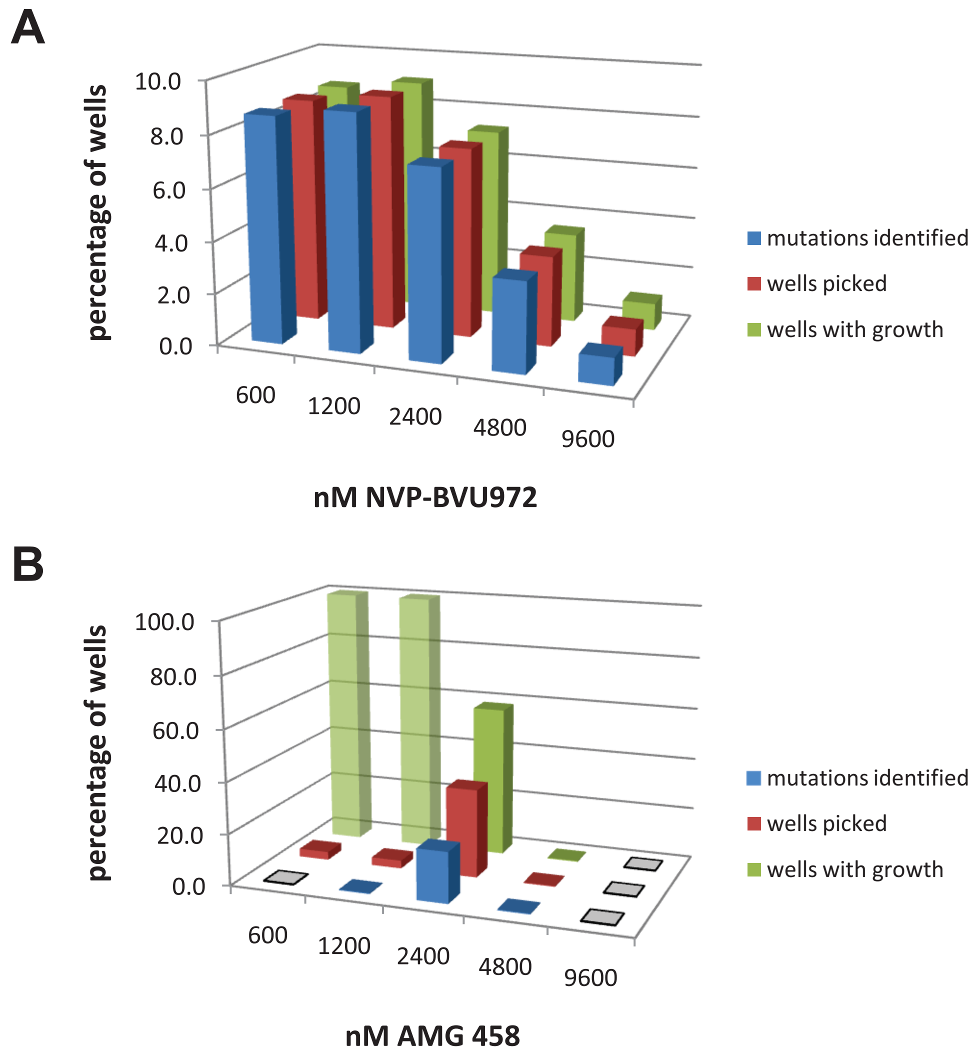
