Supplementary Methods

Cell culture and reagents

The MET-dependent adenocarcinoma line MKN-45, KYM-1 rhabdomyosarcoma line and MG-63 osteosarcoma lines were obtained from Health Science Research Resources Bank (Japan Health Sciences Foundation). The FGFR-dependent urinary bladder carcinoma RT-112 was obtained from Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen. Hs-683 glioma line and HEK 293T/17 cells, a human kidney line expressing SV40 large T antigen, were purchased from American Type Culture Collection. MKN-45 cells were cultured in RPMI + 10% fetal bovine serum (FBS). RT-112 cells were maintained in EMEM + 10% FBS. Hs-683 and HEK 293T/17 cells were maintained in DMEM +10% FBS. MG-63 were maintained in Eagle's minimal essential medium and 10% FBS. KYM-1 cells were grown in DMEM/HAM's F12K (1:1) medium with 10% FBS. Purified proteins were purchased from R&D Systems. NVP-INC280, NVP-JAA120 and NVP-BGJ398 were synthesized internally at Novartis.

Secretomics screening (detailed method description)

Supernatant production: DNA from the aforementioned clones was stamped into clear, tissue-culture treated 384-well plates (Matrix), 4ul/well at 7.5ng/ul. Stamps were stored frozen at -20C until use. On day of experiment stamped cDNA plates were thawed and equilibrated to room temperature. HEK293T/17 cells were reverse-transfected as follows: Fugene HD (Promega) was diluted in Optimem (Invitrogen) to achieve a final ratio of 4:1 (nl Fugene HD : ng DNA). Diluted transfection reagent was added to stamped DNA plates 10ul/well and allowed to incubate 30 minutes at room temperature. HEK293T/17 cells were then added at 7,000 cells/50ul/well and incubated four days under standard tissue culture conditions to allow accumulation of secreted proteins in the media supernatant.

MKN-45 Secretome screen: MKN-45 cells were plated in white, tissue-culture treated 384-well plates (Greiner) at 3000 cells/20ul DMEM +10% FBS/well and allowed to attach overnight. Supernatant from

library-transfected HEK293T/17 cells was then transferred to the MKN-45 cells at 30ul/well using a Biomek FX liquid handler (Beckman Coulter) with pipetting speeds reduced to minimize disturbance of the HEK293T/17 monolayer. As positive controls, the purified proteins rhEGF and rhNRG1-β1 (R&D Systems) were added to isolated wells on each plate for a final concentration of 150ng/ml; supernatant from mock-transfected HEK293T/17 wells were transferred as neutral controls. Following addition of supernatants and purified protein controls, NVP-JAA120 diluted in DMEM was added at 10ul/well for a final assay concentration of 100nM. After 96 hours incubation, growth was measured using the CellTiter-Glo luminescent cell viability assay system (Promega). In brief, 30ul CellTiter-Glo reagent was added to all wells, then incubated for 15 minutes at room temperature before reading luminescence on a Viewlux plate reader (Perkin Elmer).

RT-112 Secretome screen: The basic format was identical to the MKN-45 secretome screen with slight modifications. RT-112 cells were plated in EMEM+10% in white, tissue-culture treated 384-well plates at 1000 cells/20ul/well and allowed to attach overnight. Supernatant from library-transfected HEK293T/17 cells was transferred as described above for the MKN-45 secretome screen. Purified proteins rhNRG1-β1 and rhTGFα were added as positive controls, at a final concentration of 150ng/ml. Following addition of supernatants and purified protein controls, NVP-BGJ398 diluted in DMEM was added at 10ul/well for a final assay concentration of 100nM. Cell viability was measured after 72 hours using CellTiter-Glo as described above.

In both screens assay data was normalized to vector only controls using the formula:

$$Normalized\ activity = 100 \times \left(\frac{X - vector_{median}}{vector_{median}}\right)$$

where X is the raw value and $vector_{median}$ is the median of vector control wells for a given plate.

Purified protein confirmation: The assay format for purified protein confirmation was identical to the format used for primary screening, with the exception that purified proteins were added at 30ul/well in place of HEK293T/17 supernatant, for a final concentration of 100ng/ml.

