Structural and molecular insight into resistance mechanisms of first generation cMET inhibitors.

Gavin W. Collie, Cheryl M. Koh, Daniel J. O'Neill, Christopher J. Stubbs, Puneet Khurana, Alice Eddershaw, Arjan Snijder, Fredrik Mauritzson, Louise Barlind, Ian L. Dale, Joseph Shaw, Christopher Phillips, Edward J. Hennessy, Tony Cheung and Ana J. Narvaez.

Supporting information

Contents:

1. Supporting figures

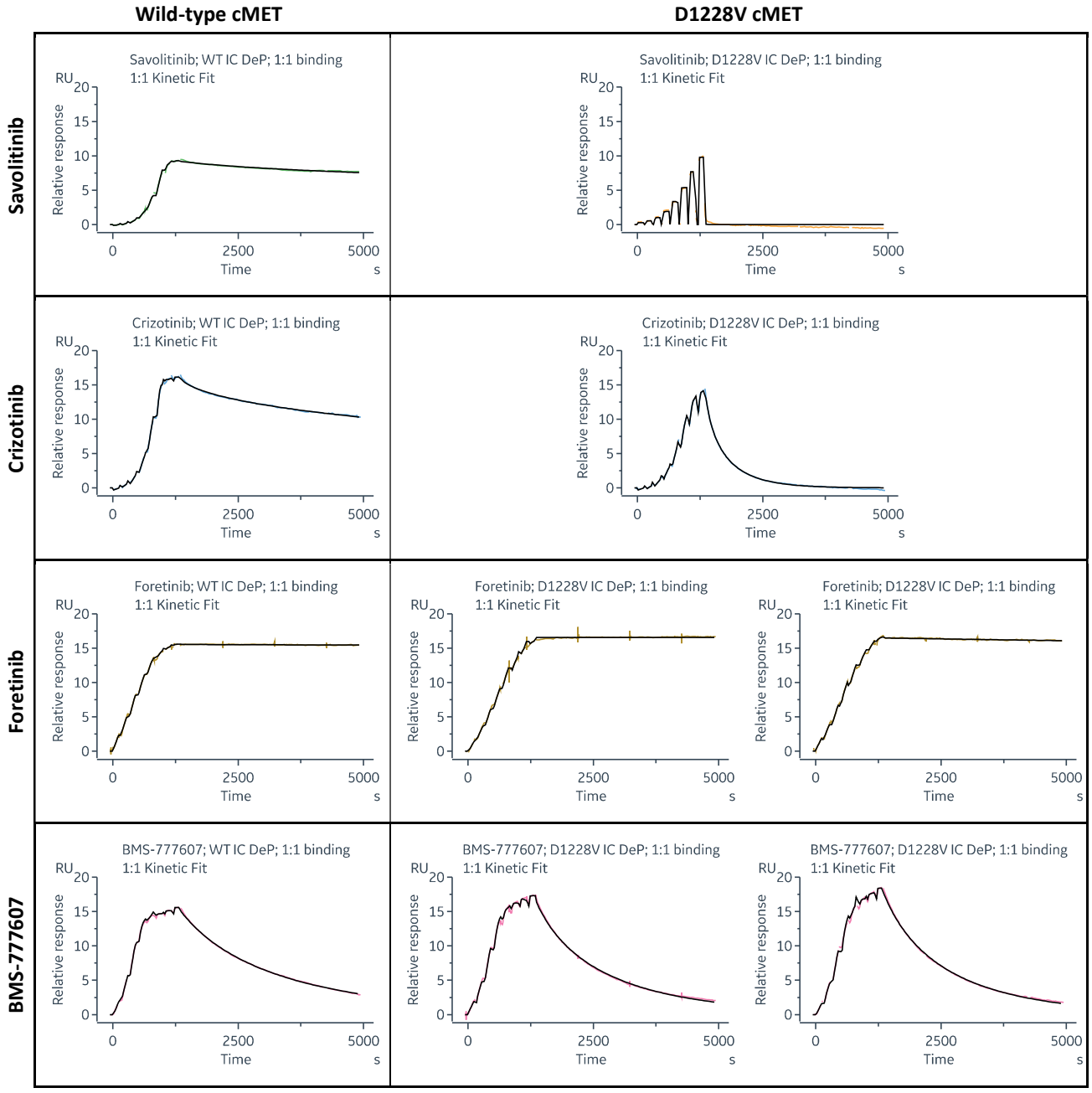

Figure S1. SPR sensorgrams showing binding of savolitinib, crizotinib, foretinib and BMS-777607 to the wild-type (left) and D1228V (right) intracellular domain of dephosphorylated cMET. Coloured lines represent the experimental data, and black lines show the best-fit to a 1:1 binding model. Affinity and kinetic parameters derived from these sensorgrams are shown in Tables S1 and S2.