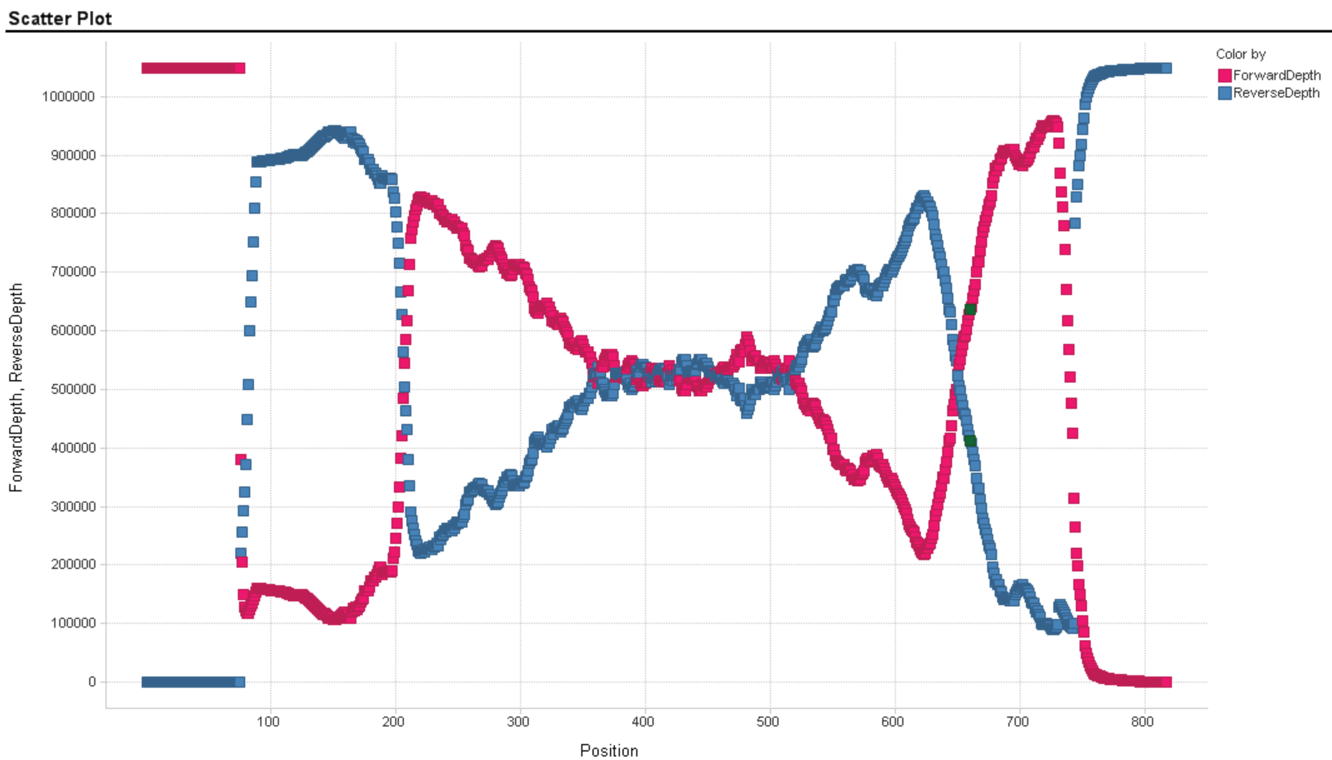