Reversal of rescue with selective inhibitors (figure 2B)

MKN-45 Dual Inhibition: MKN-45 cells were seeded at 3000 cells/20ul/well in 384-well plates and incubated overnight. Solutions of rhFGF7 and rhNRG-1 were prepared in DMEM+10%FBS, then added at 30ul/well to achieve a final concentration of 250ng/ml (one purified protein per treatment). The following single and dual inhibition treatments were prepared in DMEM and added at 10 μl/well: NVP-JAA120, NVP-INC280, NVP-BGJ398, Lapatinib, NVP-JAA120 and NVP-BGJ398, NVP-INC280 and NVP-BGJ398, NVP-JAA120 and Lapatinib, NVP-INC280 and Lapatinib. Final concentrations for each compound, whether single or combined, were as follows: 100nM for NVP-JAA120 and NVP-INC280, 500nM for NVP-BGJ398 and 1.5uM for Lapatinib. After 96 hours cell viability was measured by CellTiter-Glo as previously described.

RT-112 Dual Inhibition: The format for the RT-112 dual inhibition experiment was similar to that described for MKN-45 with the following modifications. RT-112 cells were plated at 1000 cells/20ul/well. Solutions of rhHGF and rhNRG-1 were added to achieve a final concentration of 250ng/ml. Single and dual inhibition conditions were prepared as follows: NVP-BGJ398, NVP-JAA120, NVP-INC280, Lapatinib, NVP-BGJ398 and NVP-JAA120, NVP-BGJ398 and NVP-INC280, NVP-BGJ398 and Lapatinib. Final concentrations for each compound, whether single or combined, were as follows: 100nM for NVP-BGJ398, 500nM for NVP-JAA120 and NVP-INC280, and 1.5uM for Lapatinib. After 96 hours cell viability was measured by CellTiter-Glo as previously described.

Western blots (figure 2C and 3C)

MKN-45 and RT-112 cells were treated in 6-well plates for 2 hours and 18 hours in the presence or absence of purified protein (rhFGF7, rhNRG1-β1, or rhHGF), and/or inhibitor (NVP-INC280, NVP-JAA120, NVP-BGJ398). Following wash with ice-cold PBS, cells were lysed with RIPA buffer (Thermo) containing phosphatase (Thermo) and proteinase (Roche) inhibitor cocktails. Total protein was quantified by bicinchoninic protein assay (Pierce). Aliquots of 20 µg were resolved by electrophoresis on NuPAGE SDS-PAGE 4-12% BIS-Tris gels (Invitrogen) before transfer to nitrocellulose membranes (Invitrogen). Membranes were blocked for one hour at room temperature (LI-COR blocking buffer) before overnight incubation at 4°C with the following primary antibodies (source rabbit, 1:1000 final dilution): anti-phospho-Akt (Ser473), anti-phospho-Met (Tyr1234/1235), anti-phospho-MAPK/ERK(1/2) (Thr202/Tyr204), anti-phospho-MEK1/2 (Ser217/221), anti-phospho-HER2/ErbB2(Tyr1221/1222), anti-phospho-HER3/ErbB3(Tyr1222), anti-AKT, anti-MAPK/Erk(1/2), anti-MEK1/2, and anti-α/β-Tubulin (Cell Signaling Technology). Anti-MET was probed at 1:800 final dilution (Cell Signaling Technology). An internal antibody was used for phospho-FRS2(Y346) (1:1500 dilution). Membranes were then washed 3 times in PBS + 0.1% Tween before addition of secondary antibody, IRDye 680LT goat anti-rabbit IgG (LI-COR Bioscience), dilution 1:15000. After one hour at room temperature, membranes were washed three times in PBS + 0.1% Tween followed by a final rinse in PBS. Bands were then visualized using an Odyssey Infrared Imager (LI-COR Bioscience).

Combination assays: monolayer, non-adherent, soft agar (Figure 4B)

Combinatorial anti-proliferative effects of NVP-INC280 (compound A) and NVP-BGJ398 (compound B) were measured in KYM-1, MG-63, and Hs 683 cells on 96-well-plates using the concentration matrix depicted below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	DMSO	B1	B1 + A6	B1 + A5	B1 + A4	B1 + A3	B1 + A2	B1 + A1	A1	-	-
C	-	DMSO	B2	B2 + A6	B2 + A5	B2 + A4	B2 + A3	B2 + A2	B2 + A1	A2	-	-
D	-	DMSO	B3	B3 + A6	B3 + A5	B3 + A4	B3 + A3	B3 + A2	B3 + A1	A3	-	-
E	-	DMSO	B4	B4 + A6	B4 + A5	B4 + A4	B4 + A3	B4 + A2	B4 + A1	A4	-	-
F	-	DMSO	B5	B5 + A6	B5 + A5	B5 + A4	B5 + A3	B5 + A2	B5 + A1	A5	-	-
G	-	DMSO	B6	B6 + A6	B6 + A5	B6 + A4	B6 + A3	B6 + A2	B6 + A1	A6	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

	μM
A1	1.0000
A2	0.2500
A3	0.0625
A4	0.0156
A5	0.0039
A6	0.0010

	μM
B1	1.0000
B2	0.2500
B3	0.0625
B4	0.0156
B5	0.0039
B6	0.0010

Wells labeled with hyphens were filled up with the according volume of growth medium. In some experiments, the maximal concentration of NVP-INC280 was 10-fold lower, but the dilutions steps were kept as above.

