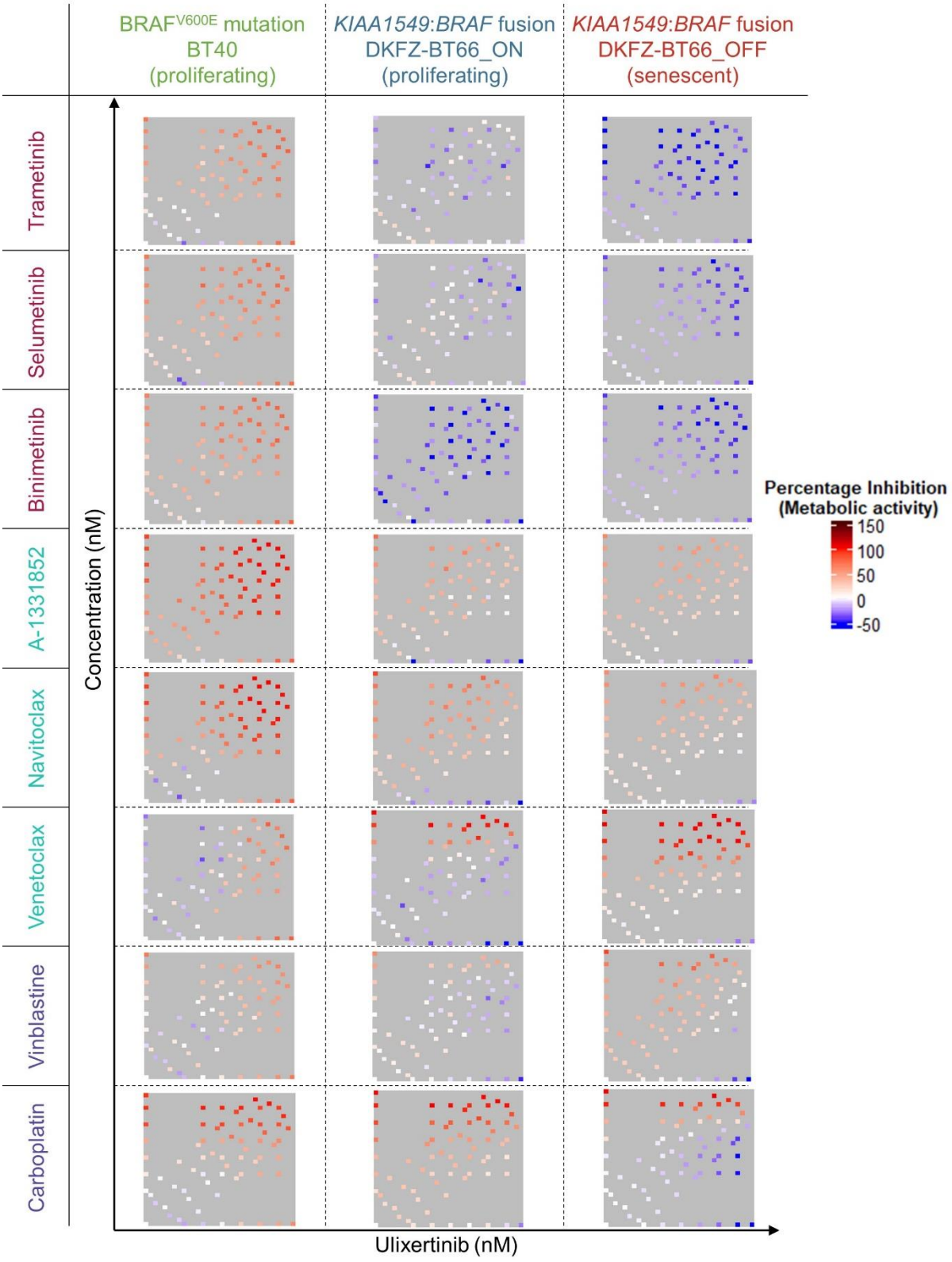




Supplementary Fig. S2: IC50 of ulixertinib's combination partners

A, Metabolic activity IC_{50} (CellTiter-Glo®) measured in BT40, DKFZ-BT66_ON, and DKFZ-BT66_OFF cells. B, MAPK reporter IC_{50} measured in BT40, DKFZ-BT66_ON, and DKFZ-BT66_OFF cells. Each dot represents the mean of three independent biological replicates. Error bars depict the standard deviation. Published pharmacokinetic data (Cmax and Ctrough, when available), zebrafish embryo maximum tolerated dose (MTD), and concentrations used in the synergy analysis are depicted.

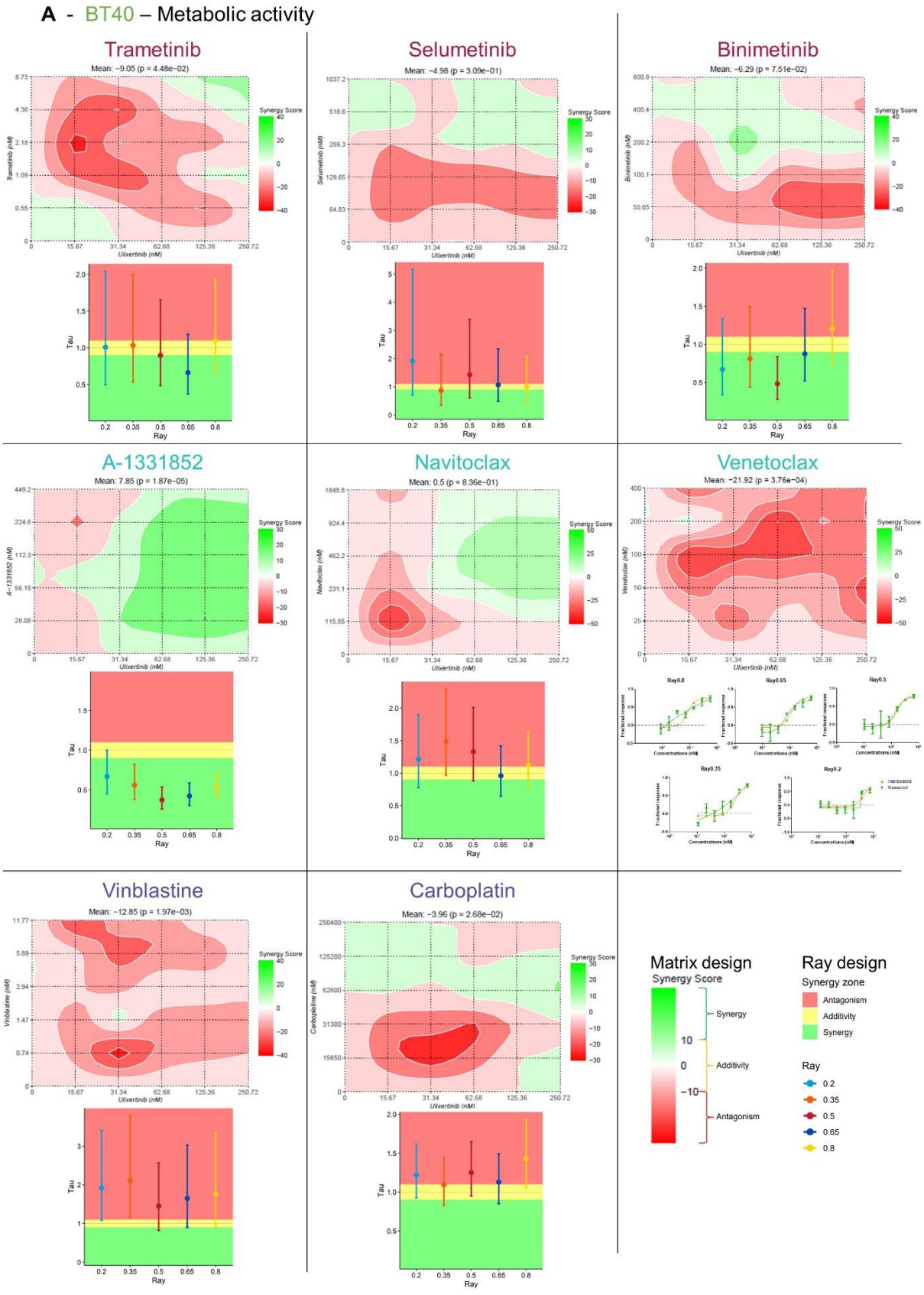
Supplementary Fig. S3



Supplementary Fig. S3: Heatmap of metabolic activity inhibition in the *in vitro* synergy screen

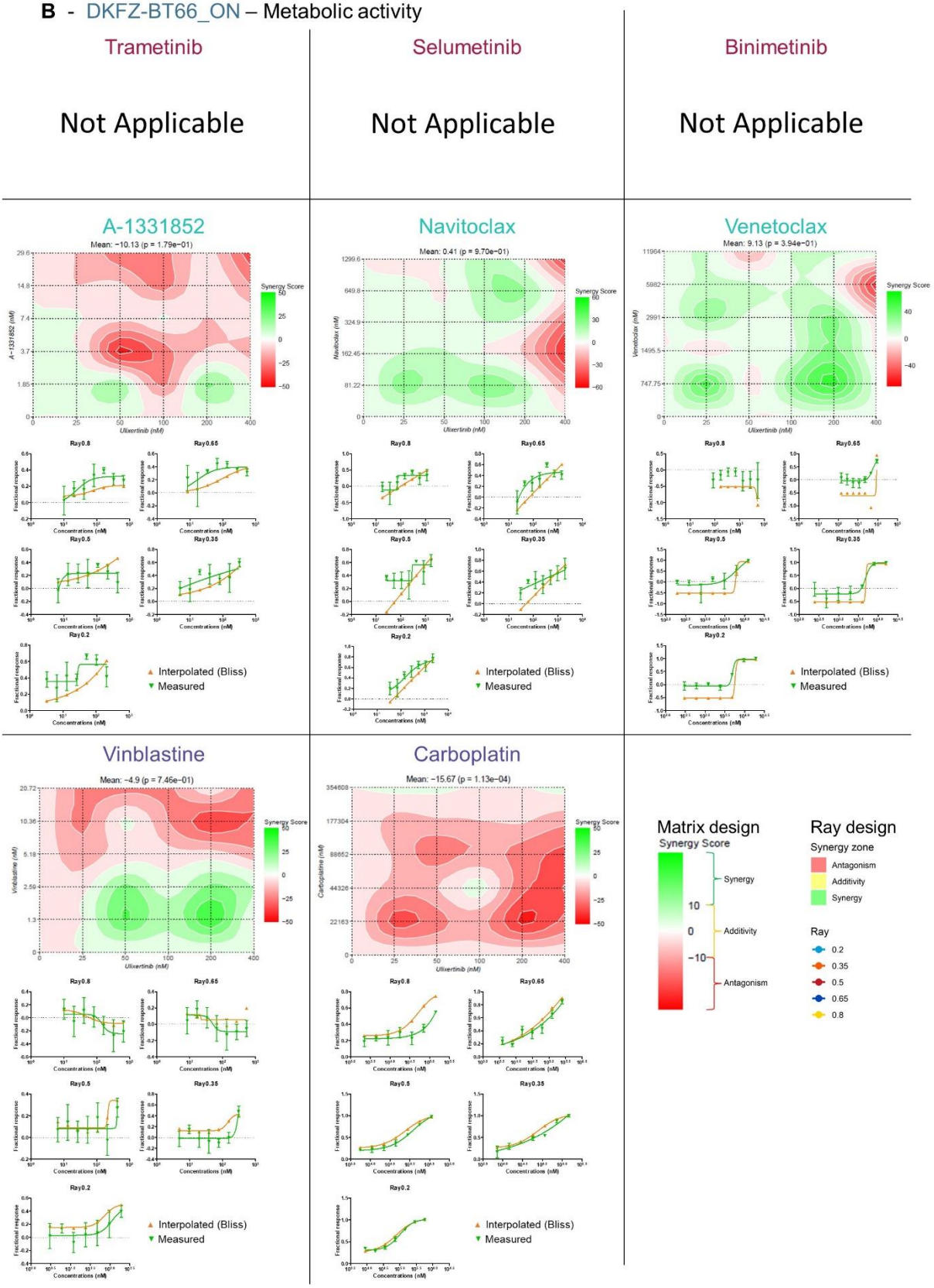
Heatmap depicting the percentage of inhibition of metabolic activity relative to DMSO control in BT40, DKFZ-BT66_ON, and DKFZ-BT66_OFF cells upon combinational treatment of ulixertinib with each combination partner. In the matrix design, each drug was combined using two-fold increment concentration ranges, centered on the corresponding drug's IC50. In the ray design, concentrations were calculated based on each drug's IC_{50}, as described previously (31). Increased inhibition is depicted with red colors, while increased metabolic activity is depicted with blue colors. Depicted are data from three biological replicates.