Supplementary Figure S1. Percentage of wells in which cell growth was observed (green), cells were picked and expanded (red) and mutations in the kinase domain were identified (blue). A total of 288 wells (three 96-well-plates) per concentration of the respective MET inhibitor were analyzed. (A) shows data for NVP-BVU972 and (B) for AMG 458. Note that with 600 and 1200 nmol/L of AMG 458 inhibition of proliferation was insufficient and growth was observed in all wells. Therefore, only a small randomly selected subset of 9 wells at each concentration was further processed, yielding only one kinase domain mutation. At 2.4 mmol/L of AMG 458 and above, the vast majority of BaF3 TPR-MET cells were killed, resulting in outgrowth of resistant clones starting after approximately 2 weeks and continuing beyond 4 weeks. A total of 168 out of 288 wells (58%) showed signs of growth, i.e. media color change and microscopically visible outgrowth of living cells. Whereas many clones picked at early time points contained kinase domain mutations, the majority of out-growing cells that appeared at later time points displayed a wild type sequence within the sequenced region. In total, 57 of the 99 analyzed clones (58%) contained a kinase domain mutation. At 4.8 mmol/L AMG 458, only one clone was obtained and found to contain a mutation, whereas no growth was observed at 9.6 mmol/L AMG 458.



Supplementary Figure S2. The depth of sequence coverage (NGS) for NVP-BVU972 at each nucleotide position across the 818 basepair Trp-Met PCR amplicon. The blue and red lines represent the forward and reverse depth of sequence coverage respectively.

Residue	Amino acid alteration	DNA alteration	Number of clones	Percent
Y1230			**75**	**88.2**
	Y1230C*	a3689g	31	36.5
	Y1230H*	t3688c	20	23.5
	Y1230N	t3688a	19	22.4
	Y1230S	a3689c	4	4.7
	Y1230D*	t3688g	1	1.2
D1228			**8**	**9.4**
	D1228N*	g3682a	5	5.9
	D1228E	c3684a	2	2.4
	D1228A	a3683c	1	1.2
V1155	V1155L	g3463t	**1**	**1.2**
F1200	F1200I	t3598a	**1**	**1.2**

Supplementary Table S1. Number and percentage of resistance mutations that were found in isolated BaF3 TPR-MET clones growing in the presence of NVP-BVU972. Amino acid and base pair numbering refer to NCBI Reference Sequence NM_000245.2. Asterisks mark mutations that were previously discovered in cancer patients without prior MET inhibitor treatment.

Residue	Amino acid alteration	DNA alteration	Number of clones	Percent
F1200			**36**	**60**
	F1200L	t3598c	17	28
	F1200L	t3600a	8	13
	F1200L	t3600g	3	5
	F1200I	t3598a	8	13
M1250	M1250T*	t3749c	**6**	**10**
M1211	M1211L	a3631c	**5**	**8**
L1195			**4**	**7**
	L1195I	c3583a	3	5
	L1195V*	c3583g	1	2
G1163			**3**	**5**
	G1163V	g3488t	2	3
	G1163E	g3488a	1	2
V1155	V1155L	g3463c	**1**	**2**
Y1159	Y1159N	t3475a	**2**	**3**
K1193	K1193I	a3578t	**1**	**2**
D1228	D1228A	a3683c	**1**	**2**
Q1258	Q1258L	a3773t	**1**	**2**

Supplementary Table S2. Number and percentage of resistance mutations that were found in isolated BaF3 TPR-MET clones growing in the presence of AMG 458. Amino acid and base pair numbering refer to NCBI Reference Sequence NM_000245.2. Asterisks mark mutations that were previously discovered in cancer patients without prior MET inhibitor treatment.

Sample Variant	Reference Nucleotide	Reference AA	AA Position	MisReadNuc	MisRead AA	Sequence Depth	Forward Depth	Reverse Depth	N	MisreadCount	MisReadFrequency	pvalue (MisReadNuc)	Original Screen
NVP-BVU972	C	L	1195	a	I	1048515	517281	531231	3	1729	0.0016	0.007387194	No
NVP-BVU972	T	F	1200	a	I	1048514	518777	529732	5	47119	0.0449	6.0698E-10	Yes
NVP-BVU972	T	Y	1230	c	H	1048509	438228	610277	4	229573	0.2190	4.99028E-09	Yes
AMG 458	G	G	1163	a	R	1048512	591932	456576	4	11803	0.0113	2.58978E-06	No
AMG 458	G	G	1163	c	R	1048512	591932	456576	4	8327	0.0079	0.00192011	No
AMG 458	G	G	1163	t	V	1048510	595128	453378	4	8340	0.0080	2.65306E-07	Yes
AMG 458	C	L	1195	g	V	1048507	507688	540816	3	36700	0.0350	6.11317E-05	Yes
AMG 458	C	L	1195	a	I	1048507	507688	540816	3	32768	0.0313	6.79954E-09	Yes
AMG 458	T	F	1200	a	I	1048496	516477	532017	2	137491	0.1311	1.96954E-13	Yes
AMG 458	T	F	1200	c	L	1048496	516477	532017	2	118594	0.1131	1.20308E-07	Yes
AMG 458	T	F	1200	a	L	1048494	513648	534843	3	143589	0.1369	1.38334E-13	Yes
AMG 458	T	F	1200	g	L	1048494	513648	534843	3	38173	0.0364	5.37502E-05	Yes
AMG 458	T	M	1250	c	T	1048450	234222	814227	1	8032	0.0077	0.00168972	Yes

