

Supplementary Information

Integrating phenotypic search and phosphoproteomic profiling of active kinases for optimization of drug mixtures for RCC treatment

Judy R. van Beijnum¹, Andrea Weiss^{2, 3}, Robert H. Berndsén, Tse J. Wong¹, Louise C. Reckman¹, Sander R. Piersma^{4,5}, Marloes Zoetemelk^{2,3}, Richard de Haas^{1,4,5}, Olivier Dormond⁶, Axel Bex^{7,8}, Alexander A. Henneman^{4,5}, Connie R. Jimenez^{4,5}, Arjan W. Griffioen¹, Patrycja Nowak-Sliwinska^{2,3*}

¹Angiogenesis Laboratory, Department of Medical Oncology, Amsterdam UMC, Vrije Universiteit Amsterdam, Medical Oncology, Cancer Center Amsterdam, De Boelelaan 1117, Amsterdam, Netherlands;

²Institute of Pharmaceutical Sciences of Western Switzerland, Faculty of Sciences, University of Geneva, Geneva, Switzerland; ³Translational Research Centre in Oncohaematology, Geneva, Switzerland;

⁴Department of Medical Oncology, Amsterdam UMC, Vrije Universiteit Amsterdam, Medical Oncology, Cancer Center Amsterdam, De Boelelaan 1117, Amsterdam, Netherlands; ⁵OncoProteomics Laboratory, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; ⁶Department of Visceral surgery, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland; ⁷Royal Free London NHS Foundation Trust, Renal Cancer Centre, UCL Division of Surgical and Interventional Science, London, UK; ⁸Netherlands Cancer Institute, Amsterdam, The Netherlands.

* Corresponding author Patrycja.Nowak-Sliwinska@unige.ch

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Drug acquisition

Ten initial drugs were selected for inclusion in the experimental steps of s-FSC that cover a broad spectrum of pathways involved in RCC development. Axitinib (targeting VEGFRs, PDGFRs and c-KIT) [1] and erlotinib (targeting EGFR) [2] were purchased from LC laboratories (Woburn, MA, USA). RAPTA-C was synthesized and purified as described previously [3]. BEZ-235 (targeting mTORC1 and mTORC2) [4] was purchased from Chemdea LLC (Ridgewood, USA). Volasertib (targeting polo-like kinase) [5], tozasertib (targeting Aurora A, B and C) [6], U-104 (targeting carbonic anhydrase IX and XII) [7,8], AZD4547 (targeting FGFR1-3) [9] and crenolanib (targeting PDGFRs) [10,11] were purchased from Selleck Chemicals (Houston, TX, USA). Dasatinib (targeting BCR/ABL) [12] was purchased from Fluorochem Ltd. (Derbyshire, UK). Sunitinib and crizotinib were not included in the s-FSC experimental steps. Sunitinib (targeting VEGFRs, PDGFRs and c-Kit) [13] was used as a control for apoptosis induction and purchased from Pfizer Inc. (New York, NY, USA). Crizotinib (targeting MET and PTK2) [14] was used to test if efficacy could be increased when added to ODCs and purchased from Selleck Chemicals. All compounds were dissolved in DMSO and stored at -20°C for short term use or at -80°C for storage up to six months with the exception of BEZ-235 which was dissolved in DMSO and stored at $+4^{\circ}\text{C}$ and RAPTA-C which was freshly dissolved in DMSO prior to each experiment. The maximum DMSO concentration for any combination was controlled in each experiment to verify its lack of activity in cell assays.

Cell culture and maintenance

786-O (human RCC), EC-RF24 (immortalized human vascular endothelial cells) and HDFA (adult human dermal fibroblasts) were maintained in RPMI cell culture medium supplemented with 1% of antibiotics (penicillin/streptomycin, Life Technologies, Carlsbad, California, USA) and 10% bovine calf serum (NBCS) (Sigma-Aldrich, St. Louis, USA). Caki-1, Caki-2, ACHN and A498 (human RCC) were maintained in DMEM supplemented with 1% of antibiotics and 10% NBCS. All cells were obtained from ATCC except for HDFA that was obtained from Cell Applications (San Diego, USA). Cells were routinely tested for the absence of mycoplasma. EC-RF24 cells were immortalized as described previously[15], and available in-house. HUVEC were freshly isolated from umbilical cords and cultured in RPMI supplemented with 10% NBCS and 10% human serum, as described previously[16]. Essential cell line characteristics are presented in **Supplementary Table S1.1**.