Supplementary Fig. S4



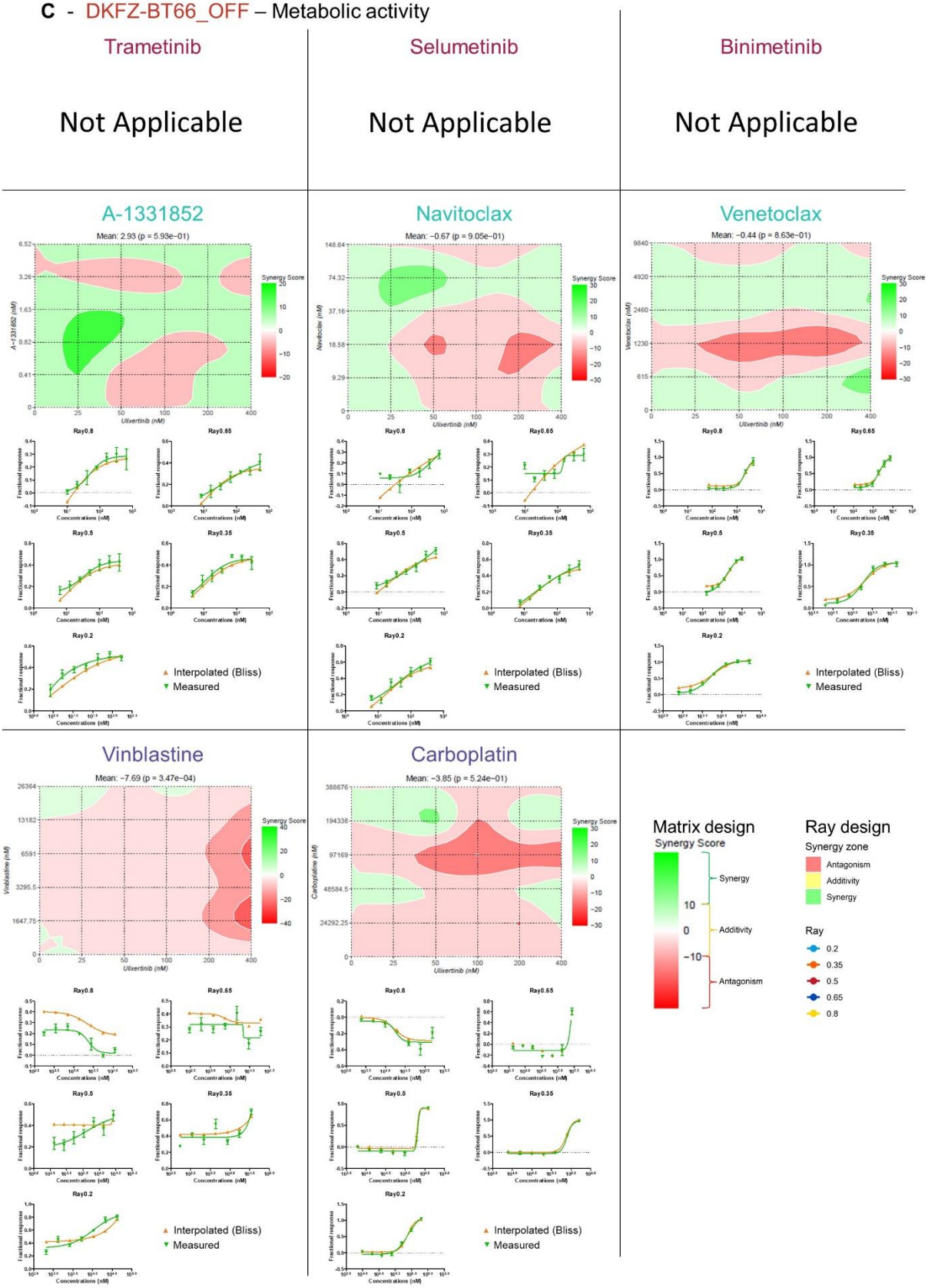
Supplementary Fig. S4

B - DKFZ-BT66_ON – Metabolic activity



Supplementary Fig. S4

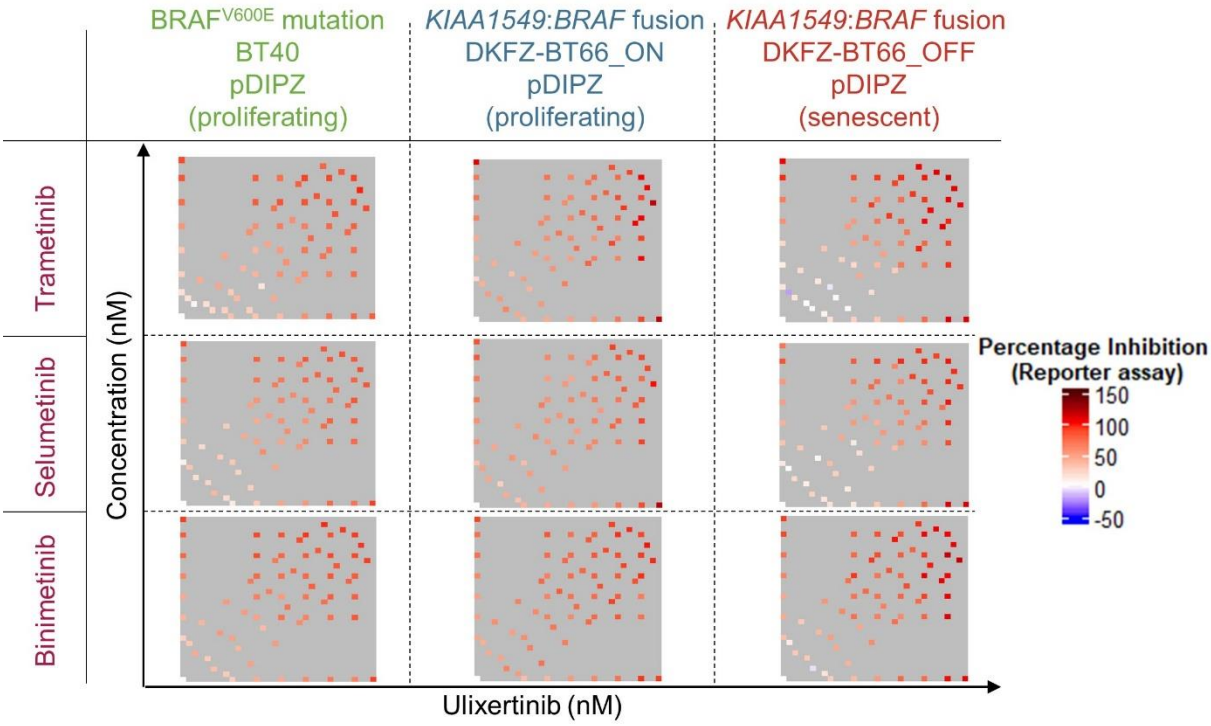
C - DKFZ-BT66_OFF – Metabolic activity



Supplementary Fig. S4: Synergy results for the matrix and ray designs using metabolic activity

Synergy maps depicting synergy score across all concentrations tested in the matrix design. Scores above 10 indicate synergy, while scores below -10 indicate antagonism. Dot plots (only where the Loewe model was applied) depict ray synergy scores and bars represent the 95% CI. Scores below 0.9 indicate synergy, scores above 1.1 indicate buffering antagonism, scores above 2 indicate antagonism. Response-curves (only where Bliss independence was applied) depict the expected effect based on the Bliss independence formula (orange curve) and actual measures (green curve). The excess over Bliss score is depicted. Scores above 0 indicate synergy, scores below 0 indicate antagonism. A, Synergy data obtained with metabolic activity in BT40 cells. B, Synergy data obtained in DKFZ-BT66_ON cells. C, Synergy data obtained in DKFZ-BT66_OFF cells. Depicted are data from three biological replicates.

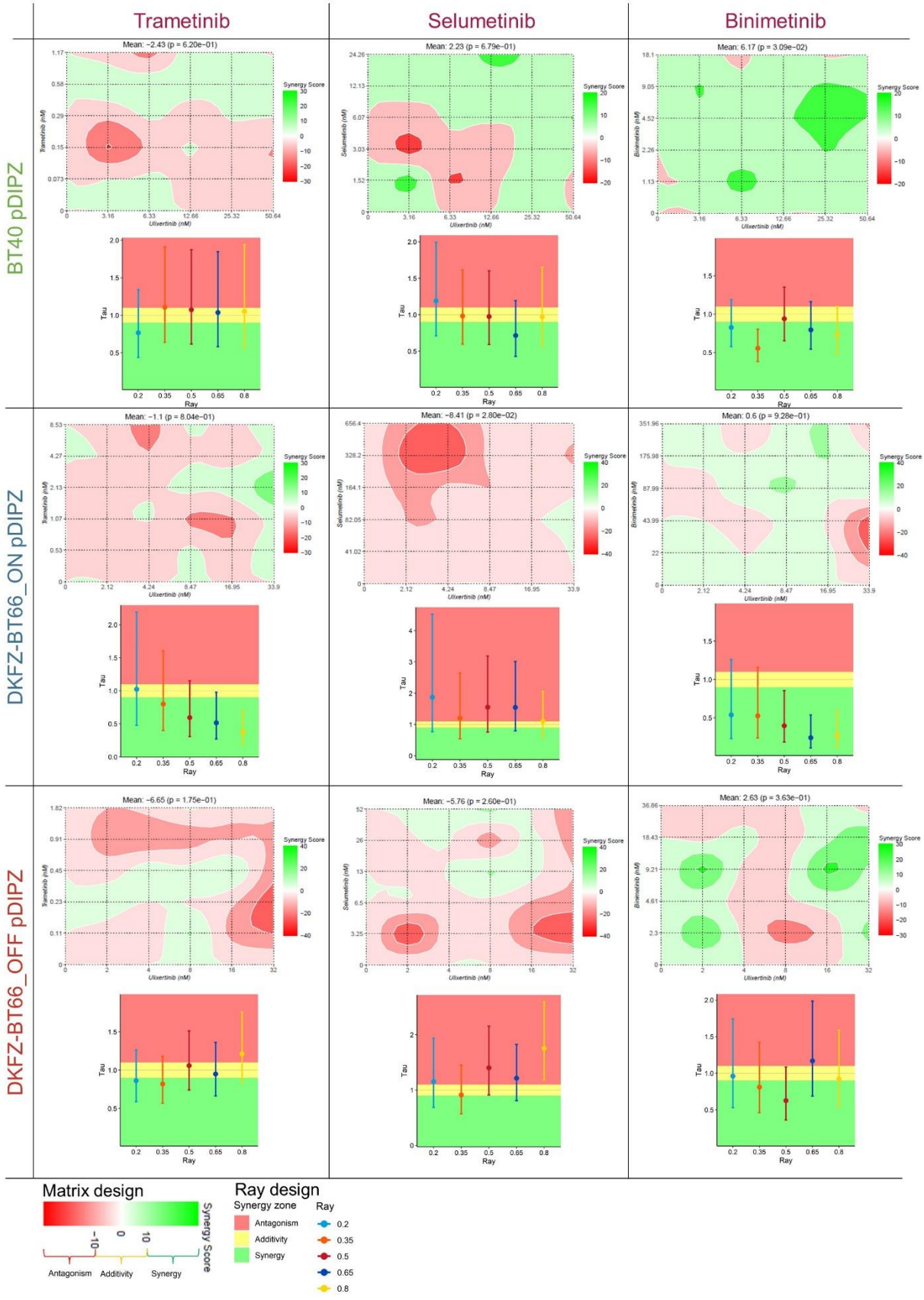
Supplementary Fig. S5



Supplementary Fig. S5: Heatmap of MAPK activity (reporter) inhibition in the *in vitro* synergy screen

Heatmap depicting the percentage of inhibition of MAPK pathway activity (assessed by MAPK reporter assay) relative to DMSO control in BT40, DKFZ-BT66_ON, and DKFZ-BT66_OFF cells upon combinational treatment with ulixertinib and each combination partner. In the matrix design, each drug was combined using two-fold increment concentration ranges, centered on the corresponding drug's IC50. In the ray design, concentrations were calculated based on each drug's IC_{50}, as described previously (31). Increased inhibition is depicted with red colors, while increased metabolic activity is depicted with blue colors. Depicted are data from three biological replicates.

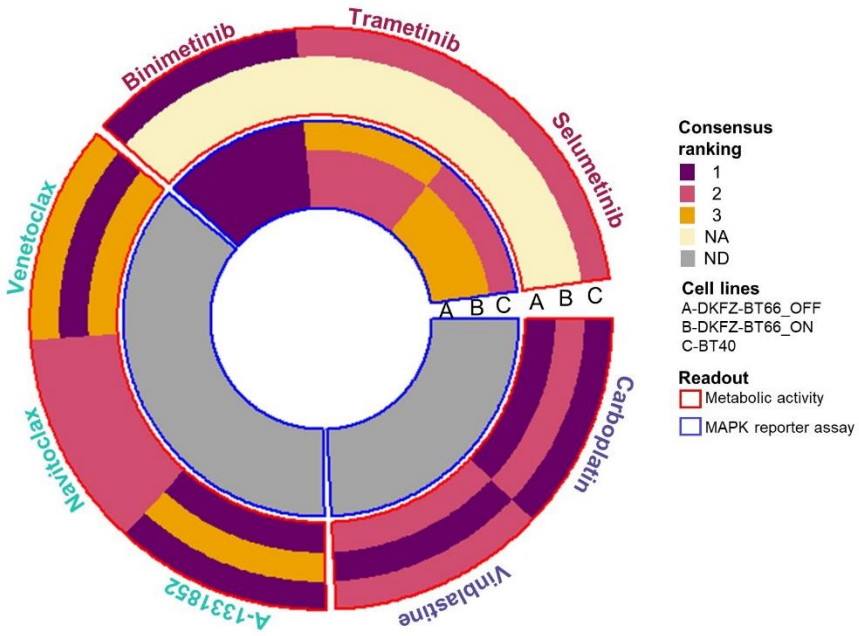
Supplementary Fig. S6



Supplementary Fig. S6: Synergy results for the matrix and ray designs using the MAPK reporter assay

Synergy maps depicting synergy scores across all concentrations tested in the matrix design. Scores above 10 indicate synergy, while scores below -10 indicate antagonism. Dot plots depict ray synergy scores and bars represent the 95% CI. Scores below 0.9 indicate synergy, scores above 1.1 indicate buffering antagonism, scores above 2 indicate antagonism. Depicted are data from three biological replicates.