Cells were grown under three different conditions: Experiments labeled “monolayer” were conducted on regular tissue culture plates, allowing cells to adhere and eventually form a monolayer. Cells were seeded on 3 plates per experiment (triplicates) in standard growth media as described above at a density of 5000 per well. Six to 8 wells on a separate plate were seeded to quantify the amount of viable cells at the point of compound addition. 24 h later, separate dilution series for each compound were prepared in growth medium at 10-fold of the final concentration starting from 10 mM DMSO stocks. DMSO-only controls were included as indicated. Aliquots of 10 μL for each compound dilution were added according to the matrix shown above, resulting in a final volume of 100 μL. At the same time, viable cells on the separate plate mentioned above were quantified using a resazurin sodium salt dye reduction readout (commercially known as AlamarBlue assay). Specifically, 10 mL of a 0.13 mg/mL stock were added per well and plates were incubated for 2 h in a cell culture incubator before measuring absorptions (excitation 560 nm, emission 590 nm). The compound-treated cells were incubated for 72 hours followed by a resazurin assay as above. Percent inhibition was calculated by (a) subtracting the readout of seeded cells at the time of compound addition and (b) setting DMSO-only treated cells to 0% inhibition and the readout of seeded cells to 100% inhibiton. Values above 100% are thus suggesting cell death over the course of incubation with compound. Quantification of Synergy was done using the methods described in (3, 4), in short by iteratively calculating the Loewe additive response ILoewe (X, Y) at each dose matrix point from the single-agent response curves, and then summing the differences between ILoewe and the

experimental data. Where the sum was larger than that from mere addition of the ILoewe data, synergy was given (X, Y are the drug concentrations of drug X and drug Y on the X and Y axis, respectively).

The experiment in KYM-1 cells labeled as “non-adherent” was conducted in the same way except that Costar ultra low adherent 96-well-plates (Corning) were used. On these plates cells were not able to attach to plastic and grew in two-dimensional aggregates. The other two cell lines did not grow under these conditions.

For experiments labeled “soft agar” the cells were embedded in semi-solid media to allow three-dimensional aggregate formation. Specifically, agarose Type VII (low gelling temperature; Sigma) was dissolved in PBS at a concentration of 2.7% by heating in a microwave. The solution was then kept at 50°C until immediately before plating and diluted with a 2-fold volume of cell line-specific growth medium as described above. Diluted agarose was then mixed with a 2-fold volume containing the respective cells and aliquots of 150 μL containing 3000 cells were quickly distributed on Costar ultra low adherent 96-well-plates. The final agarose concentration was thus 0.3%. Plates were cooled down for 5 minutes at 4°C and 5 minutes at room temperature and then transferred to an incubator. Again, wells on a separate plate were seeded to quantify the amount of cells at the point of compound addition. After 24 h, compound dilution series were prepared so that a overlay of a total of 80 μL of diluted compounds in growth media would result in the final concentrations indicated in the scheme above, resulting in a total volume of 230 μL. Seeded cells were quantified by addition of 20 μL resazurin solution and incubation for 5 h. Compound-treated cells were incubated for 7 to 10 days and colonies were quantified with resazurin. Percent inhibition and synergy were calculated as above.

Western blots (Figure 4C)

The indicated cell lines were seeded on 6-well-plates at a density of 500000 cells/well, left for 24 h to attach and then treated with a final concentration of 1 μM of the indicated compounds for another 24 h.

Growth media were then removed, cells were washed twice with ice-cold PBS and lysed in 50 mmol/L Tris pH 7.5, 120 mmol/L NaCl, 20 mmol/L NaF, 1 mmol/L EDTA, 6 mmol/L EGTA, 1 mmol/L Benzamidin, 0.2 mmol/L PMSF, 100 mmol/L sodium vanadate, 1% NP-40. The protein concentration of cleared lysates was determined with the BCA Protein Assay Kit (Novagen). 80 µg protein of each sample was separated by SDS-PAGE on NuPage 4-12% Tris-Bis Midi gels (Invitrogen), transferred to a PVDF-membrane, and probed with antibodies as listed above. After washing and incubation with secondary HRP-linked antibodies, bands were visualized using ECL detection reagent (GE Healthcare).