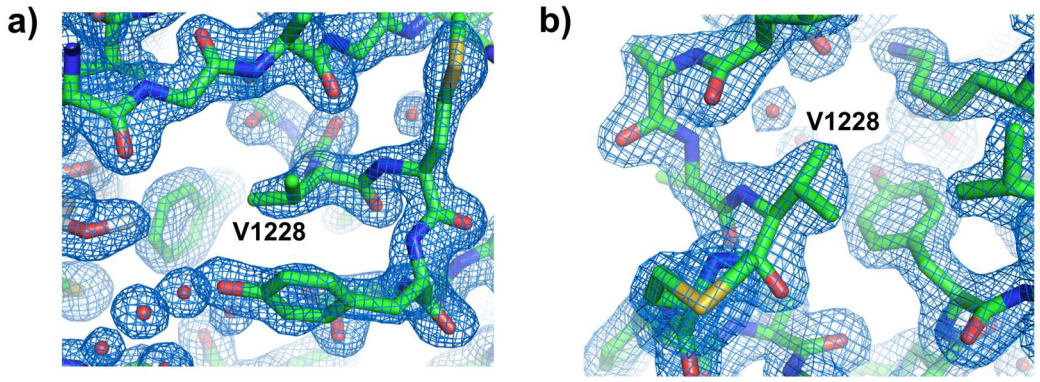

Figure S2. a) Electron density for D1228V cMET bound by foretinib centred on residue V1228. $2F_o$-F_c map at σ level 1, resolution 1.67 Å. b) Electron density surrounding residue V1228 of the D1228V cMET-BMS-777607 complex. $2F_o$-F_c map at sigma level 1.3, resolution 1.93 Å.