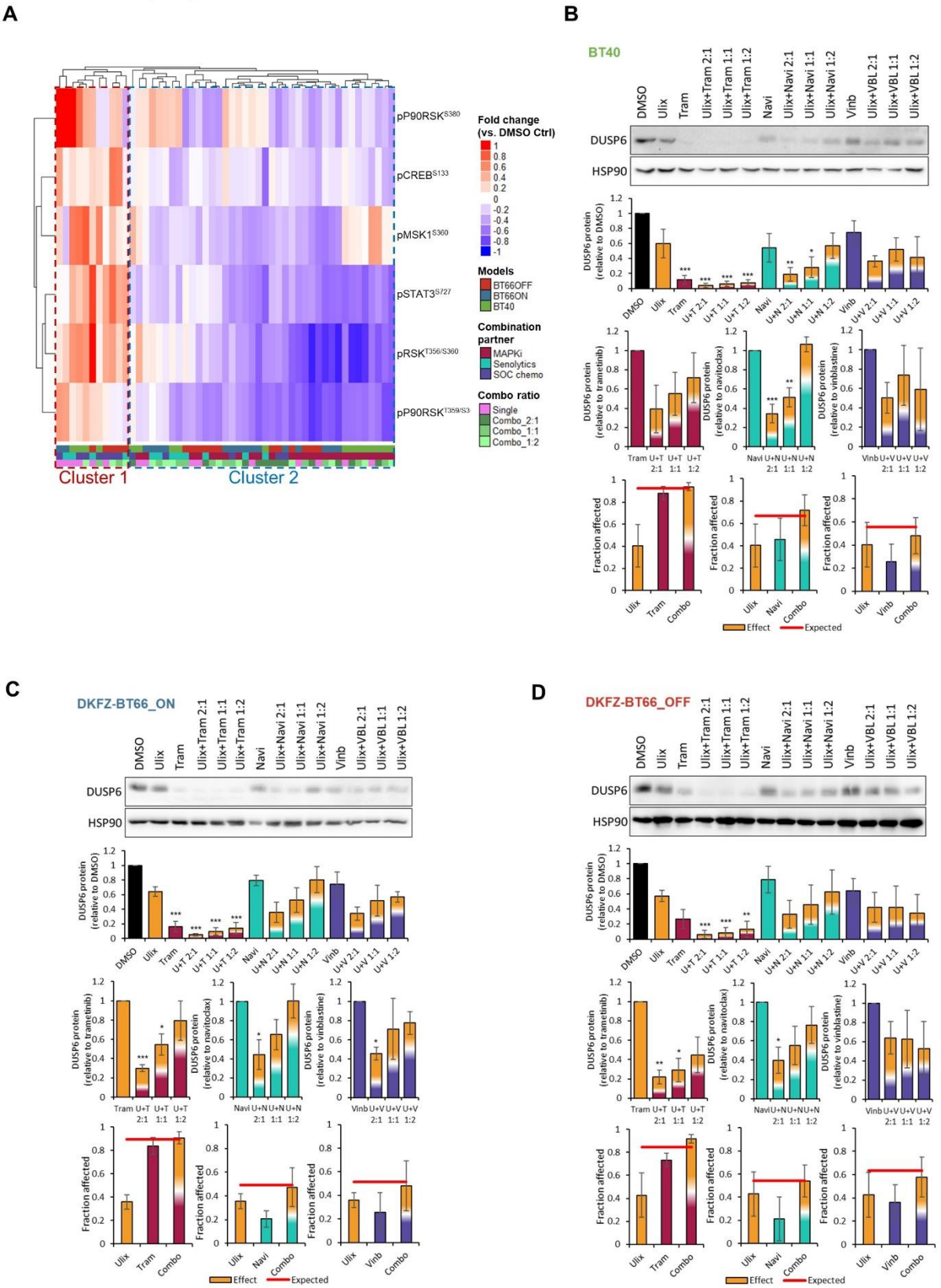
Supplementary Fig. S7



Supplementary Fig. S7: Consensus ranking of combination partners in the *in vitro* screen (metabolic activity and MAPK reporter assay)

Circular heatmap summarizing the individual drug's performance per drug class, following consensus ranking across synergy metrics. The ranking was calculated for the DKFZ-BT66_OFF, DKFZ-BT66_ON, and BT40 (designated by A, B, and C respectively), and for each readout (outermost red rings = metabolic activity; innermost blue rings = MAPK reporter assay). NA, not applicable due to readout inconsistency. ND, not determined.

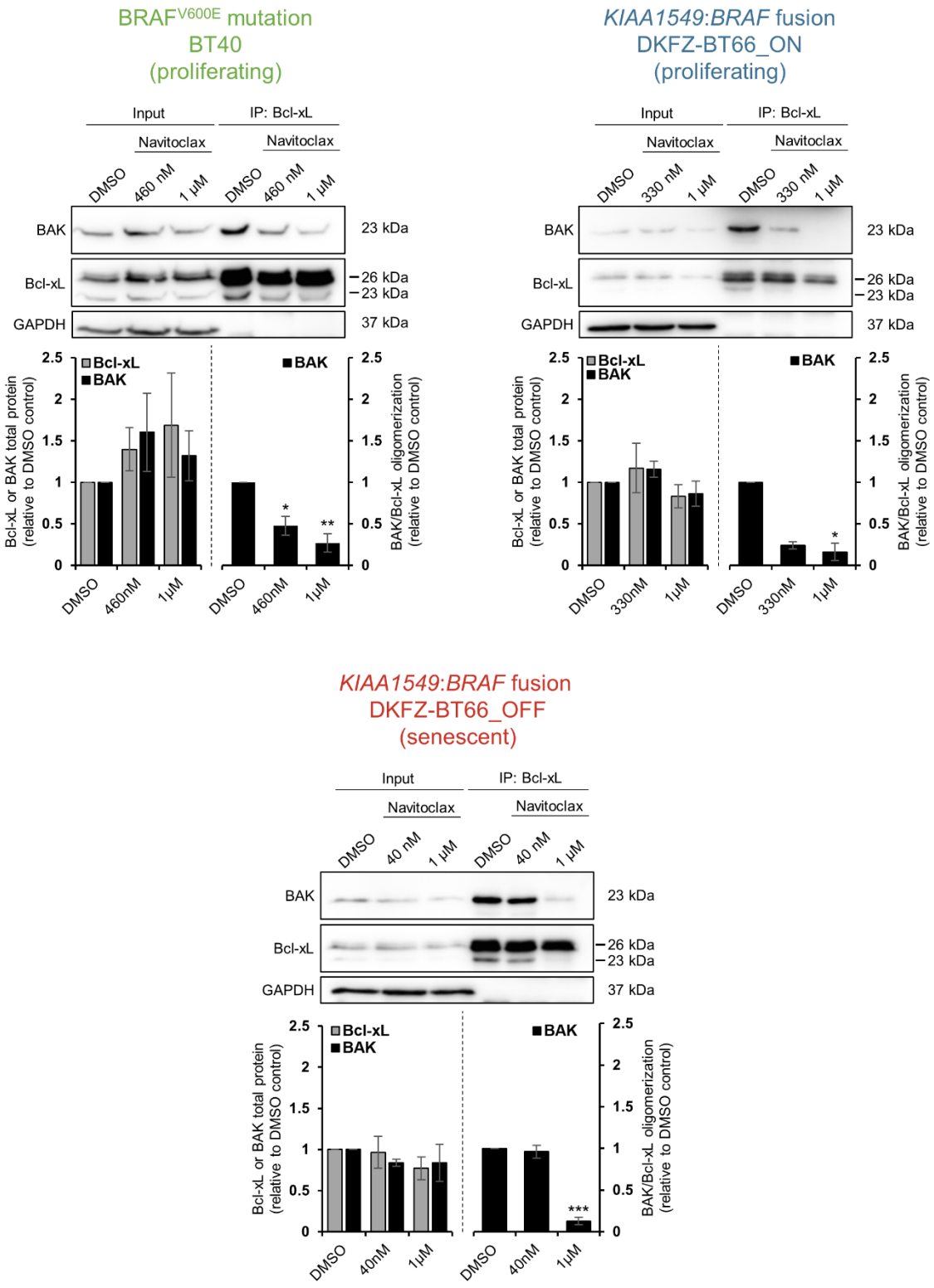
Supplementary Fig. S8



Supplementary Fig. S8: Ulixertinib on-target activity *in vitro* via RPPA and Western blot

A, Heatmap depicting phosphoprotein fold changes of selected markers upon treatment in RPPA. Fold change relative to DMSO is shown, with red colors indicating an increased phosphorylation, and blue colors indicating decreased phosphorylation. Red dotted rectangle (cluster 1) highlights the cluster of samples with an increased phosphorylation upon treatment; blue dotted rectangle (cluster 2) highlights the cluster of samples with a decreased phosphorylation upon treatment. B, C, and D, Western blot analysis of DUSP6 in BT40 (B), DKFZ-BT66_ON (C), and DKFZ-BT66_OFF (D), treated with varying concentrations of ulixertinib (2x IC_{50}, 1x IC_{50}, 0.5x IC_{50}) and a fixed concentration (IC_{50}) of the combination partner. Are depicted: quantification (n = 3 biological replicates) of all treatment conditions relative to the DMSO control; individual quantification for each classes of combination partners, depicting DUSP6 quantification relative to DUSP6 levels in the corresponding combination partner single treatment; estimation of the expected effect based on the Bliss independence formula compared to the observed effect. Depicted are data from three biological replicates. Significant differences are indicated as *, P < 0.05; **, P < 0.01; ***, P < 0.001 (ANOVA followed Tukey's honest significant test).

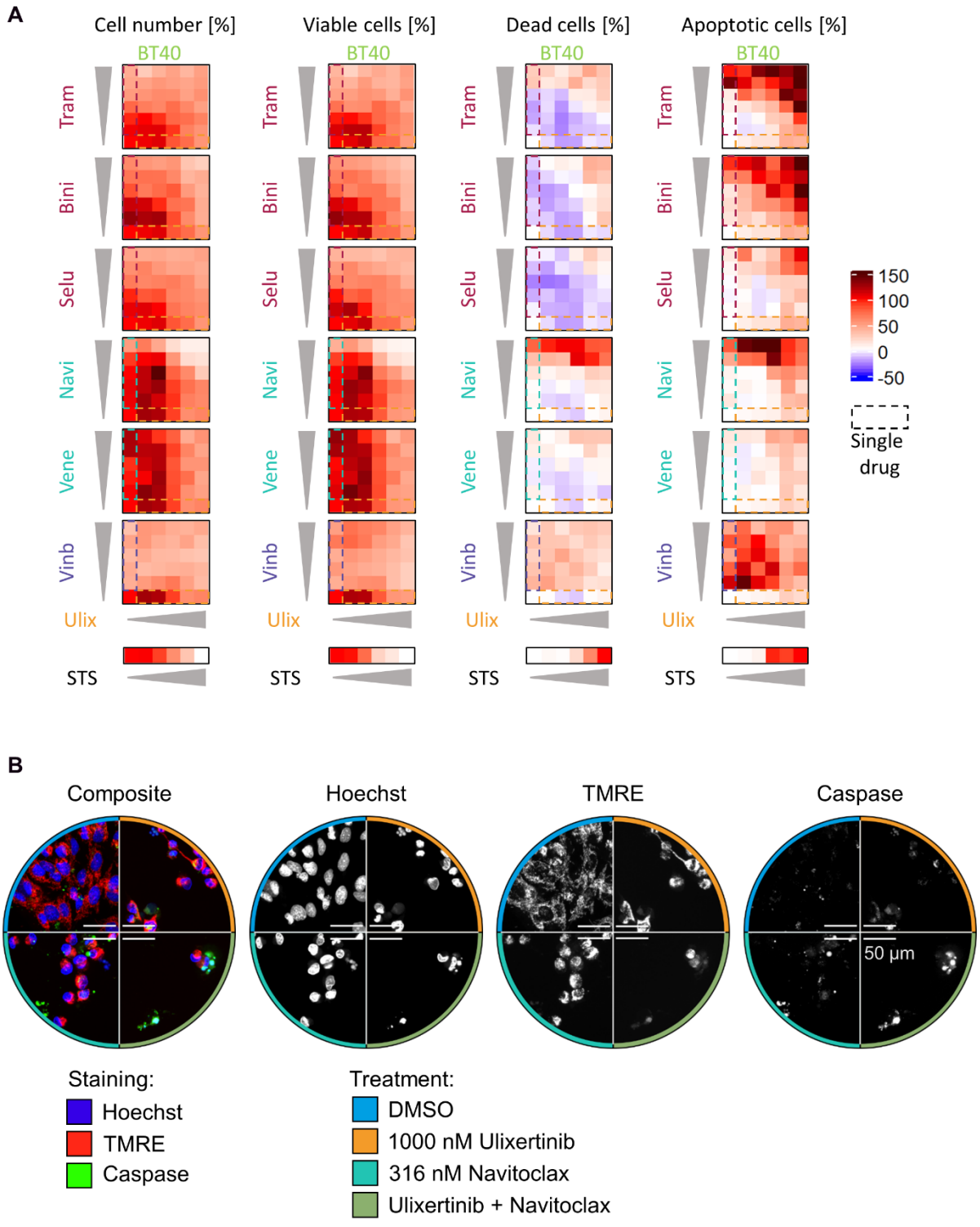
Supplementary Fig. S9



Supplementary Fig. S9: Navitoclax on-target activity *in vitro* via immunoprecipitation

BT40, DKFZ-BT66_ON and DKFZ-BT66_OFF cells were treated for 4 h with the corresponding navitoclax IC_{50}, 1 µM navitoclax or DMSO before lysis. Interaction of pro-apoptotic BAK with anti-apoptotic Bcl-xL was assessed by immunoprecipitation with a Bcl-xL specific antibody. Are depicted one representative experiment for each cell line. Is also shown Western blot quantification of 3 independent biological replicates. The left pane shows BAK and Bcl-xL normalized to the loading control GAPDH, and normalized to DMSO. The right pane shows BAK/Bcl-xL ratio, and normalized to DMSO. Significant differences are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (ANOVA followed Tukey's honest significant test).

