SUPPLEMENTARY MATERIALS AND METHODS

Microarray studies

The case studies were selected by the Gene Expression Omnibus database of the National Center for Biotechnology Information (NCBI). The data were downloaded as a "Series Matrix" file already normalized. The profile “1” (KRas-H6c7) was obtained from NCBI “GSE1955” (1). The chip “HG-U133A” was already compatible with the reference chip of MANTRA. The profile “2” (KRas12D-HPDE) was obtained from NCBI “GSE58055” (2). The chip “Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381” was processed before to make it compatible with the standard of MANTRA. We developed a method to convert Agilent probes into Affymetrix HG-U133A probes (chip reference for the network of MANTRA). The conversion was carried out in two steps. 1) From Agilent probe set_id toward Agilent gene: each probe set id was associated with the corresponding gene, based on Affymetrix annotation. If more than one probe was found to be associated with the same gene, we assigned to the gene the rank of the probe that comes first in the ranking. 2) From Agilent gene toward Affymetrix HG-U-133A probe_set_id: we associated to each gene the corresponding HG-U-133A probe set, using the Affymetrix annotation file. If a gene was associated with more than one probe set, we assigned the same ranking to all of the probes involved. For Affymetrix probes that did not generate any matches, we positioned them at the center of the ranking. We then integrated them into the drug network.

Inspection of drug-gene network by MANTRA 2.0

Drug-gene networks can be derived by computing the similarity between gene signatures generated by drug treatments in specific cell lines, with the assumption that each drug treatment generates a drug-specific signature. In this drug network, drugs can be grouped into communities composed of drugs that produce similar transcriptional profiles and, thus, may act through a similar MoA. This network-based analysis allows the identification of the MoA of novel compounds, as well as the repurposing of drugs for novel therapeutic indications (“drug repositioning”).

MANTRA 2.0 is a computational approach to predict drug MoA and drug repurposing by integrating analysis of the connectivity map *(3)*, which is a compendium of gene expression profiles, after drug treatment of human cell lines with 1309 bioactive small molecules. The MANTRA approach was based on generating a single “prototype”-ranked list of differentially

expressed genes for each drug after treatment across multiple cell lines or at different dosages *(4)*.

Gene expression profiles generated from a disease state or a genetic state (such as oncogene activation) are used to inspect the MANTRA network by computing the transcriptional similarity between each couple *(5)*. Specifically, Affymetrix microarrays .CEL files were uploaded in the MANTRA online tool (http://mantra.tigem.it) and automatically transformed into a new node in the drug network (6). MANTRA computed the transcriptional similarity quantified as a distance, which is a number greater than or equal to zero, with zero indicating identical profiles. We then considered drugs that scored below a transcriptional distance threshold of 0.85 from all of our profiles.

To reposition FDA-approved drugs that might act as inhibitors of oncogenic KRAS-dependent pathways, we queried MANTRA 2.0 for drugs with significance distance from the reverse KRAS oncogene-dependent signature, generated by means of isogenic cell line models. These reverse signatures were generated by sorting genes in reverse order of differential expression (i.e., the most downregulated ranked at the top of the signature, whereas the most upregulated ranked at the bottom) to generate a gene expression profile associated with inhibition of oncogenic KRAS-dependent pathways. Computational distance analysis of these transcriptional signatures in the MANTRA 2.0 network enabled the identification of drugs and communities with significant distance (threshold ≤ 0.85) from the oncogene-specific node.

Signature score calculation methods

The gene signature scores were calculated by subtracting the average normalized expression of "down" genes from the average normalized expression of "up" genes, defining the L-score and the S-score. The RNA sequencing data were normalized by taking the log10 ratio of expression of the sample compared to the average expression of the gene across all samples *(7)*. The L-score genes are described in *(8)*; the S-score genes are described in *(9)*. The top KRAS-dependent genes were selected as "up" genes, and the top KRAS-independent genes were selected as "down" genes. The microarray data were normalized by performing the same transformation as described for the RNA sequencing data, except on the normalized intensity values rather than TPM.