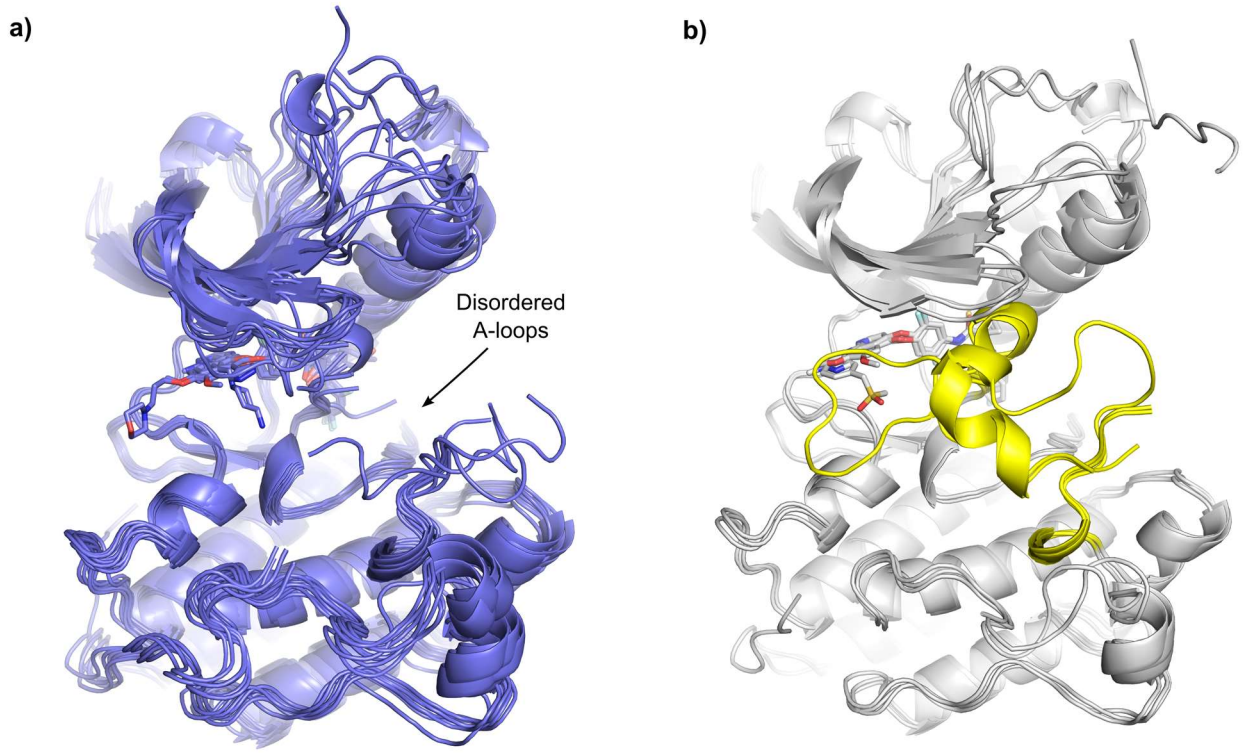

Figure S3. Comparison of type-II-cMET complexes available in the Protein Data Bank (PDB). Of the 11 crystal structures involving cMET bound by type-II kinase inhibitors that we could identify in the PDB, 8 have disordered A-loops (a) with 3 showing ordered A-loops (b) (A-loops coloured yellow in (b)). PDB entries shown here are: 3c1x, 3ce5, 3cth, 3ctj 3f82, 3l8v, 3lq8 and 4eev (a) and 3vw8, 4mxc and 5dg5 (b).

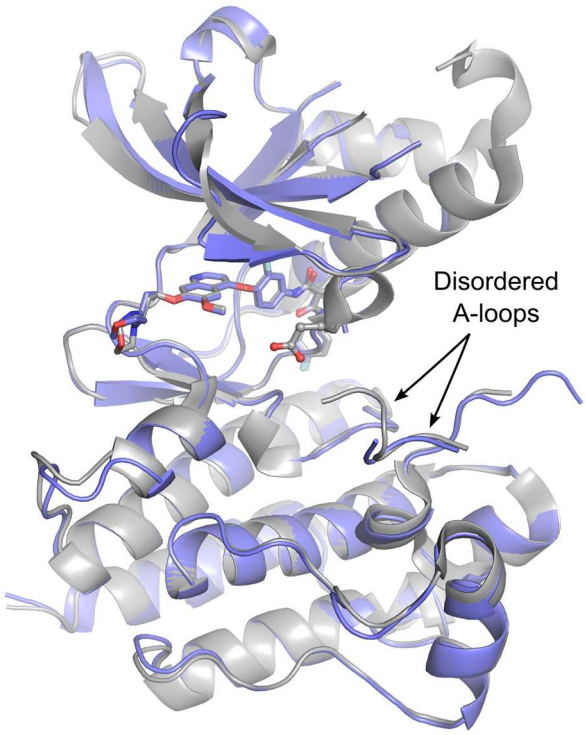

Figure S4. Structural alignment of wild-type cMET-foretinib crystal structures. Blue: crystal structure reported in this work; grey: crystal structure reported previously in alternative space group[1] (PDB entry 3LQ8). RMSD of alignment: 0.857 Å for 2046 to 2046 atoms.

2. Supporting tables

Table S1. Summary of cellular and *in vitro* inhibition studies of type-I and type-II inhibitors for wild-type and D1228V cMET

	pGI_{50} (Cell[1])		**pIC_{50} (Cell HTRF[2])**		**pIC_{50} (Biochemical[3])**		**pK_d (SPR[4])**	
cMET:	Wild-type	D1228V	Wild-type	D1228V	Wild-type	D1228V	Wild-type	D1228V
Savolitinib	8.03 ± 0.10	5.08 ± 0.42	8.70 ± 0.04	< 4.5	> 8.6	6.17 ± 0.03	10.2	5.4
Crizotinib	-	-	7.58 ± 0.14	5.99 ± 0.07	> 8.6	> 8.6	8.6	7.9
Foretinib	7.21 ± 0.07	7.28 ± 0.14	7.91 ± 0.03	7.63 ± 0.04	> 8.6	> 8.6	>9.1	>8.9
BMS-777607	-	-	6.70 ± 0.07	6.67 ± 0.02	> 8.6	> 8.6	8.7	8.4