Supplementary Table S3. NVP-BVU972 and AMG 458 variants identified by the NGS platform. There was an even distribution on the forward and reverse strand for all variants, but an upper depth limit of approximately 1 million base pairs was observed in the MAQ assembly. The MisReadFrequency was calculated by dividing the MisReadCount for a specific mismatch by the sequence depth at the particular base with variants detected at frequencies as low as 0.1%. The p values for each variant were calculated for each nucleotide as specified in the Supplementary Materials and Methods. The variants detected by the NGS platform largely correlated with the variants listed in Supplementary Tables S1 and S2 with the 3 discrepancies present at MisReadFrequencies at or below 1%.

Mutation	BVU972	PF-02341066	AMG 458	XL880
wt	172	179	794	73
Y1230H	>10’000	1814	1187	53
D1228A	>10’000	1970	3215	172
F1200I	1948	1202	3910	886
wt	104 ± 42	40 ± 7	296 ± 25	na
Y1230H	>10’000	413 ± 64	201 ± 33	na
L1195V	862	395 ± 83	3228 ± 1304	na

Supplementary Table S4. IC_{50} values in nmol/L (± standard deviation where applicable) of four structurally diverse MET inhibitors tested in proliferation assay with BaF3 TPR-MET cells containing the indicated mutations. Upper section: Mutations were generated by site-directed mutagenesis in a pMSCVpuro TPR-MET vector and expressed in BaF3 cells by retroviral transfer. Proliferation assays were performed manually with 72 h compound exposure. Values indicate average ± standard deviation (SD; N=3). Lower section: Mutations were generated by site-directed mutagenesis in a pCDNA3.1 TPR-MET vector and expressed in BaF3 cells by stable transfection. Proliferation assays were performed in triplicates in a robotic system with 48 h compound exposure. Values indicate average ± SD where N=3, no SD value where N=1. >10’000 indicates assay results where an IC_{50} was not detectable at the maximal compound concentration of 10’000 nmol/L (N=3 in both sections).

Supplementary Materials and Methods

BaF3 proliferation assays

BaF3 cells containing TPR-MET (14) or various mutants thereof were grown in RPMI 1640 medium containing 10 % fetal calf serum. For maintenance of parental BaF3 cells the medium was additionally supplemented with 10 ng/mL interleukin-3 (IL-3; Biosource). For proliferation assays, BaF3 cells were seeded on 96-well-plates in triplicates at 10000 cells per well and incubated with various concentrations of MET inhibitors for 72 hours followed by quantification of viable cells using a resazurin sodium salt dye reduction readout (commercially known as AlamarBlue assay). IC_{50} values were determined with the XLFit Excel Add-In (ID Business Solutions) using a 4-parameter dose response model.