The expression for The Cancer Genome Atlas (TCGA) PAAD samples was extracted from the UCSC Project Xena full reprocessing of TCGA next-generation sequencing data *(10)*. The KRAS mutation status of TCGA PAAD samples was also acquired from the UCSC Project Xena reprocessing of TCGA data. After filtering samples with low purity (11), the L-score and S-score

were calculated for all TCGA PAAD samples using the same analytical method presented above for calculating the L- and S-score from cell line data.

The correlation of L- and S-score to DEC EC_{50} was calculated using drug screen data for pancreatic cancer cell lines from the Cancer Therapeutics Response Portal (CTRP, https://portals.broadinstitute.org/ctrp/; *(12)* and expression data from the Cancer Cell Line Encyclopedia (CCLE) *(13)*. Correlated cell lines were also filtered for consistent doubling time, as indicated by the Expasy database (https://web.expasy.org/), to consider only those cell models that would show growth change in the high-throughput assay after drug treatment.

Reagents

5-Aza-2'-deoxycytidine (A3656), 2'-Deoxycytidine (D38979), SGI-1027 (1358), gemcitabine hydrocloride (G6493), RG108 (R8279), camptothecin (C9911), and doxorubicin hydrochloride (44584) were purchased from Sigma Aldrich. Trametinib (TRAM) (GSK1120212) was provided by Dr. Michele Milella (Regina Elena National Cancer Institute-Rome).

Cell viability assay

The viability of cells was evaluated by Cell-Titer-Glo Luminescent Cell Viability Assay (Promega). For KP4, PA-TU-8902, PA-TU-8988T, and HPDE/K-*RAS*^{G12V}, a total of 5×10^2 cells were seeded at T=0. For CAPAN-1 and HPDE cells, 1 $x10^3$ cells were seeded onto 96-well plates. After 24 hours, cells were exposed to indicate serial concentrations of drugs, and culture media and treatments were refreshed after 72 hours. Viability was measured either 72 hours or 144 hours later, as indicated, using CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions. Dose response curves and inhibitory concentration $(IC)_{50}$ values were generated using GraphPad Prism software (GraphPad Software).

Cell cycle analysis by flow cytometry

Cells (100-200 x 10^3) were seeded in 100-mm cell culture dishes. Twenty-four hours after plating, cells were treated with 1.25 μM of 5-aza-2′-deoxycytidine for 72 hours. Then, 1 x 10^6 cells were harvested, washed in 1X PBS, fixed with fridge-cold 70% EtOH, and kept at -20°C for 24 hours. Fixed cells were re-suspended in an RNase A (10 μg/mL) and propidium iodide solution (10μg/mL) for 30 minutes before analysis with flow cytometry. Samples were acquired and analyzed with C6 Accuri BD (Becton Dickinson & Co.).

Senescence-associated β-galactosidase staining

Cells (6×10^3) were plated in 12-well plates, and 24 hours after plating, cells were treated with 1.25 μM of 5-aza-2′-deoxycytidine for 5 days. For senescence-activated β-galactosidase (SA-βGal) staining, we used a Senescence-βGal Staining Kit (Cell Signaling Technology) following the manufacturer's instructions. The percentage of senescent cells (blue) was quantified using ImageJ and related to the total number cells.

Colony formation assays

The clonogenic growth of PDAC cells was analyzed by plating 500 cells/well in 6-well plates. After 24 hours, cells were exposed for 6 days and 18 days to 1.25 μM of DEC or dimethyl sulfoxide (DMSO), used as drug diluent. Cell culture media containing either DMSO or DEC were refreshed every 3 days. Before the assay, cells were washed two times with 1X PBS, and colonies were then stained with crystal violet for 1 hour. Colonies were washed with distilled water and left drying in air before colony counting. The percentage of colony was related to the total number cells.