For conventional 2D cell metabolic activity assays, 5000 (RCC) or 7500 (HDFA, EC-RF24) cells were plated in standard 96-well flat-bottom culture plates, and allowed to grow O/N. Drugs were added for 72 hours before luminescence readout with CellTiter-Glo® (G7572, Promega) in a BioTek Cytation 3 reader equipped with Gen5 Image software version 3.04.

Data analysis and modeling

A limited number of drug combinations are screened in order to develop second order linear regression models defining the relationship between drug combinations (i.e. drugs and corresponding doses) and the output response of cell viability. Drug combinations to be screened are selected based on a statistical design of experiment approach using and design matrix, specifically an orthogonal array composite design (OACD). This particular design is composed of a two-level factorial or fractional factorial design and a three-level orthogonal array[17,18]. Using these drug combinations and their corresponding efficacy as data points, regression analysis is performed using Matlab® based on a stepwise linear regression model with the following form:

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^k \sum_{j=i+1}^k \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where β_0 , β_i , β_{ii} and β_{ij} are the intercept,

linear, quadratic and bilinear (or interaction) terms, respectively; y is the response variable (i.e. cell metabolic activity, represented as a percentage of the control); x_i and x_j are independent variables (i.e. drug combinations composed of drugs and corresponding drug doses); ε is an error term with a mean equal to zero. Data is modelled using coded concentration values and data was not transformed.

Supplementary table S1.2 states the drug doses (IC_{20}) that were used for each cell line in the s-FSC procedure.

3D spheroid culture

Heterotypic 3D co-cultures were obtained by mixing the RCC cells with HDFA in a ratio of 3:1 (4500 and 1500 cells, respectively), followed by addition of 10% HUVEC (600 cells) per spheroid. Cells were suspended in cell-repellent 96-well round-bottom plates (650970, Greiner Bio-One) in 100ul medium containing 2.5% Matrigel (354230, Corning). Plates were centrifuged for 5 minutes at 400g to collect the cells at the bottom to initiate spheroid formation. Where indicated, monoculture spheroids consisting of solely RCC were produced with 6000 cells. After 24 hours, treatment was started for 72 hours.

Cell cycle analysis and apoptosis

Cell cycle distribution was assessed based on flow cytometry analysis of cellular DNA content as previously described[19]. RCC and EC-RF24 cells were seeded in a 24-well plate at $20-40 \times 10^3$ cells/well and after 24 h medium was replaced with drug containing medium. After 72 h incubation, cells were harvested and fixed in 70% ethanol for 2 h at -20 °C. Cell pellets were resuspended in DNA extraction buffer (90 parts 0.05 M Na_2HPO_4 , 10 parts 0.025 M citric acid and 1 part 10% Triton-X100, pH 7.4) and incubated for 20 min at 37 °C. Propidium iodide (PI, 20 µg/ml) was added and analysis was performed on a FACS Calibur flow cytometer (BD Biosciences) in the FL2 channel. DNA content was quantified with CellQuest Pro software (BD Biosciences). DNA content was defined as 4N (G2/M phase), 2N (G1/G0) or in between (S), and apoptotic cells were defined by subG1 DNA staining.

Western blot

Cells were cultured to near confluence before treatment with drugs for 2 or 24 hours. Cells were lysed in lysis buffer containing protease and phosphatase inhibitors as previously described[20]. Protein concentrations were evaluated in lysates using Bradford assay or micro BCA protein assay (Thermo Fischer Scientific, Waltham, MA, USA). Fifteen to fifty µg of proteins per condition was separated on 4-12% polyacrylamide gels (Invitrogen, Waltham, MA, USA) and transferred to a polyvinylidene difluoride membrane. Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) was used to block membranes and following incubation with primary and infrared secondary antibodies, bands from immunoreactive proteins were visualized by an Odyssey infrared imaging system. Images were obtained with the Licor Odyssey CLx scanner at one default exposure setting. Alternatively, secondary antibodies were HRP-labeled and detected using chemiluminescence (SuperSignal West Pico Plus; ThermoFisher Scientific), and imaged using a Uvitec Alliance chemiluminescence imaging system. Quantification of band intensity was performed with ImageJ.