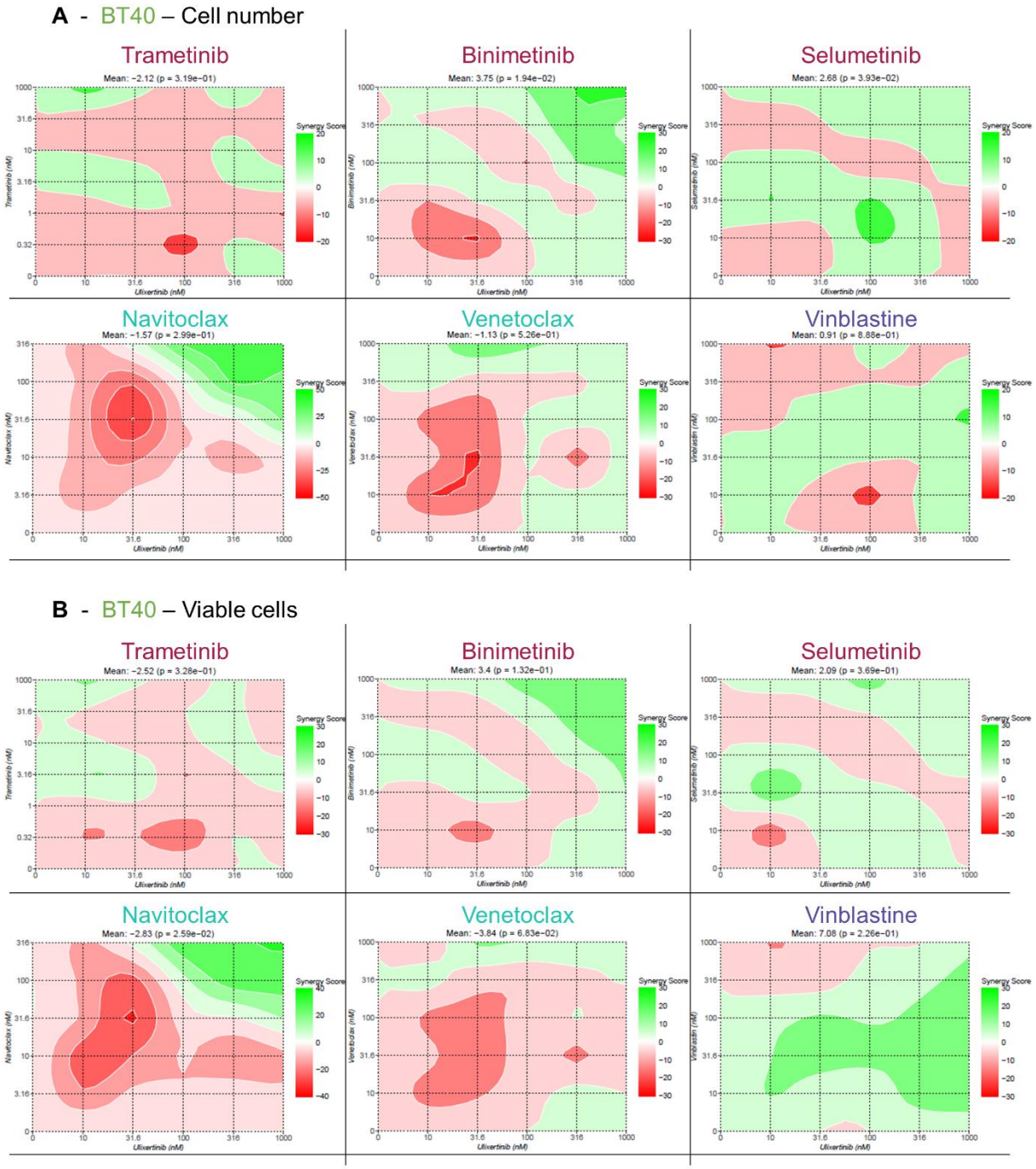
Supplementary Fig. S10



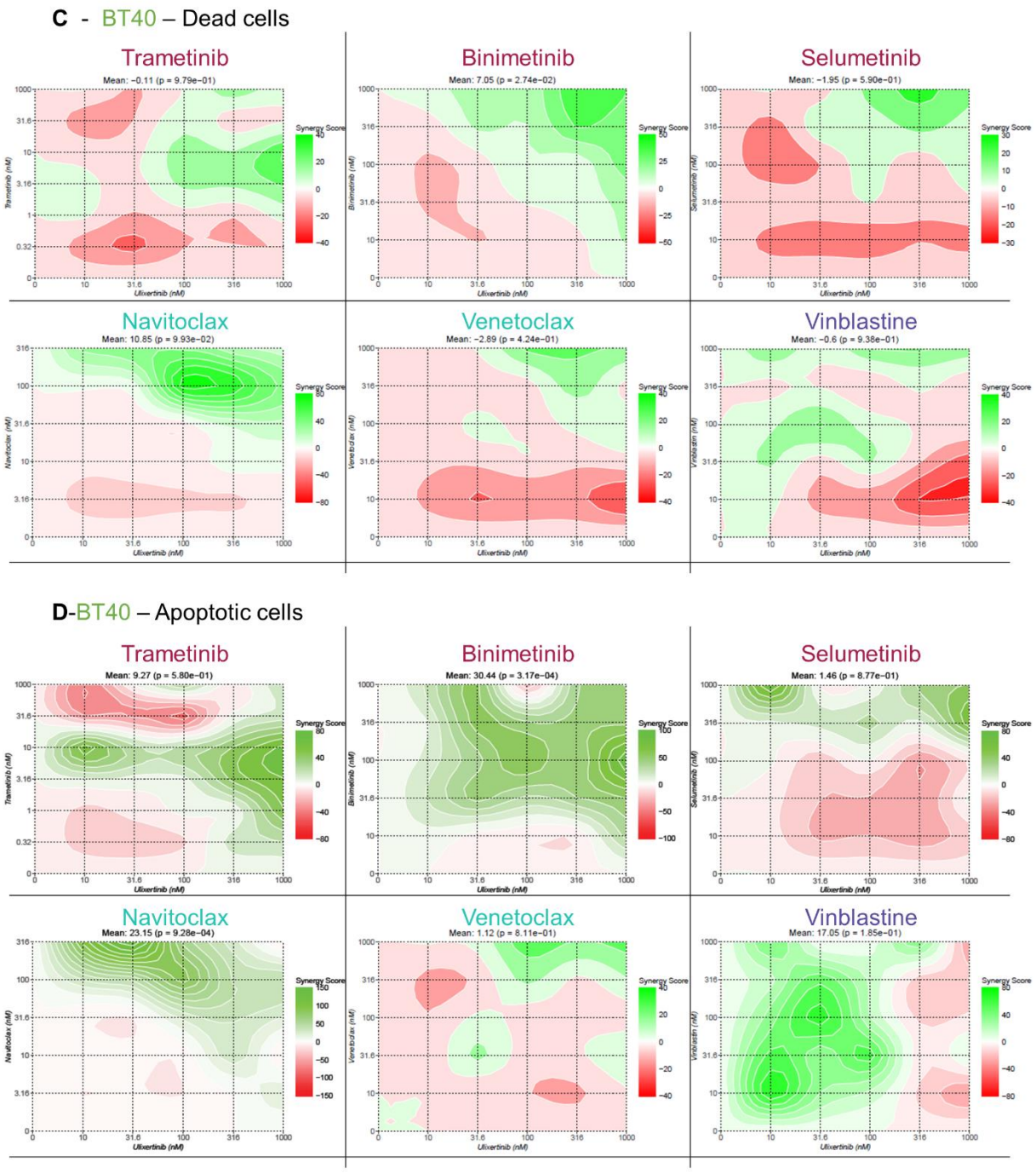
Supplementary Fig. S10: High content microscope images and analysis in BT40 cells

A, BT40 cells were treated for 72 h. Each drug was combined using concentration ranges following a 1/2 Log distribution and centered around the corresponding drug's metabolic IC_{50} rounded to the closest power of 10 (10 nM or 100 nM, as indicated): 0, 10, 31.6, 100, 316, 1000 nM ulixertinib (Ulix) alone or in combination with 0, 0.31, 1, 3.16, 10, 31.6, 1000 nM trametinib (Tram), 0, 10, 31.6, 100, 316, 1000 nM binimetinib (Bini), 0, 10, 31.6, 100, 316, 1000 nM selumetinib (Selu), 0, 3,16, 10, 31.6, 100, 316 nM navitoclax (Navi), 0, 10, 31.6, 100, 316, 1000 nM venetoclax (Vene) or 0, 10, 31.6, 100, 316, 1000 nM vinblastine (Vinb). A staurosporine (STS) concentration curve (0, 0.1, 1, 10, 100, 1000 nM) was added as death control. The following readouts were evaluated: percent cell number, percent viable cells, percent dead cells (evaluated by fragmented nuclei) and percent apoptotic cells. All BT40 readouts are normalized to DMSO and 1000 nM STS. B, BT40 cells were treated with DMSO, 1000 nM ulixertinib, 316 nM navitoclax and their combination for 72 h. Treatments are indicated by the colored border. The cells were stained with Hoechst33342 (blue), TMRE (red), and CellEvent Caspase3/7 stain (green). The scale bar represents 50 µm. Depicted are data from at least three biological replicates.

Supplementary Fig. S11



Supplementary Fig. S11

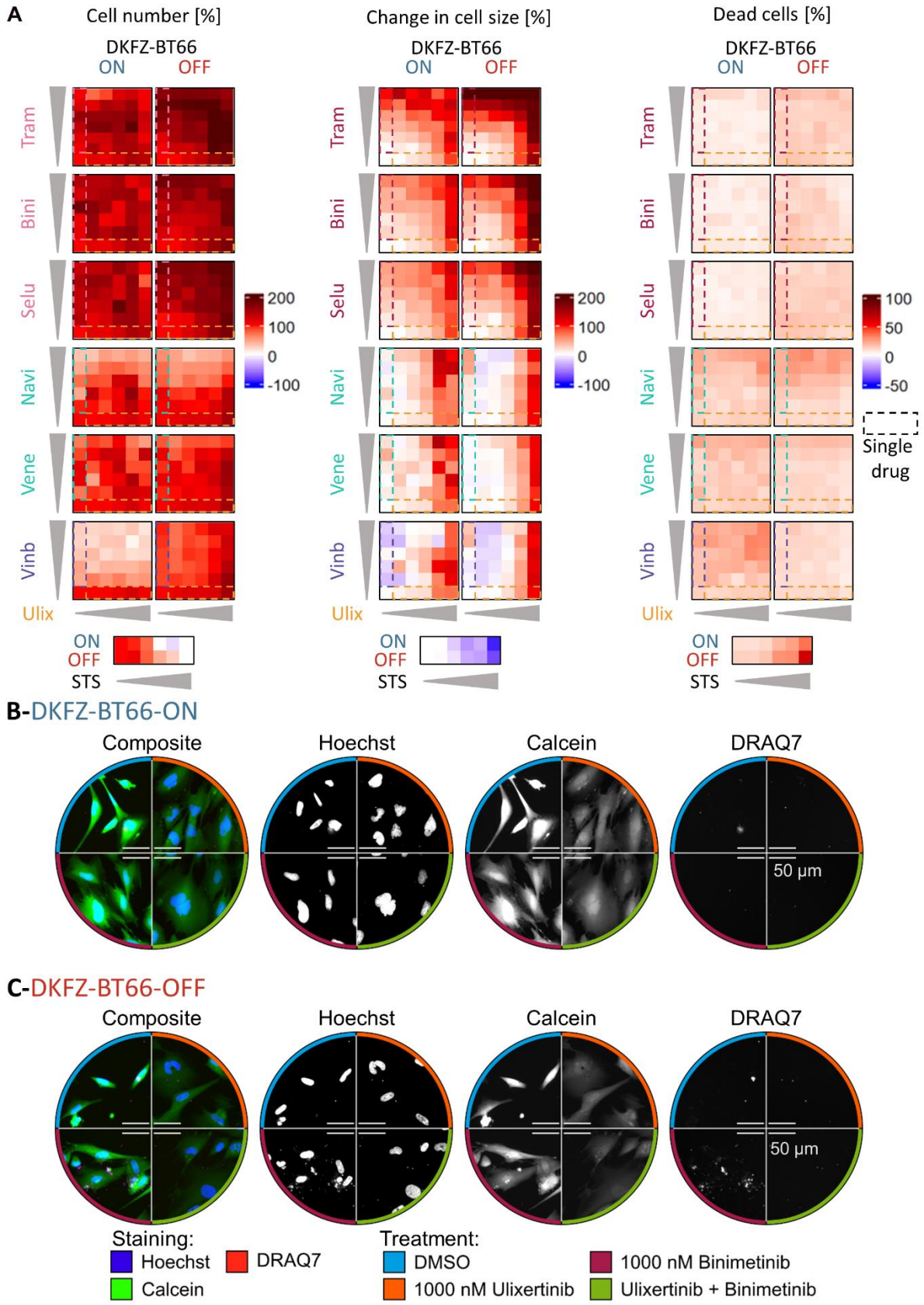


Supplementary Fig. S11: Synergy analysis of high content microscopy data in BT40 cells

Synergy analysis was calculated with www.synergyfinder.org using the data presented in Supplementary Fig. S10A. Synergy models used are described in the

372 methods section. The following readouts were evaluated: (A) Cell number, (B) viable
373 cells, (C) dead cells (evaluated by fragmented nuclei), and (D) apoptotic cells.