[1]Three-day proliferation assay using parental NCI-H1993 cells expressing wild-type cMET and CRISPR/Cas9 modified NCI-H1993 cells expressing D1228V-mutated cMET. Values shown are average of three separate experiments ± standard deviation. $pGI_{50} = -\log_{10}(GI_{50})$, M. [2]HTRF-based measurement of potency of cMET inhibitors in NCI-H1993 and NCI-H1993 D1228V cMET cells. Cells were incubated with compounds for four hours before detection of phospho-cMET (Tyr1234/1235) by HTRF. Values are expressed as the mean ± standard error of mean (s.e.m.) of at least three separate experiments. $pIC_{50} = -\log_{10}(IC_{50})$, M. [3]ADP-Glo kinase assay using dephosphorylated wild-type or D1228V cMET comprising full intracellular domains. All potencies, except that measured for savolitinib for D1228V cMET, are beyond the theoretical tight binding limit of the assay. Value shown is expressed as mean ± s.e.m of three separate experiments. [4]SPR experiments were performed using a Biacore 8K instrument using the same wild-type and D1228V mutant cMET protein constructs as described for the biochemical ADP-Glo experiments. K_d values for foretinib are reported as inequalities due to very slow dissociation rate constants preventing reliable measurement of k_{off} and subsequently K_d. $pK_d = -\log_{10}(K_d)$, M.

Table S2. SPR analysis of wild-type and D1228V cMET

	Wild-type cMET					
	k_{on} ($M^{-1}s^{-1}$)	SE(k_{on} ($M^{-1}s^{-1}$))	k_{off} (s^{-1})	SE(k_{off} (s^{-1}))	R_{max} (RU)	K_D (M)
Crizotinib	2.9×10^6	2.8×10^2	6.9×10^{-4}	6.6×10^{-8}	16.0	2.4×10^{-9}
Savolitinib	1.9×10^6	1.9×10^4	1.1×10^{-4}	7.5×10^{-7}	9.2	5.8×10^{-11}
Foretinib	1.1×10^4	4.5×10^0	$<1 \times 10^{-5}$	-	15.5	$<8.9 \times 10^{-10}$
BMS-777607	2.9×10^5	2.8×10^2	6.4×10^{-4}	4.6×10^{-7}	15.2	2.2×10^{-9}

	D1228V cMET					
	k_{on} ($M^{-1}s^{-1}$)	SE(k_{on} ($M^{-1}s^{-1}$))	k_{off} (s^{-1})	SE(k_{off} (s^{-1}))	R_{max} (RU)	K_D (M)
Crizotinib	4.1×10^5	1.2×10^3	5.8×10^{-3}	1.8×10^{-5}	15.6	1.4×10^{-8}
Savolitinib	6.2×10^4	5.6×10^2	2.3×10^{-1}	2.0×10^{-3}	13.5	3.8×10^{-6}
Foretinib (1)	7.0×10^3	4.9×10^0	$<1 \times 10^{-5}$	-	16.7	$<1.4 \times 10^{-9}$
Foretinib (2)	7.6×10^3	3.7×10^0	$<1 \times 10^{-5}$	-	16.6	$<1.3 \times 10^{-9}$
BMS-777607 (1)	2.6×10^5	2.9×10^2	1.0×10^{-3}	9.5×10^{-7}	16.8	4.1×10^{-9}
BMS-777607 (2)	2.1×10^5	4.2×10^1	1.1×10^{-3}	1.8×10^{-7}	18.0	5.1×10^{-9}

The sensorgrams in Figure S1 were fit to a 1:1 binding model (with mass transport), and the best-fit parameters (and their fit standard errors) are shown above. Dissociation rate constants (k_{off}) slower than 1×10^{-5} s^{-1} (and the related K_D values) are reported as inequalities, as they cannot be reliably measured beyond this limit. Experiments were performed using dephosphorylated protein comprising the full intracellular domain of cMET (wild-type and D1228V; full details of protein construct used for these SPR experiments can be found below).