TR-FRET biochemical assay with MET wild type and mutants

Purified recombinant enzymes were purchased from ProQinase GmbH. Constructs comprised human MET amino acids 956-1390 fused to an N-terminal glutathione-S-transferase (GST) tag. Enzyme activity was measured in a time resolved fluorescence resonance energy transfer (TR-FRET) assay, detecting tyrosine phosphorylation with a Eu-labelled anti-phospho tyrosine antibody (fluorescence donor) and allophycocyanin conjugated to streptavidin (fluorescence acceptor) which binds to a biotin on the substrate peptide. For each variant, K_m concentrations for ATP were determined in the absence of compound, and the ATP concentration in the kinase reaction was set to K_m (4 μmol/L for MET wt, 1 μmol/L for MET Y1230H and MET F1200I). Compounds were dissolved and diluted in DMSO and assayed in quadruplicate. Kinase reactions were carried out in 50 mmol/L Tris-HCl pH 7.5, 8 mmol/L $MgCl_2$, 4 mmol/L $MnCl_2$, 0.05 % Tween 20, 0.05 % bovine serum albumin, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L Na_3VO_4, in white 1536 well plates (Greiner) at room temperature. Compound and enzyme were incubated in a volume of 2 µl for 20 min, followed by the addition of 1 µl ATP and 1 µl biotinylated peptide substrate (PTK1, Promega product number V287A) to final concentrations of K_m and 1 μmol/L, respectively. Enzyme concentrations in the reactions were 5 nmol/L for MET wt, and 4 nmol/L for the F1200I and Y1230H variants. After 90 min, reactions were stopped by addition of 1 µl stop / detection solution to reach final concentrations of 10 mmol/L EDTA, 3.5 nmol/L Eu-labelled anti-phospho-tyrosine antibody PY20 (Invitrogen), and 10 nmol/L Streptavidin Allophycocyanin (Perkin Elmer). Time resolved fluorescence resonance energy transfer was measured in an Envision plate reader (Perkin Elmer, excitation 320 nm, emission 615 nm and 665 nm).

Analysis of tyrosine phosphorylation by Western blotting

BaF3 TPR-MET cells (unmutated and selected mutants) were treated with 0, 0.01, 0.1, 1 or 10 µmol/L NVP-BVU972 or AMG 458 for two hours, then washed twice with 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. Protein concentration of cleared lysates was determined with the BCA Protein Assay Kit (Novagen). 10 µg protein of each sample was separated by SDS-PAGE , transferred to a PVDF-membrane, and probed with antibodies specific for β-tubulin (T4026, Sigma), total MET (sc-10, Santa Cruz Biotechnologies) and phospho-MET Tyr1234/1235 (#3126, Cell Signaling Technology). After washing and incubation with secondary HRP-linked antibodies, bands were visualized using ECL detection reagent (GE Healthcare).

Protein kinase activities measured by the LanthaScreen TR-FRET method (kinase selectivity panel)

Based on the optimized assay conditions for the individual kinases a generic assay set-up was chosen. The assay has been run at room temperature on a liquid handling robot. To the assay plates containing 50 nL compound or control solutions, 4.5 µL of ATP mix (20 mmol/L Tris-HCl pH 7.4, 1 mmol/L DTT, 0.025 % Tween 20, 0.01 mmol/L Na_3VO_4, and 4 µmol/L ATP) were added per well. Subsequently, 4.5 µL of enzyme-substrate mix (20 mmol/L Tris-HCl pH 7.4, 1 mmol/L DTT, 0.025 % Tween 20, 0.01 mmol/L Na_3VO_4, 0.5 % BSA, and 100 nmol/L poly(EAY)) were added, with individual concentration for kinase and divalent cations. The final reaction volume is 9.05 µL with final reagent concentrations of 20 mmol/L Tris-HCl pH 7.4, 1 mmol/L DTT, 0.025 % Tween 20, 0.01 mmol/L Na_3VO_4, including a generic concentration of 2 µmol/L ATP and 50 nmol/L of substrate poly(EAY). Enzyme concentrations and divalent cations were adjusted to the individual enzyme requiremants. After 1 hour of incubation the kinase reactions have been stopped by the addition of 4.5 µL of stop solution (20 mmol/L Tris-HCl pH 7.4, 1 mmol/L DTT, 0.025 % Tween 20, 0.01 mmol/L Na_3VO_4, 50 mmol/L EDTA) immediately followed by 4.5 µL of antibody solution (20 mmol/L Tris-HCl pH 7.4, 1 mmol/L DTT, 0.025 % Tween 20, 0.01 mmol/L Na_3VO_4, 1.72 µg/ml Tb-PY20). After an incubation time of 45 min in the dark, the plates were transferred into a fluorescence reader and counted in time resolved fluorescence mode (settings according to the reagent supplier's recommendation). The effect of compound on the enzymatic activity was obtained from end point measurement.