Supplementary Fig. S12

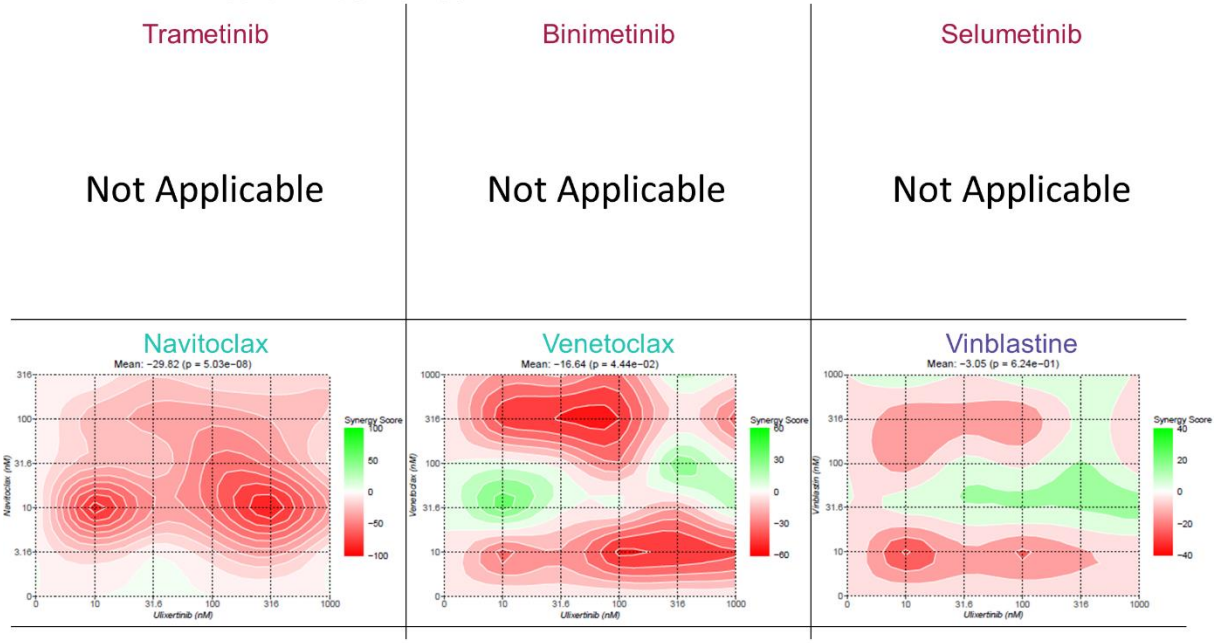


Supplementary Fig. S12: High content microscope images and analysis in DKFZ-BT66 cells

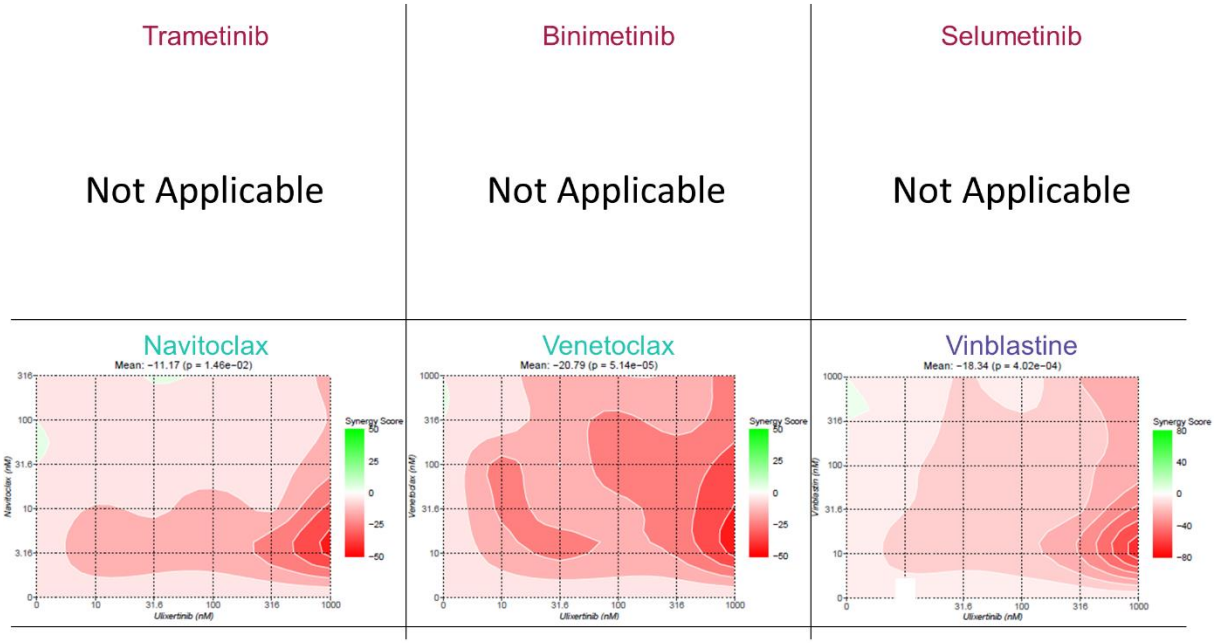
A, DKFZ-BT66_ON and DKFZ-BT66_OFF cells were treated for 72 h. Each drug was combined using concentration ranges following a 1/2 Log distribution and centered around the corresponding drug's metabolic IC_{50} rounded to the closest power of 10 (10 nM or 100 nM, as indicated): 0, 10, 31.6, 100, 316, 1000 nM ulixertinib (Ulix) alone or in combination with 0, 0.31, 1, 3.16, 10, 31.6, 1000 nM trametinib (Tram), 0, 10, 31.6, 100, 316, 1000 nM binimetinib (Bini), 0, 10, 31.6, 100, 316, 1000 nM selumetinib (Selu), 0, 3,16, 10, 31.6, 100, 316 nM navitoclax (Navi), 0, 10, 31.6, 100, 316, 1000 nM venetoclax (Vene) or 0, 10, 31.6, 100, 316, 1000 nM vinblastine (Vinb). A staurosporine (STS) concentration curve (0, 0.1, 1, 10, 100, 1000 nM) was added as death control. For DKFZ-BT66 the readouts used were in percent: cell number (normalized to DMSO and 1000 nM staurosporine), change in cell size (normalized to DMSO), and dead cells (evaluated as percent of DRAQ7 positive nuclei). B-C, DKFZ-BT66 were treated for with DMSO, 1000 nM ulixertinib, 1000 nM binimetinib, and their combination for 72 h. Treatments are indicated by the colored border. The cells were stained with Hoechst33342 (blue), calceinAM (green), and DRAQ7 (red). The scale bar represents 50 µm. Depicted are data from at least three biological replicates.