In vivo mouse study (figure 5)

The in vivo anti-tumor efficacy study in the patient-derived non-small cell lung cancer xenograft LXFL 1121 was carried out at Oncotest GmbH (Freiburg, Germany). Xenografts were grown subcutaneously in nude mice. Eight tumor-bearing mice each were treated with either vehicle control, INC280 alone, BGJ398 alone or both drugs in combination a the doses and frequencies indicated. Due to body weight loss in the combination group, dosing frequency of INC280 was arbitrarily reduced to once daily after 2 weeks. The study was continued in this setup until day 18.

INC280 was formulated for oral administration by solving the compound in half the final volume of water using a water bath sonicator (1 hour), then adding the other half final volume of 0.5% methylcellulose/0.1% Tween80. Therefore, final concentrations were 0.25% Methylcellulose:0.05% Tween 80. BGJ398 was formulated by dispensing the compound in acetic acid/acetate buffer pH 4.68 (50% of final formulation volume), sonicating for 15 min until a milky suspension was obtained, adding PEG300 (50% of final formulation volume), and sonicating/vortexing until a clear solution was obtained. Note that the frequency of NVP-INC280 administration was reduced from twice to once daily at study day 14 after body weight loss in the combination group was observed. In the combination group, one animal died after study day 7 and one after study day 18 for unknown reasons. Tumor volumes were measured

on the indicated days and synergy of the combination treatment was assessed by the method of Clarke (2).

For pharmacokinetic/pharmacodynamic analysis, animals of the vehicle and INC280-only groups were sacrificed after study day 21, half of the animals each at 2 h or 12 h after last administration. Plasma and tumor samples were collected. Treatment in the two remaining groups was stopped after day 21 and tumors were allowed to re-grow in order to obtain sufficient material for pharmacodynamic analysis. On day 61, a final dose of BGJ398 or BGJ398/INC280 combination were given and half of the remaining mice were sacrificed after 2 h. A second dose of INC280 was administered to the combination group 12 h after the first, and the remaining animals were sacrified 24 h after BGJ398 = 12 h after the last INC280 administration.

Snap-frozen tumor samples were pulverized by hand in a steel mortar that was cooled with liquid nitrogen. Protein extracts were then prepared in lysis buffer (100 mM Tris-HCl pH 7.5, 1% NP-40, 150 mM NaCl, 25 mM NaF, 40 mM β-glycerol phosphate) that had been freshly supplemented with 200 µM sodium vanadate, 1 mM DTT, 100 µM PMSF, 1 mM benzamidine and 1 µM microcystin. To that end, tumor powder was mixed with complete lysis buffer in a ratio of 1:10 (weight : volume). After one freeze-thaw cycle, insoluble debris was removed by centrifugation and protein lysates transferred to fresh tubes and kept at -80°C until further analysis. Total protein content of the homogenates was measured by using Coomassie Plus protein assay kit (Pierce cat #1856210). To quantify the phospho-Met / total Met levels, a MSD 96-well MULTI-SPOT Phospho (Tyr1349)/Total Met Assay (Meso Scale Discovery, Gaithersburg, Maryland, USA, cat # K15126D-2) was used according to the manufacturer's instructions. Briefly, tumor lysates (100 µg of total protein) were added to pre-spotted MSD plates with anti phospho-Met, total Met and BSA wells, which were blocked with 3% BSA. After a 2 hour incubation with shaking at room temperature, the wells were washed 3 times with TBS-T (50 mM Tris HCl pH 7.4, 150 mMNaCl, 0.02% Tween 20). Then, 25 µL of diluted SULFOTAG anti- total Met (1:50 in dilution buffer provided in

the MSD kit) were added per well and the plate was incubated at room temperature with shaking for 1 hour. Finally, the wells were washed 3 times with TBS-T and 150 µL of MSD Read buffer T (1:4 diluted in nanopure water) were added to each well before reading on a SectorImager 6000 (Meso Scale Discovery). For graphic representation, the ratio of phospho-MET to total MET was calculated for all tumor samples. Other (phospho-)proteins were visualized by Western blot analysis as described before. Concentration of INC280 and BGJ398 in plasma and tumor lysates were determined by LC-MS following standard procedures.

Supplementary References

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