Table S3. X-ray data collection and refinement statistics

	Structure 1	Structure 2	Structure 3	Structure 4
cMET form	Wild-type	D1228V	Wild-type	D1228V
Compound	savolitinib	foretinib	foretinib	BMS-777607
Crystallisation conditions	15 % 2-propanol, 15 % PEG4K, 0.2 M PCPT[1] pH 7.5	15 % 2-propanol, 10 % PEG4K, 0.1 M NaHEPES pH 8	15 % 2-propanol, 17 % PEG4K, 0.1 M NaHEPES pH 8	8 % ethanol, 20 % PEG8K, 0.1 M PCPT[1] pH 7.5
Data Collection				
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell				
a, b, c (Å)	42.13, 43.36, 156.41	42.57, 80.40, 91.31	41.88, 73.73, 91.47	57.96, 67.81, 90.05
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	43.36 – 2.49 (2.56 – 2.49)	42.57 – 1.67 (1.70 – 1.67)	28.70 – 2.35 (2.49 – 2.35)	54.17 – 1.93 (1.96 – 1.93)
R_{meas} (%)	15.1 (175.9)	17.0 (140.2)	11.3 (48.6)	10.6 (145.2)
$CC_{1/2}$ (%)	99.7 (42.7)	98.2 (44.3)	99.7 (60.8)	99.8 (62.6)
I / σ	8.8 (1.0)	6.3 (1.1)	13.20 (3.43)	8.9 (1.2)
Completeness (%)	99.1 (94.5)	99.3 (91.6)	97.5 (85.7)	99.6 (96.9)
Reflections (total)	68729	225396	73251	170752
Reflections (unique)	10493	36878	12092	27276
Redundancy	6.5 (6.5)	6.1 (4.4)	6.1 (4.8)	6.3 (5.6)
Refinement				
Resolution (Å)	41.78 – 2.49	23.45 – 1.67	28.70 – 2.35	23.30 – 1.93
R / R_{free} (%)	20.3 / 27.3	20.1 / 24.2	21.0 / 27.0	21.0 / 23.0
Overall B-factor (Å²)	69.55	24.88	42.38	35.83
R.m.s. deviations				
Bond lengths (Å)	0.010	0.010	0.010	0.010
Bond angles (°)	1.07	0.97	1.08	0.94
PDB code	6SDE	6SDC	6SD9	6SDD

[1]PCPT = Sodium propionate, sodium cacodylate trihydrate, bis-tris propane.

3. Supporting materials and methods

Chemistry

All small molecule kinase inhibitors studied in this work were either synthesised following published procedures or purchased from commercial suppliers, and were shown to be >95 % pure based on NMR, LC-MS and HPLC methods.

Generation of CRISPR knock-in cells

For genome editing, NCI-H1993 cells harbouring wt-cMET were transfected by FuGENE HD (Promega) according to the manufacturers protocol with a plasmid encoding both Cas9-T2A-GFP and a guide RNA specific to the Exon 19 insertion site (TTCTTTATCATACATGTCTC). A synthetic single-strand DNA oligo donor with homology arms to cMET Exon 19 and harbouring the required nucleotide insertion was added to the transfection at a ratio of 1:1 by weight. The oligo donor (Ultramer oligo, IDT) was designed to harbour a silent mutation in the PAM site and an additional silent mutation to aid in downstream screening of single cell clones, and has the following sequence:

TTGTCCTTTCTGTAGGCTGGATGAAAAATTCACAGTCAAGGTTGCTGATTTTGGTCTCGCGAGAGTCATGTATGATAAAGAATACTAT
AGTGTACACAACAAAACAGGTGCAAAGCTGCCAGTGAAGTGGATGGCTTTGG

Transfected cells were positively selected for Cas9 expression by FACS sorting GFP positive cells and grown in the presence of 10 nmol/L savolitinib for 2 weeks before single cell cloning. Single cell clones were collated into 96 well plates, DNA extracted in Direct PCR Lysis Reagent (Viagen #301C) with 0.4 mg/ml proteinase K (Viagen #501-PL) and incubation at 85 ^{o}C for 40 mins. Samples were then diluted 1:5 in dH_2O and subject to ddPCR using primers: cMET ddPCR_F TTCTATTTCAGCCACGGGTAA and cMET ddPCR_R GCAGACTTTCCAAAGCCATCC and detection probes D1228V cMET FAM-TTGGTCT[C]GC[G]AGAG[T]CATGTA and wt-cMET HEX-TCTTGC[C]AG[A]G[A]CATGTATGATA. Positive clones were confirmed by Sanger sequencing and full exome sequencing.