The antibodies used: pAKT (Ser473, 4060, Cell Signaling Technology [CST]), AKT (2920, CST), pMAPK or pERK1/2 (Thr202/Tyr204, 9101, CST), MAPK or ERK1/2 (4696, CST), S6 Ribosomal Protein (2217, CST) (Phospho-S6 Ribosomal Protein (Ser235/236, 4858, CST or M3500, Spring Bioscience Corporation), EPHA2 (6697, CST), pEPHA2 (Tyr588, 12677, CST), MET (8198, CST), pMET (Tyr1234/1235, 3077, CST) and actin (A2228, Sigma Aldrich). Antibody dilutions were according to the manufacturers' instructions.

Caki-1 subcutaneous tumor xenografts in Swiss nu/nu mice

Experiments were conducted according to licence GE-2-17. Briefly, male and female Swiss nu/nu mice at age 6-8 weeks (Charles River, Ecully, France) received an injection of 5×10^6 Caki-1 cells suspended in 100 µL DMEM and 1% FCS in the left flank. Treatment with CTRL, drug combinations or single drugs was started on day 4 when palpable tumor formation could be confirmed. Erlotinib was freshly dissolved in 0.5% methylcellulose (Sigma-Aldrich)/0.1% Tween-80 in sterile H₂O. Dasatinib and AZD-4547 were freshly dissolved first in 30% PEG300 (Sigma-Aldrich)/5% Tween-80 followed by addition

of sterile H₂O to 100%. Drug concentration in mg/kg was based on average mice weight and treatment consisted of 100 µL/mouse by oral gavage. Mice were weighted and scored daily according to standard parameters to monitor health and tumor diameters was measured daily and tumor volume was determined with the formula “smallest diameter² + biggest diameter / 2”. When a tumor reached 1000 mm³ or 15 mm in diameter (around day 13) experimental endpoint was reached and all mice were euthanized.

In two independent *in vivo* experiments, several single drug doses were tested to determine adequate dose ranges and various combinations based on *in vitro* dose relations between the drugs. Drug doses *in vivo* were based on previously published studies in mice, as selected in such way that no or minimal tumor inhibition was expected when applied as monotherapies[9,21–23]. In order to compare these doses with *the in vitro* doses used in our study, we used data from dose-escalation studies[24–26] . We first established a non-linear dose response curve of the drug concentration in patient blood in these studies. Second the *in vivo* doses used in the mouse models here, were converted to human doses and interpolated in the non-linear dose response curve of patient data, to arrive at corresponding concentrations. Data were analysed using 2-way ANOVA in GraphPad Prism.

Phosphopeptide enrichment

Cells were washed once in phosphate-buffered saline (PBS) prior to lysis in lysisbuffer (9M urea buffer containing 20 mM HEPES pH 8.0 and phosphatase inhibitors)and were processed as described previously[27]. Cells were scraped and sonicated on ice using a Soniprep 150 tip sonicator. Subsequent centrifugation of the lysate was performed for 15 min at maximum speed. The cleared lysate was aliquotted and stored at -80 °C until further use. Lysates were reduced in 4 mM dithiotreitol (DTT) for 20 min at 60°C, cooled to room temperature and subsequently alkylated in 10 mM iodoacetamide for 15 min in the dark. After dilution to 2M urea using 20 mM HEPES buffer pH 8.0, the lysate was digested with 20 µg Sequencing Grade Modified trypsin/mg protein by overnight incubation at 22°C. Digestion was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 1%. Samples were then incubated for 15 min on ice, centrifuged for 5 min at 1800 x g and transferred to a new tube. Tryptic digests were desalted using 6 cc HLB Oasis cartridges (Waters, Milford, MA) placed on a vacuum system. Bound