Protein kinase activities measured by the Caliper method (kinase selectivity panel)

All assays were performed in 384 well microtiter plates. Each assay plate contained 8-point serial dilutions for 40 test compounds, as well as two 16-point serial dilutions of staurosporine as reference compound, plus 16 high- and 16 low controls. Liquid handling and incubation steps were done on a Thermo CatX workstation equipped with a Innovadyne Nanodrop Express. Between pipetting steps, tips were cleaned in wash cycles using wash buffer. Plates with terminated kinase reactions were transferred to the Caliper LC3000 workstations for reading. Phosphorylated and unphosphorylated peptides were separated using the Caliper microfluidic mobilitishift technology and kinase activities were calculated from the amounts of formed phospho-peptide.

Kinase reactions were prepared in 384 low volume plates by the following sequence:

1. 0.05 µl compound (start with 1.8 mmol/L in 90 % DMSO/10 % H2O)
2. + 4.5 µl 2x peptide/ATP solution
3. + 4.5 µl 2x enzyme solution
4. Incubate for 60 min at 30 °C
5. + 16 µl stop/run buffer

Independent of the kinase, all reactions were performed in 50 mmol/L HEPES, pH 7.5, 1 mmol/L DTT, 0.02 % Tween 20, 0.02 % BSA, and 0.6 % DMSO. Other components were assay specific and optimized for each kinase individually.

MET autophosphorylation measured by AlphaScreen® detection

The human gastric carcinoma cell line GTL-16 shows an approximately 10-fold amplification of the non-rearranged Met gene resulting in overexpression of the MET receptor that is constitutively phosphorylated on tyrosine (1). GTL-16 cells were plated in 384-well plates at a density of 10'000 cells in 20 µl complete growth medium (DMEM high glucose supplemented with 10 % (v/v) heat inactivated fetal FCS and 1 mM sodium pyruvate) and were incubated at 37°C / 5 % CO2 / 95 % humidity for 20h. The cells were washed and the 30 µl assay buffer (DMEM high glucose, 1 mM sodium pyruvate, 0.1 % BSA) was added. Compounds were diluted in 384-well compound plates to

obtain 8-point serial dilutions for 40 test compounds in 90 % DMSO, as well as a reference compound plus 16 high- and 16 low (inhibited) controls. These compound plates were prediluted 1:200 in assay buffer into a compound predilution plate and 10 μl of prediluted compound solution were transferred to the cell plate using a 384-well pipettor, resulting in a final DMSO concentration of 0.11 %. Cells were incubated for 1 h at 37°C / 5 % CO2 / 95 % humidity. The supernatant was removed, the cells were lysed in 25 μl of RIPA lysis buffer supplemented with 2 mM sodium vanadate and a protease inhibitor cocktail, and cell plates were stored at -80°C.