Proliferation assay

30 µl of NCI-H1993 or NCI-H1993 cMET D1228V cells (5.5 x 10^4 cells/ml) were seeded into 384-well plates (Corning 3712) using a Wellmate Multidrop dispenser and allowed to attach overnight. The next day, the cells were Echo dosed with compounds (10 µM highest concentration, 10-point, half-log dilutions). The cells were incubated for 3 days, and the number of viable cells was measured using Cell Titer Glo Luminescent Viability Assay (Promega G7572).

Pathway analysis by western blotting

NCI-H1993 and NCI-H1993 cMET D1228V cells were treated with savolitinib or foretinib (1 µM final concentration) for 4 hours. The cells were lysed in RIPA buffer (Cell Signaling #9086) with protease inhibitors (Sigma 5892970001) and phosphatase inhibitors (Sigma 4906845001). Protein lysates were prepared in NuPAGE LDS Sample buffer (ThermoFisher NP0008) with reducing agent, heated at 95 ^{o}C for 10 mins and separated on NuPAGE 4-12 % Bis-Tris protein gels (ThermoFisher NP0321) for 45 mins at 200 V. The proteins were transferred to PVDF membranes (ThermoFisher LC2005) by semi-dry transfer (Biorad Trans-Blot SD Semi-Dry Transfer Cell, #1703940) for 30 mins at 15 V. The membranes were blocked for 1 hour at room temperature with 5 % BSA (Cell Signaling #9998) in TBST (Cell Signaling #9997S). The membranes were incubated with the following primary antibodies overnight (1:1000 in TBST with 1 % BSA): Actin (Cell Signaling #5057), MET (Cell Signaling #8198), pMET (Tyr1234/1235) (Cell Signaling #3077), AKT (Cell Signaling #9272), pAKT (Cell Signaling #4060). The membranes were washed 3 x 10 mins in TBST, and incubated with the secondary antibody, Anti-rabbit IgG, HRP linked Antibody (Cell Signaling #7074) (1:10000 in TBST with 1 % BSA) for 2 hours at room temperature. The membranes were washed 3 x 10 min in TBST. Proteins were visualized using SignalFire ECL Reagent (Cell Signaling #6883), and images were obtained on a GE ImageQuant.

HTRF assay

NCI-H1993 and NCI-H1993 cMET D1228V cells were plated into tissue culture treated white HiBase low volume 384 well plates (Greiner BioOne) at 800 and 3,000 cells/well respectively. Cells were allowed to adhere overnight by incubation under standard tissue culture conditions before treatment with test compounds, with additions made using an HP300 non-contact dispenser (Tecan). Following a 4 hour incubation, medium was removed from wells using a BlueWasher centrifugal plate washer (Blue Cat Bio) and 5 µl of phosphor-cMET (Tyr1234/1235) HTRF lysis/detection reagent added (CisBio, AstraZeneca custom assay) using a Multidrop Combi dispenser (Thermo Fisher Scientific). Plates were lidded and incubated at room temperature in the dark for a minimum of 2 hours before detection of HTRF signal (excitation 337nm, emission 620/665nm) using a Pherastar FS multimode reader (BMG Technologies).

Protein expression and purification

For biochemical assays and SPR analysis, wild-type and D1228V cMET constructs comprising residues 956-1390 each with an N-terminal HN-tag and avi-tag and a TEV protease recognition site were synthesised and cloned into pFastBac vectors. Protein expression was performed in Sf21 cells grown in wave-bioreactors or Thomson Optimum Growth flasks at 27 °C. An increase in cell diameter was confirmed for all growths indicating successful viral infection. Sf21 cells were harvested after 48 hours by centrifugation at either at 2500 or 3400 g for 15 mins. Cells were lysed by freeze-thawing in lysis buffer (100 ml/L cultured cells). The lysate was clarified by 60 min centrifugation at 35000 g at 4 °C. The supernatant was supplemented with 5 ml Ni-NTA superflow incubated for 2 hours, and washed with wash buffer. After elution, cMET containing fractions were further purified using size-exclusion chromatography on a superdex 200 column. Where applicable, cMET was biotinylated using BirA biotinylation kit (BirA-500, avidity) according to protocols described by the manufacturer. Samples were dephosphorylated using λ-phosphatase, where a 1:20 molar ratio of phosphatase to cMET was applied. After confirmation of dephosphorylation by MS, cMET was purified either by captoQ purification or by an additional size-exclusion purification.