Supplementary Fig. S13

A - DKFZ-BT66-ON – Cell number

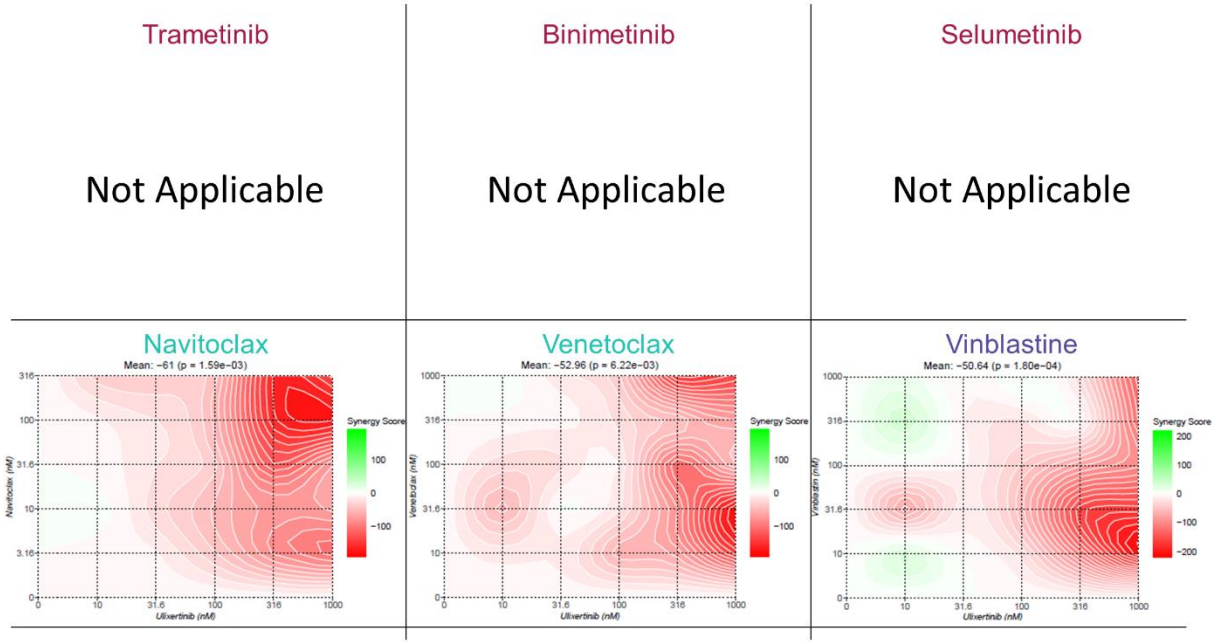


B - DKFZ-BT66-OFF – Cell number

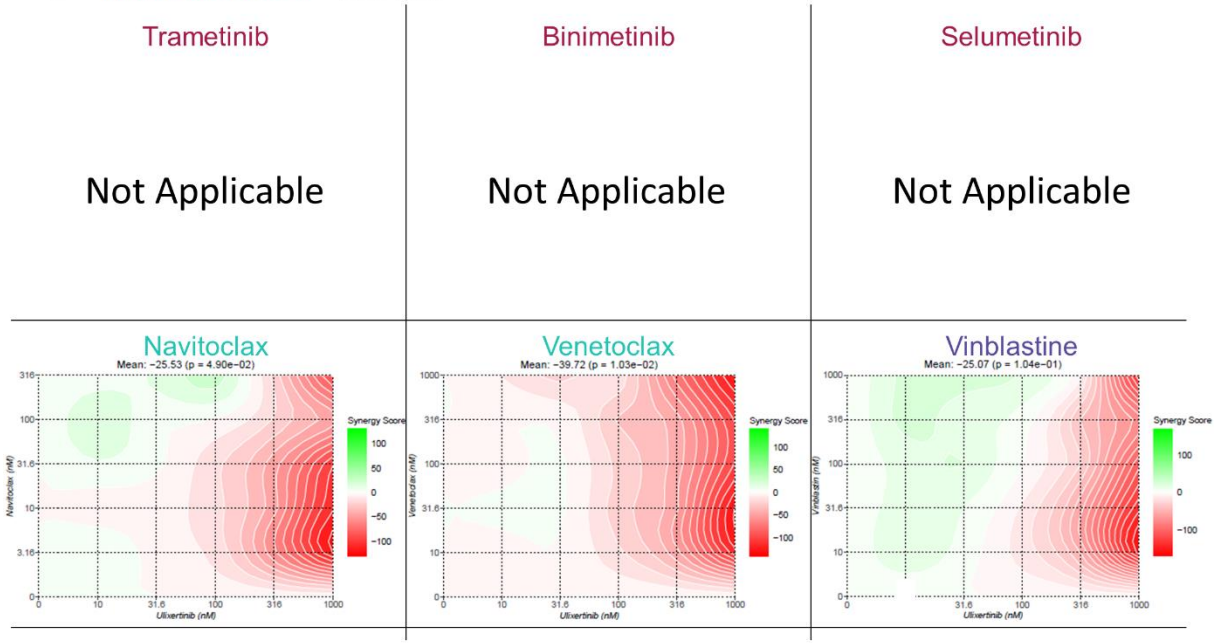


Supplementary Fig. S13

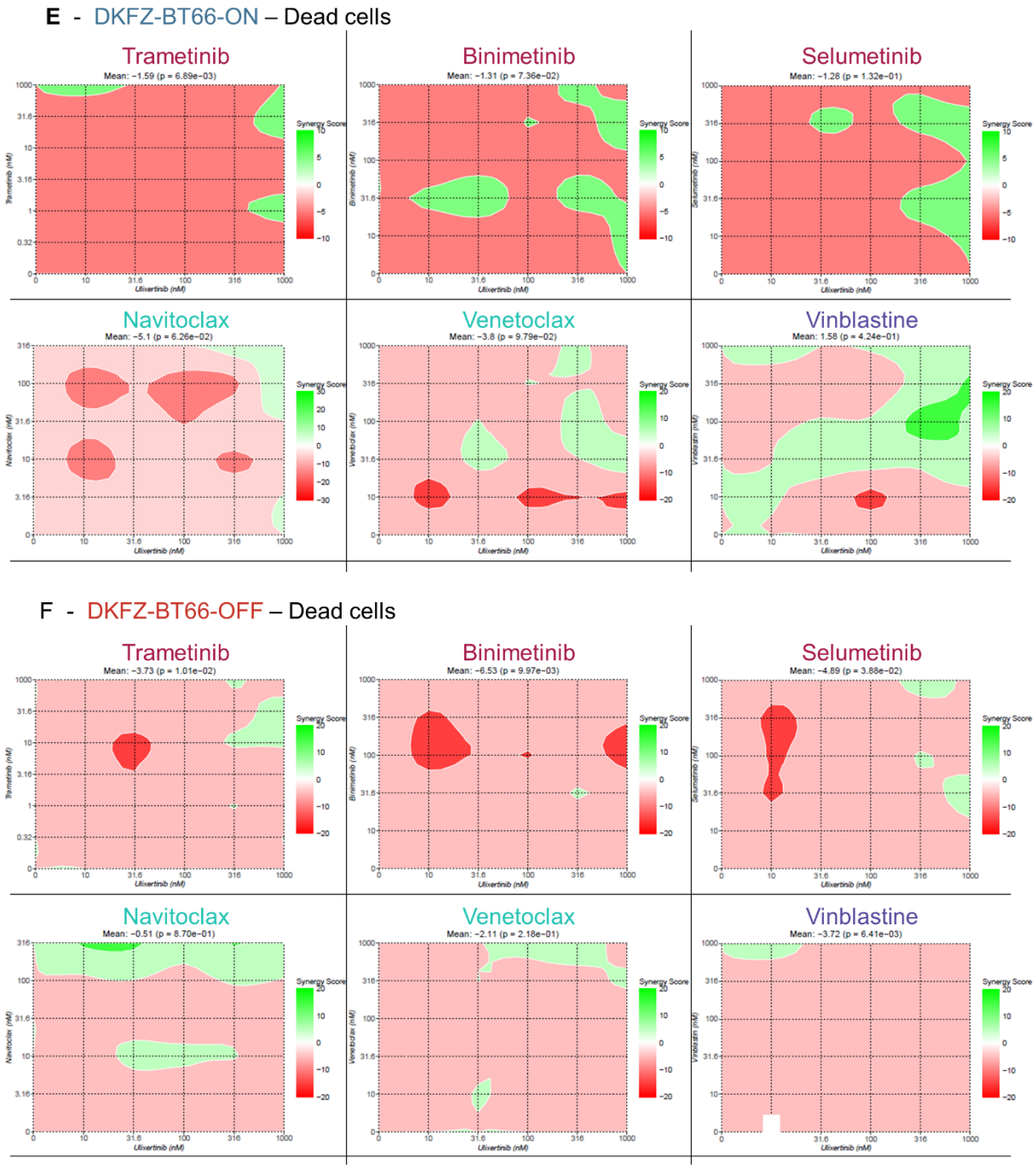
C - DKFZ-BT66-ON – Cell size



D - DKFZ-BT66-OFF – Cell size



Supplementary Fig. S13

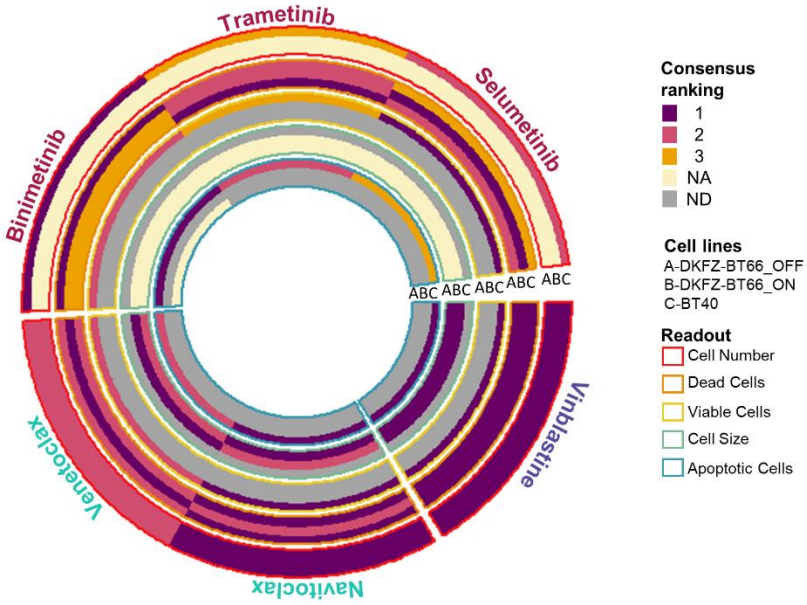


Supplementary Fig. S13: Synergy analysis of high content microscopy data in DKFZ-BT66 cells

Synergy analysis was calculated with www.synergyfinder.org using the data presented in Supplementary Fig. S12A. Synergy models used are described in the methods section. The readouts used were: (A-B) cell number, (C-D) change in cell

size, and (E-F) dead cells (evaluated as percent of DRAQ7 positive nuclei). Synergy scores for cell number and cell size could not be calculated for trametinib, binimetinib, and selumetinib in both DKFZ-BT66 cell lines.

Supplementary Fig. S14



405

406 **Supplementary Fig. S14: Consensus ranking of combination partners in**

407 **the high content microscopy *in vitro* screen**

408 Circular heatmap summarizing the individual drug's performance per drug class,

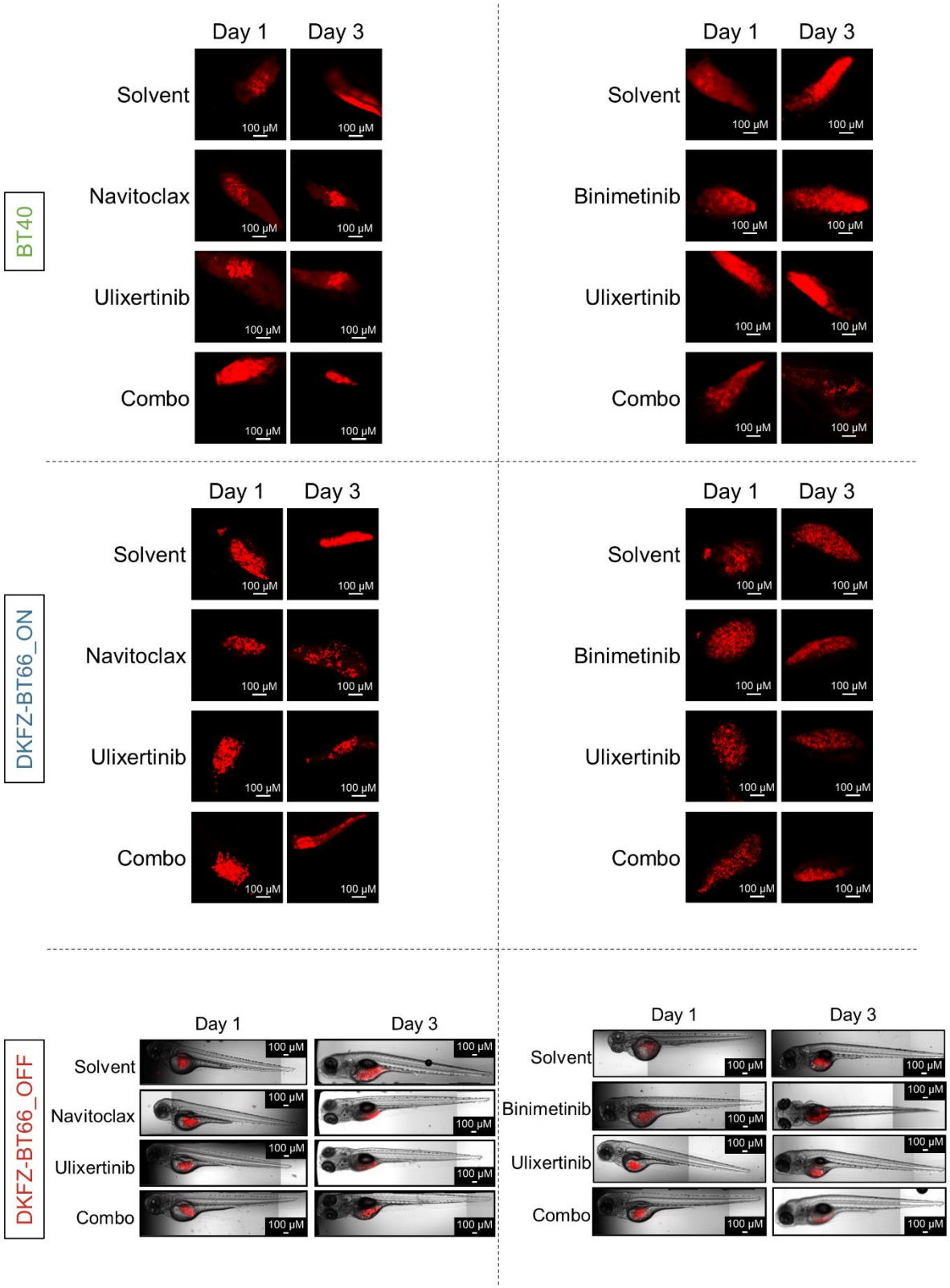
409 following consensus ranking across synergy metrics. The ranking was calculated for

410 the DKFZ-BT66_OFF, DKFZ-BT66_ON, and BT40 models (respectively designated

411 by A, B, and C) and for each HCM readout (from outermost rings to innermost rings:

412 cell number, dead cells, viable cells, cell size, and apoptotic cells).

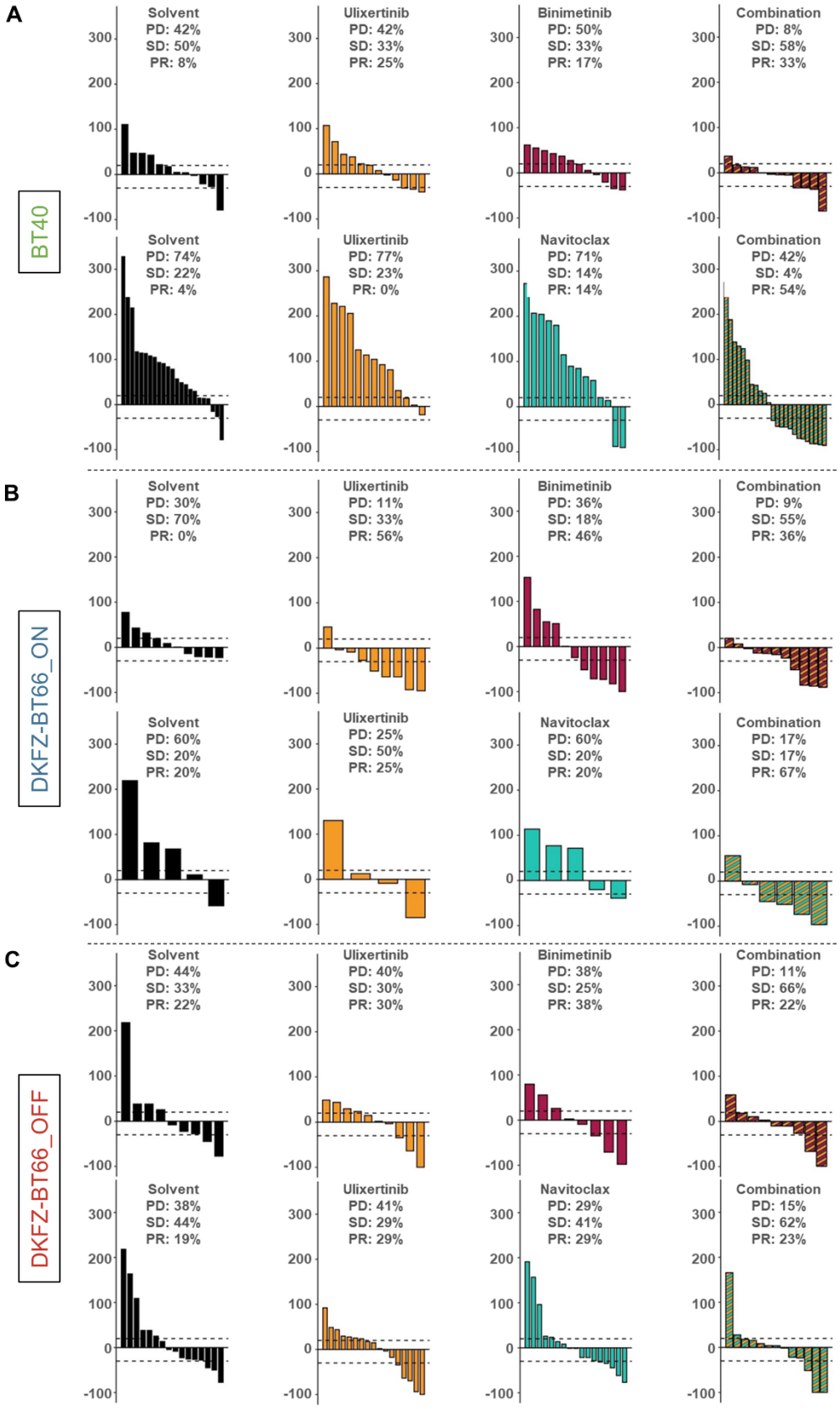
Supplementary Fig. S15



Supplementary Fig. S15: pLGG cell lines transplantation in zebrqfish embryo *in vivo*

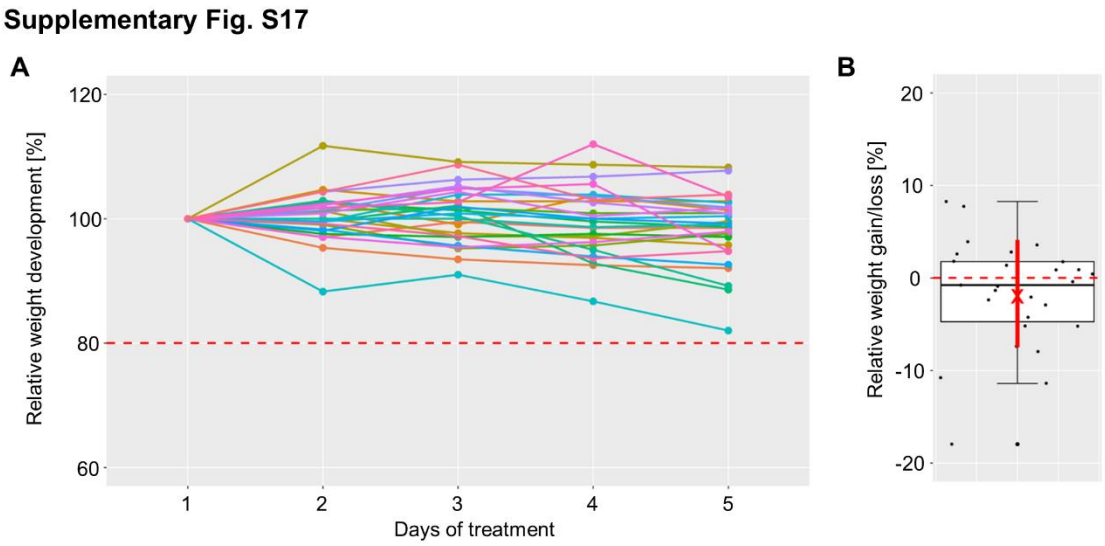
Exemplary pictures of pLGG cell line transplantation in zebrafish embryos. Are shown picture from one representative embryo transplanted with BT40, BT66_ON, or DKFZ-BT66_OFF, after 1 day (baseline) and 3 days (endpoint) of treatment as indicated. The scale bar represents 100 µm.