peptides were washed using 0.1% TFA before elution to glass vials in 40% ACN/0.1% TFA. Eluates were lyophilized to dryness for 48 hours and stored at -80 °C until further use. Enrichment for tyrosine phosphorylated phosphopeptides was performed using the anti-phosphotyrosine antibody P-Tyr-1000 coupled to agarose beads (PTMScan, Cell Signaling Technology). Briefly, 5 mg lyophilized phosphopeptides were dissolved in IAP buffer (20 mM Tris-HCl pH 7.2, 10 mM sodium phosphate and 50 mM NaCl) and incubated with 2 µl P-Tyr-1000 beads per mg protein at 4 °C for 2 hours. After sequential washes in cold IAP buffer and MQ, peptides were eluted from the beads in two steps in 0.15% TFA, desalted in 20 µl StageTips (1mm 3M SDB-XC membrane) using 0.1% TFA and eluted with 80% ACN/0.1% TFA into glass-lined autosampler vials. Eluates were dried in a speedvac at 45°C and redissolved in 20µl loading solvent (4% acetonitrile in 0.5% TFA) and stored at 4° C until LC-MS/MS measurement on the same day. For all pTyr samples corresponding lysate (1 µg on column) were also analysed for global protein expression.

LC-MS/MS

Global proteomics analysis and phosphoproteomics analysis were conducted according to established pipelines, as previously described [27]. Briefly, peptides separation was performed using nano liquid chromatography (Dionex U3000, Amsterdam, The Netherlands). Peptides were separated on a Reprosil Pur (Dr Maisch GMBH, Ammerbuch-Entringen, Germany) C18 column (40 cm x 75µm) by applying a 90 minute acetonitrile gradient (2-32% in 0.1% formic acid) resulting in an inject-to-inject time of 120 minutes. Peptides were analyzed on-line on a Q Exactive-HF Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). After ionization at 2 kV, MS1 masses were measured at R=70.000 (AGC 3E6) and MS2 masses at R=15.000 (AGC 1E6, MaxIT 64 ms). Peptides with charges >+1 were fragmented (isolation-width 1.4 Da) at NCE of 25 in a top-15 experiment. Dynamic exclusion time was 30 sec with a repeat-cont of 1.

Phosphopeptide and protein identification

MS/MS spectra were searched against Swissprot human proteome (canonical_and_isoforms, downloaded February 2019, 42417 entries) using MaxQuant 1.6.4.0. Enzyme specificity was set to trypsin and up to two missed cleavages were allowed. Cysteine carboxamidomethylation (Cys, +57.021464 Da) was treated as fixed modification and serine, threonine and tyrosine phosphorylation (+79.966330 Da), methionine oxidation (Met, +15.994915 Da) and N-terminal acetylation (N-terminal, +42.010565 Da) as variable modifications. Peptide precursor ions were searched with a maximum mass deviation of 4.5 ppm and fragment ions with a maximum mass deviation of 20 ppm. Peptide, protein and site identifications were filtered at an FDR of 1% using the decoy database strategy. The minimal peptide length was 7 amino-acids and the minimum Andromeda score for modified peptides was 40 and the corresponding minimum delta score was 6 (default MaxQuant settings). Peptide identifications were propagated across samples with the match between runs option checked.

Phosphopeptides were quantified by counting MS/MS spectra (spectral counts) or by their extracted ion intensities ('Intensity' in MaxQuant). For pTyr IP data for each sample the phosphopeptides/and site intensities were normalized on the corresponding lysate summed spectral counts. Proteomics data have been deposited in ProteomeXchange via the PRIDE repository [28] with accession number PXD016475.