For detection of phopspho-MET, an AlphaScreen® -based assay was used. AlphaScreen® (Amplified Luminescent Proximity Homogeneous Assay, ALPHA, Perkin Elmer) is a non-radioactive bead-based proximity assay technology to study biomolecular interactions in a homogenous microtiter plate format. This technique has been adapted to measure the phosphorylation of endogenous cellular proteins in cell lysates by the use of specific antibody pairs against the total protein and a phospho-specific epitope. 5 μl of cell lysate was transferred to 384-well low volume Proxiplates for detection using a 384-well pipettor. First, 5 μl of a premix of an anti-cMet antibody (0.25 μg/mL f.c.), a biotinylated PY20 antibody (0.05 μg/mL f.c.) and AlphaScreen® Protein A-coupled acceptor beads (10 μg/mL f.c.) in RIPA buffer supplemented with 0.25 % (v/w) TOP BLOCK, 2 mM sodium vanadate, and a protease inhibitor cocktail and was added, the plate was sealed, and incubated on a plate shaker for 2 hours at room temperature. Second, 2 μl of dilution buffer containing AlphaScreen® Streptavidin-coated donor beads (10 μg/mL f.c.) in RIPA buffer was added, and the plate was incubated on plate shaker as above for a further 2 hours. The plate was read on an AlphaScreen® compatible plate reader, using standard AlphaScreen® settings.

Measurement of HGF-induced MET phosphorylation in A549 cells by Multispot ELISA

In order to increase the maximal phospho-MET signal, a subclone of A549 cells was generated by stable transfection that overexpresses full length MET. These cells were maintained in RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, and 1 mg/ml G418. For MET phosphorylation assays, cells were seeded at a density of 30'000 per well. On the following day, the attached cells were washed and then kept in serum-free medium (where FCS was replaced with 0.1 % bovine serum albumine). After 24 h of starvation, cells were exposed to a dilution series of the test compound (final concentration of 0.1 % DMSO) for 90 minutes, followed by stimulation with 200 ng/mL human recombinant HGF (Peprotech) for 10 minutes. Controls without HGF stimulation and with HGF but no compound were prepared to determine the multiple by which MET phosphorylation was stimulated. After HGF exposure, cells were washed two times with ice-cold PBS and lysed in 50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L sodium chloride, 20 mmol/L sodium fluoride, 1 mmol/L EDTA, 6 mmol/L EGTA, 15 mmol/L sodium pyrophosphate, 1 mmol/L benzamidine, 0.1 mmol/L PMSF, 0.5 mmol/L sodium vanadate, 1 % NP-40 supplemented with Complete protease inhibitor cocktail (Roche) and Phosphatase Inhibitor I and II (Sigma). A Multi-spot Phospho-(Tyr 1349), total Met Assay (Meso Scale Discovery) was used according to the manufacturer's instructions to determine the ratio of phosphorylated to total MET in each sample. In samples not stimulated with HGF, phospho-Met was barely above the detection limit, whereas HGF increased Y1349 phosphorylation by more than 100-fold. IC_{50} values for inhibition of MET phosphorylation were determined with the XLFit Excel Add-In (ID Business Solutions) using a 4-parameter dose response model.

Cellular proliferation assays (GTL-16, EBC-1, MKN-45)

The MET-amplified gastric cancer cell lines GTL-16 and MKN-45 were grown under standard cell culture conditions in DMEM (high glucose) and RPMI 1640, respectively. EBC-1 MET-amplified lung cancer cells were propagated in MEM-EBS. All media were freshly supplemented with 10 % heat-inactivated fetal calf serum and 2 mmol/L L-glutamine. For proliferation assays, cells were seeded at a density of 3000 per well in 96-well-plates. 24 h later, a 10-point dilution series of compound (3-fold steps, ranging from 10 mM to 0.5 mM plus vehicle control) was prepared in DMSO. Compound was then diluted 1000-fold in growth media in two steps and added to cells in triplicates, resulting in a final volume of 100 μL per well and maximal final compound concentrations of 10 μmol/L. Cells were incubated for 72 h and the amount of viable cells was then measured by adding 20 μL of MTS reagent (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega), further incubation for 30’, and reading of the optical density at 490 nm. IC_{50} values were determined with the XLFit Excel Add-In (ID Business Solutions) using a 4-parameter dose response model.