Supplementary Fig. S16



Supplementary Fig. S16: Zebrafish embryo validation of ulixertinib's combination partners

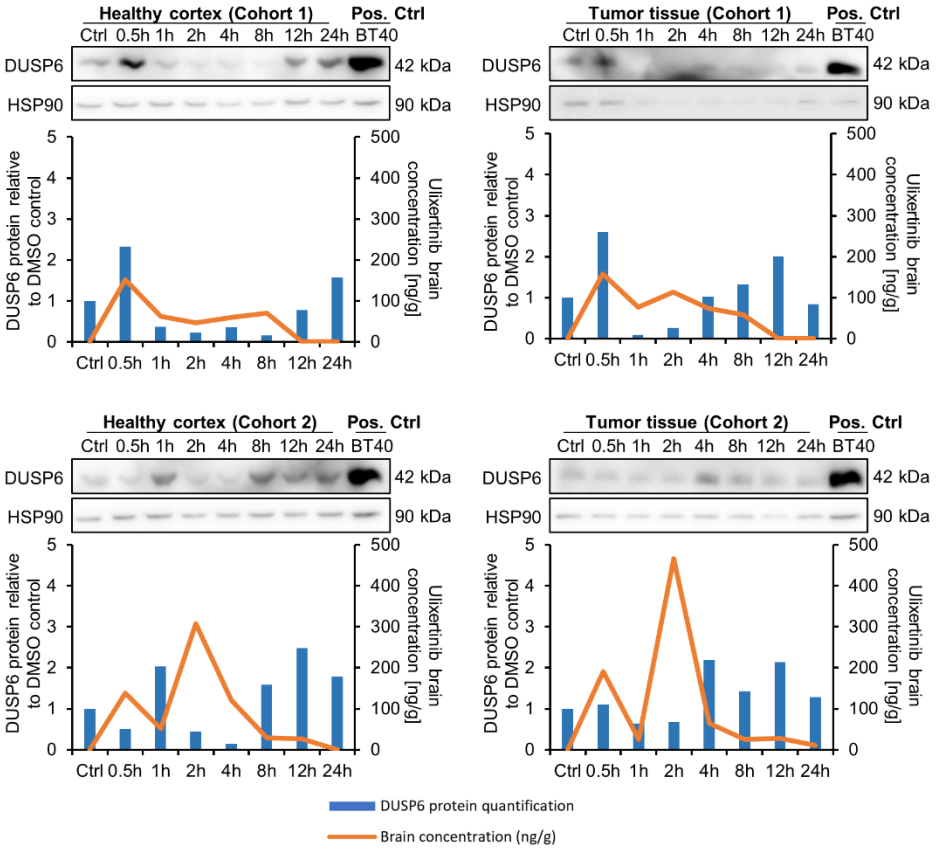
Waterfall plots showing the change from baseline (in %) for tumor volume upon treatment for each individual tested zebrafish embryo with A) BT40, (B) DKFZ-BT66_ON, or (C) DKFZ-BT66_OFF xenografts. Drugs were applied to the embryo-surrounding buffer solution in the following concentrations: ulixertinib (1 µM), binimetinib (2 µM), and navitoclax (5 µM). PD, progressive disease; SD, stable disease; PR, partial response. n= 4 – 23 embryos/group.



Supplementary Fig. S17: Relative weight development as surrogate for in vivo toxicity during ulixertinib treatment in the pharmacokinetic study.

A, BT40-PDX mice were treated with ulixertinib (p.o. 80 mg/kg, 2x/day for five days, n=28) and relative weight development was monitored for the treatment period. Different colors represent individual mice. Exclusion criterion of a maximal weight loss of 20% is indicated as red line. B, Relative weight development was plotted after calculating the average difference of animal's weight before the dosing and at the end of treatment. Initial weight is indicated by the red dashed line. Mean (red cross) ± S.D. (red line) are highlighted.

Supplementary Fig. S18



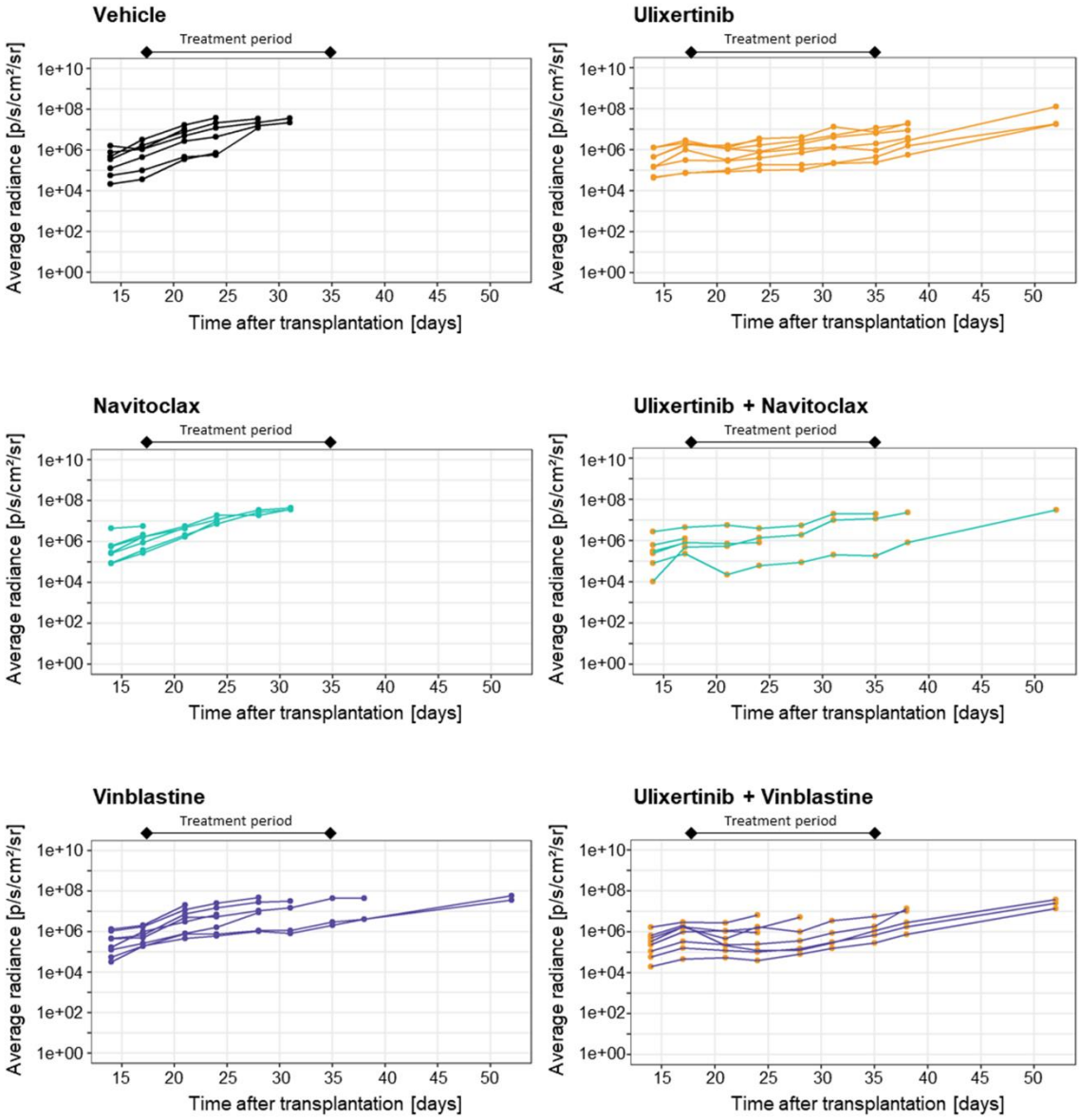
441

442 **Supplementary Fig. S18: Ulixertinib on-target activity in vivo.**

443 Western blot analysis of the DUSP6 and HSP90 proteins in tissue samples from both
444 cohorts and both tissues (healthy and tumor). Depicted blots represent data from a
445 single mouse per time point. Western blot quantification is shown (blue bars),
446 depicting DUSP6 signal normalized to its corresponding HSP90, and normalized to
447 the DMSO control.

448

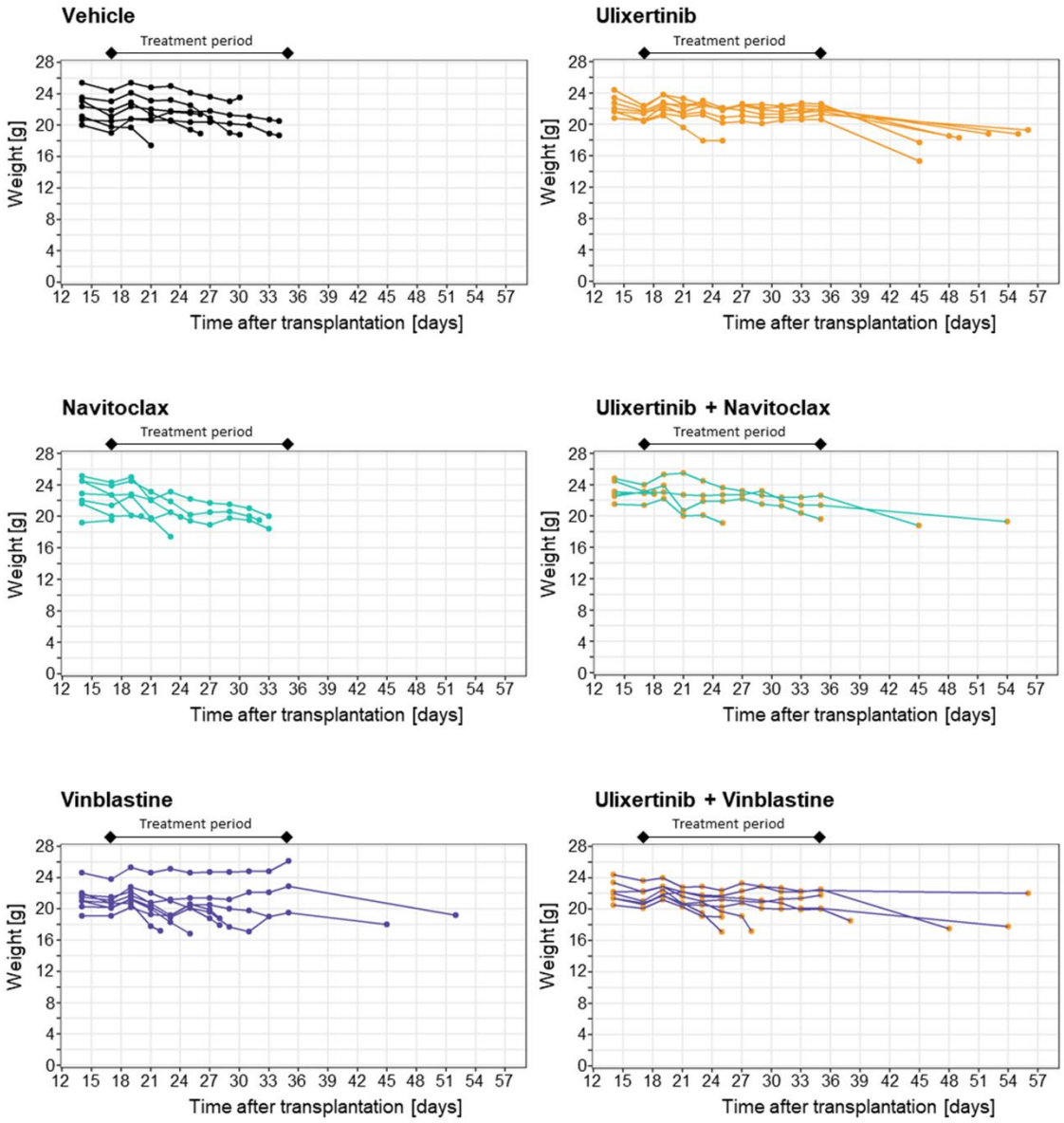
Supplementary Fig. S19



Supplementary Fig. S19: Bioluminescence signals representing tumor volumes in PDX mice

Tumor volumes according to bioluminescence measurements from individual animals during treatments as indicated (n=8 mice per group). Luciferase signal were measured in photons/sec/cm²/steradian over the entire treatment- and observation-period. Ultimately censored animals are not depicted.

Supplementary Fig. S20



456

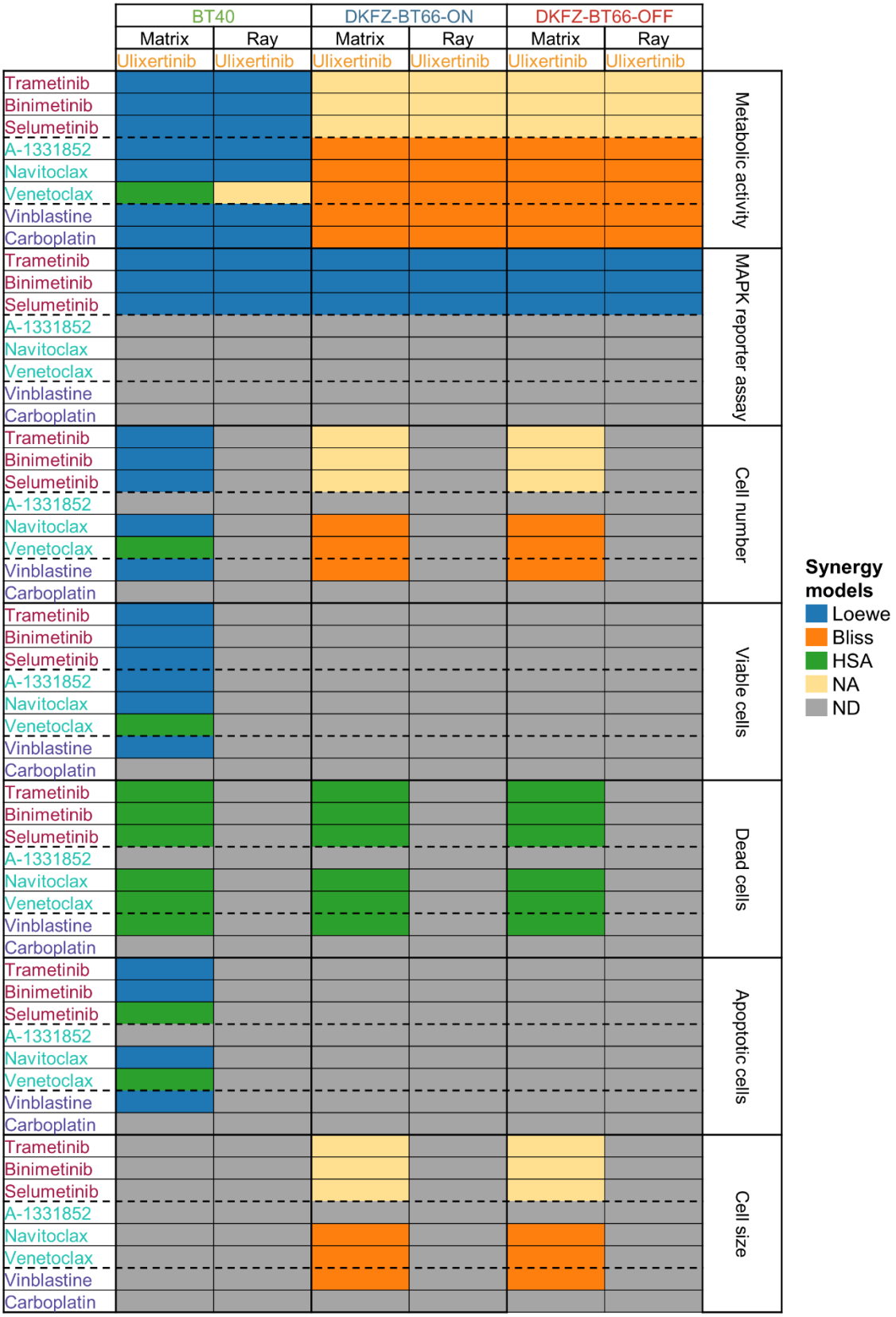
457 **Supplementary Fig. S20: Mouse weight development during the**

458 **preclinical *in vivo* study**

459 Weights of animals clustered by treatment as indicated (n=8 mice per group).

460 Ultimately censored animals are not depicted.

Supplementary Fig. S21



Supplementary Fig. S21: Summary of the synergy models used *in vitro*

Summary of all synergy models (Loewe, Bliss, Highest single agent HSA) used per cell line, per combination, per readout, in the *in vitro* screen and in the high content microscopy validation. NA, not applicable due to readout inconsistency. ND, not determined.