Kinase-drug database analysis

Using Proteomicsdb.org [29], all available drugs targeting the top 20 INKA kinases in 786-O were retrieved, along with their reported EC₅₀. Unsupervised clustering and heatmap analysis were performed to display all drugs targeting this set of kinases with a EC₅₀ below 2μM. Data are presented in **Supplementary Figure 8** and show that a subset of drugs show inhibitory activity to multiple kinases while others are rather specific for a single kinase

Supplementary Figure legends

Supplement 1: s-FSC of RCC lines

Figure S1.1.1: Drug selection

Schematic drawing of ten initial drugs covering a broad targeting spectrum in a cell: **axitinib** (VEGFR's, PDGFR and c-KIT); **erlotinib** (EGFR inhibitor); **RAPTA-C** (chromatin inactivator); **BEZ-235** (inhibitor of both of the main protein complexes of mTOR (mechanistic target of rapamycin), mTORC1 and mTORC2); **volasertib** (inhibitor of PLK- polo-like kinase, which is found in the nuclei of dividing cells, and controls multiple stages of cell cycle and division); **dasatinib** (inhibitor of BCR/ABL and Src, binding both active and inactive forms of ABL kinase); **tozasertib** (VX-680, inhibitor of Aurora A, B, C, which play a role in mitosis and meiosis during proliferation (between G2 to M phase); **U-104** (inhibitor of carbonic anhydrase IX/X inhibitor II. CAIX expression is regulated by VHL protein, and VHL mutation loss is associated with clear cell RCC, so CAIX expression is related to clear cell RCC); **AZD4547** (TKI targeting FGFR 1-3 and also showing a weak activity against FGFR4), and **crenolanib** (designed to inhibit PDGFRs but shown to act via cell cycle modulation as well).

Figure S1.1.2 Monotherapy dose response curves

Dose response curves based on cell metabolic activity were generated for 5 RCC lines (786-O, A498, Caki-1, Caki-2 and ACHN) and the endothelial cell line EC-RF24, for all ten drugs used in the s-FSC screen. For presentation purposes, non-linear fitting was performed on the data (at least triplicate measurements). Experiments were performed in triplicate and repeated 2-4 times per condition.

Figure S1.2.1-6 Screening for optimized drug combinations

Data are presented for all RCC lines and EC-RF24. A) Efficacy of the tested drug combinations in search 3. Colored squares below the Y-axis denote drug combinations, with complete fills representing the indicated (IC_{20}) concentrations and fills with "X" represent half of the drug concentration. White squares indicate absence of the drug. In green the ODC (with a maximum of 3 drugs out of 4) is indicated. Experiments (N=2) were performed in triplicate and mean \pm SEM are shown. B) Coefficients from the

second order linear regression analysis applied to search 3, composed of the final 4 drugs remaining in the screen and leading to the selection of the ODC. Modeling was based on the data presented in (A). Negative values for single drug linear effects indicate that increasing the dose of that drug increases the inhibition of cell metabolic activity. Negative two-drug interaction values denote synergy, whereas positive values indicate antagonism. Quadratic single drug interactions are a measure of sensitivity to dose changes. C) Response surfaces modelling the interactions between two drugs and their effect on cell metabolic activity while other compounds are held at a constant dose. On the x-axis 1= highest concentration, 0 = lower concentration, -1 = no drug added, and the remaining cell metabolic activity (y-axis) is color coded.

Figure S1.3.1 Spheroid morphology after drug treatment

Heterotypic spheroids consisting of a 1:3 mixture of fibroblasts and RCC, supplemented with 10% HUVEC, were treated with either ODC or monotherapy for 72hrs before analysis. Due to cell line specific morphology, size was noted not to accurately correlate with metabolic activity (shown in Figure 1).

Figure S1.3.2 Dose sensitivity in 3D mono- and coculture systems

Homotypic spheroids were formed from RCC lines only and were treated with monotherapies and ODC either at half or at full dose. Similar inhibition is seen with the majority of cell lines and drugs. Dose-sensitivity is only evident in Caki-2 where reducing the dose of the monotherapies considerably impacts the response. However, the ODC effect is apparently not affected by halving the doses of the individual drugs. Experiments (N=3) were performed in triplicate and mean \pm SEM are shown.