BaF3 cellular selectivity panel

Selectivity profiling in BaF3 cell models transformed with activated tyrosine kinases has been done as described (2). Parental BaF3 cells as well as BaF3 strains containing the following oncogenic kinase variants were tested: BCR-ABL, NPM-ALK, ERBB2(V659E), TEL-FGFR3, FLT3(ITD), TEL-IGF1R, KIT(D816V), TPR-MET, PTC3-RET, TEL-PDGFRβ, TEL-INSR1, TEL-JAK2, TEL-KDR, JAK2(V617F).

Generation of mutant BaF3 TPR-MET strains by site directed mutagenesis

A QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used to generate the desired mutations in TPR-MET that had been cloned into the eukaryotic expression vector pDNA3.1 (Invitrogen; sequence of construct available on request). The resulting constructs were then transfected with a Nucleofector device (Lonza) into parental BaF3 cells, followed by selection with 800 μg/mL G418 (Gibco) and stepwise withdrawal of IL-3. Alternatively, TPR-MET was cloned into the MSCVpuro TPR-MET retroviral vector (Clontech) and mutations were introduced as before. The resulting constructs were then transfected into EcoPack 2-293 cells (Clontech) to produce retrovirus-containing supernatant with which parental BaF3 cells were infected. Presence of the respective mutations in the resulting IL-3 independent strains was confirmed in by PCR amplification and sequencing of the MET kinase domain as above.

Generation of resistant BaF3 cell pools that were analyzed by NGS

In an attempt to streamline the process of resistance mutation profiling by application of next generation sequencing technology, a second screen was set up in which resistant BaF3 TPR-MET cells were selected in a pool without isolation of single clones. Unexpectedly, no outgrowth of viable cells was observed in large flasks when NVP-BVU972 was applied at concentrations of 600 nmol/L or above. Lowering the concentration to 120 nmol/L (~1-2 times IC_{50}) resulted in an outgrowing pool of viable BaF3 cells after approximately 2 weeks. Isolation of DNA and amplification of the MET kinase domain by PCR was done as for single clones. BaF3 cells resistant to 2.4 μmol/L AMG 458 were selected in 96-well-format as before, but all wells that showed signs of growth were pooled prior to isolation of genomic DNA and PCR.

NGS protocol

PCR amplification of the MET kinase domain was done as before. One µg of each 818 base pair TPR-MET amplicon was sheared to 200-250 base pair fragments using a Covaris S2 instrument. These fragments were prepared for the Illumina NGS platform using the Illumina genomic DNA prep kit (cat # FC-102-1001). A 75 base pair single read reaction was performed with standard Illumina reagents, instrumentation, and conditions. Approximately 25 million reads per sample were assembled using the MAQ software (http://maq.sourceforge.net/index.shtml), and the resultant data was used to generate mutation frequencies with associated p-values at each position for all possible nucleotide substitutions in all 4 samples (Supplementary Material and Methods).

cMet Resistence Mutation Calling (NGS)

1) Calculate freq of a misread in both forward and reverse for each nucleotide at each position. Smooth data denominator by to avoid devision by zero

Ex: fAFreq = fACounts/(ForwardDepth+1)

2) Calculate a mean of the forward and reverse frequency for each nucleotide at each position

Ex: mAFreq = (fAFreq+rAFreq)/2

3) Transform all frequencies to log space to produce a more normal distribution

4) Calculate p.values by calculating the z score for each frequency relative to a ctrl distribution (Sample = Trp_Met-ENU) and and then looking up the probability of that z.score from the **cumulative normal distribution function** of the normal distribution.

Supplementary references

1. Giordano S, Ponzetto C, Di Renzo MF, Cooper CS, Comoglio PM. Tyrosine kinase receptor indistinguishable from the MET protein. Nature. 1989;339:155-6.
2. Boulay A, Breuleux M, Stephan C, Fux C, Brisken C, Fiche M, et al. The Ret receptor tyrosine kinase pathway functionally interacts with the ERalpha pathway in breast cancer. Cancer Res. 2008;68:3743-51.