Supplementary Tables

Supplementary Table S1: Seeding densities for in vitro assays

Cell line	Experiment type	Plate type	Seeding densities (cells/well)
BT40	Metabolic activity measurement	384-well	300
DKFZ-BT66_ON	Metabolic activity measurement	384-well	300
DKFZ-BT66_OFF	Metabolic activity measurement	384-well	4 800
BT40-pDIPZ	MAPK reporter activity measurment	384-well	3 000
DKFZ-BT66_ON-pDIPZ	MAPK reporter activity measurment	384-well	1 500
DKFZ-BT66_OFF-pDIPZ	MAPK reporter activity measurment	384-well	6 000
BT40	High-content microscopy measurments	384-well	500
DKFZ-BT66_ON	High-content microscopy measurments	384-well	1 000
DKFZ-BT66_OFF	High-content microscopy measurments	384-well	8 000
BT40	On-target activity measurement (WB/RPPA)	6-well	500 000
DKFZ-BT66_ON	On-target activity measurement (WB/RPPA)	6-well	500 000
DKFZ-BT66_OFF	On-target activity measurment (WB/RPPA)	6-well	1 000 000
BT40	On-target activity measurement (IP)	10 cm dish	5 000 000
DKFZ-BT66_ON	On-target activity measurement (IP)	10 cm dish	3 000 000
DKFZ-BT66_OFF	On-target activity measurment (IP)	10 cm dish	5 000 000

Supplementary Table S2: Markers conditions for high content microscopy analysis

Cell line	Marker	Supplier	Cat. No.	Concentration
BT40	Hoechst33342	ThermoFisher Scientific	H3570	10 µg/ml
BT40	TMRE	Abcam	ab113852	1 µM
BT40	CellEvent Caspase3/7	ThermoFisher Scientific	C10723	8 µM
DKFZ-BT66	Hoechst33342	ThermoFisher Scientific	H3570	10 µg/ml
DKFZ-BT66	CalceinAM	ThermoFisher Scientific	65-0853-39	1.25 µM
DKFZ-BT66	DRAQ7	ThermoFisher Scientific	D15106	0.6 µM

476 **Supplementary Table S3: In vitro synergy screens datasets**

477 (See Excel file)

478 **Supplementary Table S4: Antibodies used for WB, IP and RPPA analyses**

Antibody	Species	Clonality	Supplier	Cat. No.	Dilution	Application
DUSP6	Rabbit	Poly-clonal	Cell Signaling	50945	1:1000	*In vitro* validation
DUSP6	Mouse	Mono-clonal	Santa Cruz	sc-377070	1:1000	*In vivo* validation
GAPDH	Mouse	Mono-clonal	Merck	MAB374	1:1000	*In vitro* & *in vivo* validation
BCL-XL	Rabbit	Mono-clonal	Abcam	ab32370	5µg	Immuno-precipitation
BAK	Rabbit	Mono-clonal	Abcam	ab32371	1:1000	Immuno-precipitation

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481 **Supplementary Table S5: Antibodies used for RPPA and RPPA analyses**

Protein name	MW (kDa)	Function	Cascade position
MEK1/2 S217.S221	45	MEK1/2 is activated by a wide variety of growth factors and cytokines and also by membrane depolarization and calcium influx *S298 phosphorylated by PAK1, facilitates signal transduction from Raf to MEK1 and Erk2 *T386 ERK-mediated phosphorylation, interferes with PAK phosphorylation of MEK1 *S217/S221 phosphorylation and activation by RAF	Upstream activator
ERK1/2 T202.Y204	42, 44	Kinases involved in proliferation, differentiation, motility, and death => phosphorylated and activated by MEK1/1	Target
p90RSK T359.S363	90	Widely expressed Ser/Thr kinases, activated by MAPK pathway *T359/S363 phosphorylated by ERK1/2 and ERK5	Cytosolic target
p90RSK S380	90	Widely expressed Ser/Thr kinases, activated by MAPK pathway *S380 docking site for the constitutively active Ser/Thr kinase PDK1, which in turn phosphorylates p90RSK at Ser221 within the N-terminal kinase domain activation loop, resulting in full enzymatic activation of p90RSK	Cytosolic target
RSK3 T356.S360	90	Widely expressed Ser/Thr kinases, activated by MAPK pathway	Cytosolic target
STAT3 Y705	79,86	Signaling molecule for many cytokines and growth factor receptors; activated in a number of human tumors and possesses oncogenic potential and anti-apoptotic activities *Y727 regulates transcriptional activity through MAPK or mTOR pathways	Cytosolic target
Bim S69	26	Pro-apoptotic protein belonging to the BH3-only group of Bcl-2 family; induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family	Cytosolic target
MSK1 S360	90	Mitogen and stress activated protein kinase => phosphorylation and activation by Erk, p38 MAPK in response to growth factors and cellular stress, respectively	Nuclear target
Elk-1 S383	62	Transcription factor => direct target of the MAPK pathway, phosphorylated and activated by ERK1/2	Nuclear target

CREB S133	43	Transcription factor that activates target genes through cAMP response elements, promoting neuronal survival, precursor proliferation, neurite outgrowth, and neuronal differentiation => phosphorylated and activated by Erk, p90RSK, MSK, CaMKIV, and MAPKAPK-2	Nuclear target
c-Myc S62	62	Transcriptional regulators; proliferation, differentiation and apoptosis => Phosphorylation controls proteasomal-dependent degradation. Mitogens, mitosis, or cellular stress induce phosphorylation at Ser62, which serves as a priming site for GSK-3 phosphorylation of Thr58	Nuclear target
DUSP6	44	Downstream effectors of Erk-MAPK pathways; invovled in Erk negative feedback loop	Downstream target
Beta actin	45	Loading control	Loading control

482

483

Supplementary Table S6: Clinically achievable concentrations, metabolic and MAPK activity IC50, zebrafish embryos tested concentrations, and MTDs

	Clinical data		BT40		DKFZ-BT66_ON		DKFZ-BT66_OFF		Zebrafish embryos		
	Cmax (nM)	**Ctrough (nM)**	**Metabolic IC50 (nM)**	**MAPK IC50 (nM)**	**Metabolic IC50 (nM)**	**MAPK IC50 (nM)**	**Metabolic IC50 (nM)**	**MAPK IC50 (nM)**	**Concentration range tested (nM)**	**MTD (nM)**	**LD (nM)**
Ulixertinib 6,**adult**	4 892.3	3 461.6	62.7	12.7	*NA*	8.5	*NA*	8.0	1 - 50 000	2 500	25 000
Trametinib 7, **ped.**	19.2	3.6	2.2	0.3	*NA*	2.1	*NA*	0.5	0.1 - 50 000	100	1 000
Selumetinib 8, **ped.**	1 296.0	ND	259.3	6.1	*NA*	164.1	*NA*	13.0	5 - 2 500	> 2 500	> 2 500
Binimetinib 9, **adult**	619.0	136.0	200.2	4.5	*NA*	88.0	*NA*	9.2	5 - 5 000	> 5 000	> 5 000
A-1331852	*ND*	*ND*	112.3	*NA*	7.4	*NA*	1.6	*NA*	*ND*	*ND*	*ND*
Navitoclax 10,**adult+ped**	994.2	400.0	462.2	*NA*	324.9	*NA*	37.2	*NA*	1 - 50 000	10 000	50 000
Venetoclax 10,**adult+ped**	1 612.1	725.4	*NA*	*NA*	2 991.0	*NA*	2 460.0	*NA*	0.1 - 10 000	10 000	>10 000
Vinblastine 11,**ped**	30.8	*ND*	2.9	*NA*	5.2	*NA*	6 591.0	*NA*	5 - 65 000	5 000	25 000 000*
Carboplatin 12,**ped**	58 989.9	*ND*	62 593.0	*NA*	88 652.0	*NA*	97 169.0	*NA*	*ND*	*ND*	*ND*

NA: Not applicable
ND: Not determined

* slight toxicity, not lethal

Supplementary Table S7: Navitoclax concentrations *in vivo* in PDX-BT40 tumor tissue as measured by UPLC-MS/MS

Animal ID	Treatment group	Navitoclax concentration [ng/g]
#1	Navitoclax monotherapy	3930
#3	Navitoclax monotherapy	232
#4	Navitoclax monotherapy	99
#5	Navitoclax monotherapy	1980
#6	Navitoclax monotherapy	1910
#7	Navitoclax monotherapy	118
#9	Ulixertinib + navitoclax combination	1960
#10	Ulixertinib + navitoclax combination	146
#11	Ulixertinib + navitoclax combination	111
#13	Ulixertinib + navitoclax combination	191
#15	Ulixertinib + navitoclax combination	3.7
#16	Ulixertinib + navitoclax combination	25

In green are concentrations higher than the effective concentration; in red concentrations lower than the effective concentration (effective concentration: *in vitro* metabolic activity IC_{50} in BT40 = 462.2 nM = 0.45 µg/mL).

494 **Supplementary Table S8: Drugs conditions for *in vitro* assays**

Drug name	Supplier	Cat. No.	Stock concentration (mM)	Class	Solvent	Storage
Ulixertinib	BioMed Valley Discoveries	NA	50	ERK inhibitor	DMSO	-80°C
Trametinib	Selleckchem	S2673	10	MEK inhibitor	DMSO	-80°C
Selumetinib	Selleckchem	S1008	10	MEK inhibitor	DMSO	-80°C
Binimetinib	Selleckchem	S7007	10	MEK inhibitor	DMSO	-80°C
Navitoclax	Selleckchem	S1001	50	Senolytic	DMSO	-80°C
Venetoclax	Selleckchem	S8048	10	Senolytic	DMSO	-80°C
A-1331852	Selleckchem	S7801	10	Senolytic	DMSO	-80°C
Vinblastine	MedChemExpress	HY-13780	10	SOC chemotherapy	DMSO	Room temperature under nitrogen atmosphere
Carboplatin	Pharmacy of the university hospital of Heidelberg	NA	25	SOC chemotherapy	Saline solution	4°C (away from light)
Staurosporine	TargetMol	T16680	10	Protein kinase inhibitor (death control)	DMSO	-80°C

495

Supplementary Table S9: CellProlifer pipelines for high content microscopy analysis

(See Excel file)

Supplementary Table S10: Drugs and solvents used in BT40-PDX mouse model in vivo

Drugs	Concentration (mg/kg)	Solvent	Cat. No.	Supplier
Ulixertinib	75-80	1% CMC	NA	BioMed Valley Discoveries
Navitoclax[13]	100	10% Ethanol 30% Polyethylen-glycol 400 60% Phosal 50	201970	MedKoo Biosciences
Vinblastine[14]	0.5	0.9% NaCl	S4505	Selleckchem

Solvents	Cat. No.	Supplier
Carboxymethylcellulose (CMC sodium - medium viscosity)	C4888	Sigma-Aldrich
Ethanol	32205	Sigma-Aldrich
Polyethylenglycol 400	0144.1	Carl Roth
Phosal 50	368315	Lipoid
NaCl	2350748	B.Braun

Supplementary Table S11: Optimized MS/MS parameters for the detection of ulixertinib using heated electrospray ionization and selected reaction monitoring in the positive ion mode

Parameter	Value
Capillary voltage [kV]	1
Cone voltage [V]	50
Source temperature [°C]	150
Desolvation temperature [°C]	500
Cone gas (N2) flow [L/h]	150
Desolvation gas (N2) flow [L/h]	1000
Ulixertinib mass transition [*m/z*]	433.2 à 262.1
D7-ulixertinib mass transition [*m/z*]	439.2 à 268.1
Collision gas (Ar) flow [mL/min]	0.15
Collision energy [V]	16

Supplementary Table S12: Optimized MS/MS parameters for the detection of navitoclax using heated electrospray ionization and selected reaction monitoring in the positive ion mode

Parameter	Value
Capillary voltage [kV]	1.2
Cone voltage [V]	20
Source temperature [°C]	150
Desolvation temperature [°C]	600
Cone gas (N2) flow [L/h]	150
Desolvation gas (N2) flow [L/h]	1000
Navitoclax mass transition [*m/z*]	487.5 à 742.0
Venetoclax mass transition [*m/z*]	434.8 à 636.2
Collision gas (Ar) flow [mL/min]	0.15
Collision energy [V]	14

Supplementary References

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