Figure S1.3.3 Monotherapies and combinations of drugs in Caki-1 xenografts in mice

Caki-1 tumor volume growth curves of subcutaneous tumor xenografts treated for 13 days with vehicle control, single drugs and drug combinations at various doses in N=2 independent experiments. Error bars represent the SEM. *** $p < 0.001$ shown in the graph legend represents the comparison with the vehicle control at day 13 as determined by a two-way ANOVA with post-hoc Dunnett's multiple comparisons test.

All erlotinib doses tested were already highly effective *in vivo*. Doses between 5-40 mg/kg, corresponding to 0.26-2 μM *in vitro* inhibited tumor growth by over 60%. For dasatinib and AZD4547, s-FSC-based doses showed no significant tumor growth inhibition. The *in vivo* dominance of erlotinib was further confirmed in the drug combinations where the activity was driven mostly by erlotinib. However, together, the ODCs show potent effects *in vitro* and *in vivo*.

Supplement 2: Characterization of ODC effects in RCC

Figure S2.1 Effects of ODC on cell cycle profile

A) RCC were treated with monotherapies or ODC for 72hrs and subject to flow-cytometry based cell cycle analysis by propidium iodide staining for cellular DNA content. In general, ODC treatment induced apoptosis in the cell lines. Most monotherapies do not affect the distribution of the different cell cycle phases as compared to control, with the exception of axitinib and crenolanib that seem to induce apoptosis. Significant changes in the different phases are indicated at the top of the graph. B) For a direct comparison, the sub-G1 fraction was classified as apoptotic fraction. In these experiments, sunitinib (10 μM) was used as a positive control as this compound is among first-line treatments for RCC. ccRCC lines showed apoptosis induction by sunitinib at comparable levels to ODC, whereas the papillary RCC lines were totally insensitive. Of note, crenolanib alone clearly induced cell death in Caki-2, however in combination with other drugs this effect is reduced. N=5 independent experiments were performed and results shown represent mean \pm SEM.

Figure S2.2 Mechanism of action of drug combinations

Quantification of western blots. Bands shown in Figure 2 were quantified densitometrically.

Monotherapies tend to have only limited effect on phosphorylation of the analyzed kinases, whereas the ODC is most effective in inhibiting MAPK (ERK1/2) phosphorylation. A notable exception is 786-O where the ODC does not impact the phosphorylation of AKT and MAPK. Duplicate experiments were performed.

Supplement 3: Phosphoproteomics analysis of RCC lines

Figure S3.1.1 Duplicate INKA analysis of RCC lines

INKA ranking plots of duplicate samples for all RCC lines. Kinases targeted by drugs in the ODC are color-coded according to their respective legends.

Figure S3.1.2 Overlap of top 20 INKA kinases in 5 RCC lines

Top 20 kinases from the INKA analysis were derived from the average scores per cell line. Venn diagram shows that 14 are commonly present in this top 20, and that 8 more are present in the top 20 of 2 or more cell lines. Protein-protein interaction analysis shows the close interconnectivity of these kinases.

Figure S3.1.3 Phosphoproteomics analysis of EC-RF24

A) INKA ranking plots of duplicate samples of untreated EC-RF24 cells. Drug targets in the top 20 are color-coded. B) Phosphokinase expression levels, expressed in relation to those in RCC lines shown in Figure 3.

Figure S3.2.1-6 INKA network plots of RCC lines and EC-RF24

Kinase-substrate relationship of top 20 INKA kinases and their observed substrates in duplicate samples. Nodes are color-coded for targeting by drugs in the ODC. Drug targets are shaped according to INKA evidence. Nodes are depicted as a hexagon (observed kinases, identified through one or more phosphopeptides), a pentagon (inferred kinases lacking direct observation, but linked to phosphorylation of one or more observed phosphopeptides), or as a circle (non-kinase substrates). Edge widths correlate with the associated substrate site “phosphosignal”, and edge colors indicate the analysis on which the kinase–substrate relationship was based (orange: PSP, blue: NWK, green: both).