Immunofluorescence analysis

Cells (3125 cells/well) were plated on glass coverslips in 24-well plates and treated after 24 hours with 1.25-μM DEC for the indicated time. Then, cells were washed three times with 1X PBS and fixed using 3.7% formaldehyde for 20 minutes at room temperature. Fixed cells were washed with 1X PBS and permeabilized using 0.25% Triton X-100/1X PBS solution for 5 minutes. After permeabilization, cells were washed three times with 1X PBS and then blocked using 0.5% bovine serum albumin (Sigma)/1X PBS solution, for 45 minutes, at room temperature. Cells were washed three times with 1X PBS and then incubated with primary antibody (pH2AX- H05636 Millipore) diluted 1:500 in blocking solution for 1 hour at room temperature. Then, cells were washed three times with 1X PBS and incubated with an anti-mouse secondary antibody (Alexa Fluor 555-Cell Signalling) diluted 1:500 in blocking solution for 45 minutes at room temperature. After three washes with 1X PBS, cells were stained with HOECHST (Sigma Aldrich) diluted 1:10000 in 1X PBS for 10 minutes. Then, cells were washed with 1X PBS, and coverslips were mounted on the microscopy slides with 1:1 PBS-glycerol solution and washed three times with 1X PBS. After 24 hours, stained cells were acquired using a DMi8 Leica inverted microscope. For quantitative analysis, images were processed using ImageJ, and the percentage of positive cells was quantified in relation to the total number cells.

mRNA and quantitative real-time polymerase chain reaction analysis

Total RNA extraction was carried out using RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA was then reverse transcribed into complementary DNA by a QuantiTect Reverse Transcription Kit (Qiagen) with random hexamers oligo. The complementary DNA was diluted 1:3 and subjected to quantitative real-time polymerase chain reaction (qRT-PCR) analysis using Light Cycler (Applied Biosystem) with SYBR Green PCR Master MIX Kit (Applied Biosystem).

KRAS reverse- and DEC-dependent signatures were analyzed by MANTRA to identify the 250 most upregulated and downregulated genes in the HG-U133A Affymetrix array. Genes found in both KRAS-reverse- and DEC-dependent signatures were selected for experimental validation. The primer sequences for the PCR analysis are available upon request.

Metabolomics analysis of polar metabolites

PA-TU-8902 and PA-TU-8988T (86 x 10^3 cells) were seeded in 100-mm cell culture dishes. Twenty-four hours after plating, cells were treated with 1.25 μM of 5-aza-2′-deoxycytidine; 24 hours after treatment, cells were harvested for metabolomic analysis. Two hours before collection, media was refreshed with drugs or DMSO. The medium was aspirated, and immediately 4 ml of 80% methanol (-80 C°) on dry ice was added and incubated at -80°C for 15 minutes. Then, cells were scraped on dry ice with a cell scraper, and the cell lysate/methanol mixture was transferred to conical tubes (maintained on dry ice) and centrifuged at full speed for 5 minutes in a cold room. The supernatant was transferred to a new 15-ml conical tube maintained on dry ice. After the pellet was suspended in 500 μl of 80% methanol and the mixture was transferred to a 1.5-mL Eppendorf tube on dry ice, the sample was centrifuged at full speed for 5 minutes in a cold room, and the supernatant was transferred to a 15-ml conical tube. Centrifugation and suspension steps were repeated two more times. After pooling the three extractions, the samples were completely dried using the nitrogen superficial influx desiccation method. Dried metabolite samples were stored at -80 °C. Samples were then submitted to the Beth Israel Deaconess Medical Center Mass Spectrometry Core Facility for analysis.

Targeted Mass Spectrometry

Samples were re-suspended using 20μl HPLC grade water for mass spectrometry. 5-7μl were injected and analysed using a hybrid 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 262 endogenous water-soluble metabolites for steady-state

analyses of samples. Some metabolites were targeted in both positive and negative ion mode for a total of 298 SRM transitions using positive/negative ion polarity switching. ESI voltage was +4950V in positive ion mode and –4500V in negative ion mode. The dwell time was 3ms per SRM transition and the total cycle time was 1.55 seconds. Approximately 10-14 data points were acquired per detected metabolite. Samples were delivered to the mass spectrometer via hydrophilic interaction chromatography (HILIC) using a 4.6 mm i.d x 10cm Amide XBridge column (Waters) at 400µl/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0-5 minutes; 42% B to 0% B from 5-16 minutes; 0% B was held from 16-24 minutes; 0% B to 85% B from 24-25 minutes; 85% B was held for 7 minutes to re-equilibrate the column. Buffer A was comprised of 20mM ammonium hydroxide/20 mM ammonium acetate (pH=9.0) in 95:5 water:acetonitrile. Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.1 software (AB/SCIEX).

Metabolite Set Enrichment Analysis

The Metabolite Set Enrichment Analysis (MSEA) is a metabolomic version of the popular Gene Set Enrichment Analysis software. MSEA directly investigates if a group of functionally related metabolites are significantly enriched. It has the potential to identify "subtle but consistent" changes among a group of related compounds. MSEA graphs were generated with the web tool MetaboAnalyst *(14)* (www.metaboanalyst.ca). Pathway-associated metabolite sets consisted of significantly altered metabolites with an adjusted P value of less than 0.1. The threshold P value for significant pathways was fixed at less than 0.05.

Drugs combination index calculation

To determine the nature (synergism, additivity, and antagonism) of TRAM and DEC interaction, we used the method proposed by Chou and Talalay *(15,16)*. Cells were treated with a combination of TRAM and DEC using the method of constant ratio drug combination. The two drugs were used at a constant ratio of their concentrations. The concentrations used corresponded to 0.25, 0.5, 1, 2, and 4 times the IC_{50} of each agent (TRAM: IC_{50}=2.5 nM; DEC: IC_{50}=100 nM for PA-TU-8902, and IC_{50}=180 nM for HPAF-II). This method, using the combination index (CI) equation, allows quantitative determinations of drug interactions at increasing levels of cell kill. The CI value allows classification of the anti-tumor activity of the drug combination: a CI of less than, equal to, or more than 1 indicates synergic, additive, or antagonistic effect, respectively. *Fa* is the fraction of cell death induced by drug treatment and ranges from 0 to 1, with 0 meaning no cell killing and 1 representing 100% cell killing. Dep-PDAC PA-TU-8902 and HPAF-II cells were treated with serial

dilutions of each drug alone or in combination at a fixed ratio of 1:40 (TRAM:DEC) in three independent experiments with triplicate samples.

Immunohistochemistry

After formalin fixation, tumours were cut into 5 mm thick slice and embedded in paraffin cut surface down. To optimize the detection of microscopic metastases and ensure random sampling, lungs were cut transversally into 2.0 mm thick parallel slabs, resulting in 5-8 slabs. The slabs were then embedded in paraffin cut surface down. All the sections were stained with Haematoxylin and Eosin and were independently evaluated by two pathologists. Immunohistochemical staining on formalin fixed paraffin-embedded (FFPE) tissue from human pancreas tumour xenografts, was performed using the following primary antibodies: anti-phospho-H2AX mouse monoclonal antibody (Ser139) (JBW301) (Millipore) and anti-human Ki67 mouse monoclonal antibody (MIB-1) (Dako).

The percentage of phospho-H2AX and Ki67 positive cells was evaluated on digital images of tumours (5 microscopic fields at X 200 magnification per tumour) acquired by Leica DMRD optical microscope equipped with a DFC550 camera: brown nuclei were regarded as positive cells and the percentage of positive cells (number of positive cells/total cells x100) was calculated for each field, by two pathologists, independently, and in a blind fashion.

Statistical analysis

All *in-vitro* experiments were performed in triplicate wells and repeated multiple times using independent biological replicates (n). Student's *t*-test was used to test for statistical significance of the differences between different groups and controls. P values < 0.05 were considered statistically significant. Sample correlations were calculated using Spearman's correlation coefficient.

For metabolomics analysis, data collected for cell lines PA-TU-8902 and PA-TU-8988T were measured on seven biological replicates ($n = 7$). A paired *t*-test was used to test the statistical significance between samples (cells treated with DEC and DMSO-treated controls). Adjusted P values for multiple comparisons (FDR) were computed to select the top significant metabolites. The threshold for the adjusted P value was fixed at less than 0.1. Log-fold change was calculated to show the metabolites significantly changed between the two cell lines using the Volcano plot. All analyses were carried out in the R environment.

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