Supplement 4: A common drug combination for RCC lines

Figure S4.1.1-2 Protein drug interaction maps

Protein drug interaction maps (www.proteomicsdb.org) using the median concentrations of the 4 drugs presented in Table S4.1. Combinations of 3 and 4 drugs are shown, and reveal a number of multitargeted kinases. Protein kinase targets (nodes) exceeding default effective inhibition >33% are colored in blue and have dark grey edges. Thickness of the edges is proportional to the reported EC50 of the drug-kinase interaction.

Supplement 5: Phosphokinase alterations in 786-O after ODC

Figure S5.1.1 Phosphokinases (pTyr) downregulated after ODC

A) Phosphoproteomics analysis was performed on pTyr-IP'ed proteins from 786-O treated with the different monotherapies (dasatinib 150nM, erlotinib 10uM, axitinib 7.5uM), ODC, or left untreated for 2 hours. Phosphopeptides were assembled to phosphogenes and subsequently phosphorylated kinases as described in the materials and methods section. Normalized spectral counts were used as quantifiers and kinases showing >1.6 fold downregulation following ODC were counted in the replicate experiments. 21 phosphokinases were consistently downregulated. B) Average normalized spectral counts of phosphorylated kinases consistently inhibited by the ODC. Due to ambiguity in phosphopeptide identification, multiple protein identifiers are mentioned for several of the hits. Most notable are the MAPK family, SRC family, ABL family and EPHA family kinases. C) Protein-protein interaction network of the ODC inhibited kinases shows intricate interactions between them. Color intensity of the nodes is proportional to expression level.

Figure S5.1.2 Phosphokinases (pTyr) upregulated after ODC

A) Phosphoproteomics analysis was performed on pTyr-IP'ed proteins from 786-O treated with the different monotherapies (dasatinib 150nM, erlotinib 10uM, axitinib 7.5uM), ODC, or left untreated for 2 hours. Phosphopeptides were assembled to phosphogenes and subsequently phosphorylated kinases as described in the materials and methods section. Normalized spectral counts were used as quantifiers and kinases showing >1.3 fold upregulation following ODC were counted in the replicate experiments. 10 phosphokinases were consistently upregulated. B) Average normalized spectral counts of phosphorylated

kinases consistently induced by the ODC. Due to ambiguity in phosphopeptide identification, multiple protein identifiers are mentioned for several of the hits. Most notable are the CDK family and DYRK family kinases. Induction of putative resistance mediating kinases (JAK, MET, AXL) is also noted. C) Heatmap of induced kinases, showing that kinase alterations are dominantly mediated by dasatinib. D) Protein-protein interaction network of the ODC induced kinases. The MET-JAK2-AXL axis showed significant enrichment for the KEGG pathway “EGFR tyrosine kinase inhibitor resistance” ($p=0.00079$). Color intensity of the nodes is proportional to expression level.

Figure S5.2 INKA profiles after monotherapies and ODC in 786-O

INKA ranking plots of duplicate samples for treated and untreated 786-O. Kinases targeted by drugs are color-coded according to their respective legends. For untreated 786-O all drug targets are indicated in their respective colors. These plots allow for the relative ranking of kinases before and after treatment.

Figure S5.3.1-5 INKA network plots of monotherapies and ODC in 786-O

Kinase-substrate relationship of top 20 INKA kinases and their observed substrates in duplicate samples. Nodes are color-coded for targeting by drugs in the ODC. Drug targets are shaped according to INKA evidence. Nodes are depicted as a hexagon (observed kinases, identified through one or more phosphopeptides), a pentagon (inferred kinases lacking direct observation, but linked to phosphorylation of one or more observed phosphopeptides), or as a circle (non-kinase substrates). Edge widths correlate with the associated substrate site “phosphosignal”, and edge colors indicate the analysis on which the kinase–substrate relationship was based (orange: PSP, blue: NWK, green: both).

Supplement 6: Effects of addition of crizotinib on ODC performance

Figure S6.1 Protein-drug interaction maps

Protein drug interaction maps (www.proteomicsdb.org) for the combinations of erlotinib, dasatinib, crizotinib and AZD4547 or axitinib, reveal a number of multitargeted kinases. Protein kinase targets

(nodes) exceeding default effective inhibition >33% are colored in blue and have dark grey edges.

Thickness of the edges is proportional to the reported EC₅₀ of the drug-kinase interaction.

Figure S6.2 Limited additional effect of crizotinib addition in 3D cultures

A) 2 μ M crizotinib was selected for addition to the ODC. Applied as monotherapy, crizotinib displayed <10% inhibitory activity in heterotypic 3D cultures. When added to the original ODC, no further inhibition of cell metabolic activity is observed, as the ODC displayed more potent inhibitory activity in 3D as compared to 2D in vitro assays. Experiments (N=2-4) were performed in triplicate and mean \pm SEM is shown. B) Representative images of spheroids after treatment.

Supplement 7: Analysis in EC-RF24

Figure S7.1 ODC in EC-RF24

A) Dasatinib, AZD4547 and BEZ-235 were selected as ODC in EC-RF24. Alone, they inhibit cell metabolic activity by <25%, whereas in combination their effect is >85%. N=3 independent experiments were performed and results shown represent mean \pm SEM. B, C) Cell cycle analysis (B) and apoptosis analysis (C) reveals an increase in apoptosis after ODC treatment, which is in part mediated by AZD4547. N=2 independent experiments were performed and results shown represent mean \pm SEM. D, E) Western blot analysis shows a dominant action of dasatinib and BEZ-235 on phospho-AKT and phospho-RPS6 expression.

Supplement 8: Drug-kinase efficacy

Figure S8 Heatmap of EC₅₀ of drugs inhibiting most active 786-O kinases

Drugs with reported EC₅₀ <2 μ M against the top 20 INKA kinases in untreated 786-O were retrieved from proteomicsdb.org, and used for clustering and heatmap generation. Lighter colors indicate higher activity (higher -Log₁₀(EC₅₀) values) against a given kinase. It shows that many drugs are effective against multiple kinases while others are relatively specific for a single kinase.

Supplements 9-10: Western blot

Figure S9.1-4: Original Western blot images and reference to Figure 2 and Figure S7.1

Figure S9.1 – Detection of phosphor-AKT, phosphor-MAPK and phosphor-RPS6 in 786-O and A498 as presented in Figure 2

Figure S9.2 - Detection of phosphor-AKT, phosphor-MAPK and phosphor-RPS6 in Caki-1 and Caki-2 as presented in Figure 2

Figure S9.3 - Detection of phosphor-AKT, phosphor-MAPK and phosphor-RPS6 in duplicate samples RF24 of which the left is presented in Figure S7.1

Figure S10.1-2: Original Western blot images and reference to main figure 6

Original blots. Lane numbers are indicated and may be mirrored in the presentation of figure 6.

Furthermore, for adequate presentation in main figure 6, contrast was globally adjusted.

Figure S10.1 - MET: For phosphoMET, exposure of 6' is presented in Fig 6. It must be noted that due to the low expression of phosphoMET, blots were developed using a Uvitec Alliance chemiluminescence imager whereas the remainder of the blots were imaged using Licor Odyssey CLx scanner. Therefore, a separate (light) image is shown of the blot to show the molecular weight marker.

Figure S10.2 – EPHA2: For phosphoEPHA2, exposure of 12' is presented in Fig 6. It must be noted that due to the low expression of phosphoEPHA2, blots were developed using a Uvitec Alliance chemiluminescence imager whereas the remainder of the blots were imaged using Licor Odyssey CLx scanner. Therefore, a separate (light) image is shown of the blot to show the molecular weight marker. The correct MW band of EPHA2 is indicated with “>”.

Figure S10.3 – RPS6

Figure S10.4 – Actin: Two blots are shown as separate blots were probed for total and phosphoprotein in the experiments.

Supplementary Tables

Table S1.1: IC20 drug doses used per cell line in s-FSC

Table S1.2: Characteristics of the cell lines used

Table S2: Cell viability in s-FSC iteration rounds for the common RCC drug combinations

Table S3.1: Normalized spectral counts of phosphokinases

Table S3.2: INKA scores of phosphokinases

Table S3.3: Effective inhibitory % of ODC for expressed kinases per cell line in ccRCC

Supplementary References

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