For crystallographic studies, synthetic DNA coding for residues 1038-1346 of the kinase domain of human wild-type cMET with an N-terminal HN-tag followed by a TEV protease cleavage site was cloned into a pACYC Duet vector together with DNA encoding for catYopH(164-468)[2]. A similar construct was made for the D1228V human cMET mutant. Plasmids were transformed into *E. coli* cells (LOBSTR (Kerafast EC1001) or One Shot™ BL21 (DE3) Star™ (Thermo Fisher Scientific C601003)). Proteins were produced using 5 L Bioreactor-fed batch cultures with IPTG induction according to methods described in Soini et al., 2008[3]. In brief, 3.5 L minimal salt media was supplemented with 1 x BME vitamins (Sigma-Aldrich B6891), 40 g glucose per liter, 34 µg/ml chloramphenicol, Struktol J 673-A antifoam and a trace elements solution with additional $NaSeO_3$, $NaMoO_4$ and $NiCl_2$. The fermenter was inoculated at a starting OD of 0.2 and grown at a temperature of 37 °C for about 12 hours. Dissolved oxygen was maintained at 30 %, while a pH of 7 was maintained using 14 % ammonium hydroxide solution. The feed solution contained 50 % glucose/L and was supplemented with $MgCl_2$ (approx. 7.4 g/L) and 34 µg/ml chloramphenicol. After 12 hours the temperature was lowered to 12 °C with protein production induced using 1 mM IPTG after a 2 hour equilibration period. Cells were harvested after 48 hours by centrifugation. About 200 g of cells were resuspended at room temperature in 500 ml lysis buffer (50 mM tris-HCl pH 8.3, 150 mM NaCl, 25 mM imidazole, 10 % (v/v) glycerol, 1 mM TCEP, 1 mM PMSF, 0.5 % (w/v) triton X-100 plus protease inhibitors) and subsequently passed through a Constant Systems cell disruptor at 25 kPsi. The lysate was clarified by centrifugation at 15000 g for 30 mins at 4 °C. The HN-tagged protein was batch bound overnight to 10 ml of Ni-NTA superflow resin (Qiagen, 30410) and subsequently washed with wash buffer (50 mM tris-HCl pH 8.3, 150 mM NaCl, 25 mM imidazole, 10 % (v/v) glycerol, 1 mM TCEP). cMET was eluted using the same buffer supplemented with 300 mM imidazole. After proteolytical removal of the purification tag, a subtractive IMAC was run. Samples were further purified using size exclusion chromatography on a superdex 75 gel filtration column in 20 mM tris-HCl pH 8.3, 100 mM NaCl, 10 % (v/v) glycerol, and 1 mM TCEP. cMET containing fractions were pooled and concentrated to about 10 mg/ml. MS analysis confirmed that the cMET proteins were fully dephosphorylated. The final protein yield per liter of culture medium was in the range of 5-15 mg.

Biochemical enzyme inhibition studies

The ADP-Glo Kinase Assay (Promega) was used to determine the half maximal inhibitory concentration (IC_{50}) of crizotinib, savolitinib, foretinib and BMS-777607 for wild-type (WT) and D1228V cMET. Details of protein constructs including expression and purification can be found above. The assay buffer was common for both proteins and was composed of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.1 % Pluronic-127 and 5 mM glutathione. ATP was used at K_m^{app} concentrations of 40 µM and 22 µM for WT cMET and D1228V cMET respectively. Poly(L-glutamic acid-L-tyrosine) was added at five times its K_m^{app}, i.e. 430 µg/mL and 850 µg/mL for WT cMET and D1228V cMET respectively. The phosphorylation reaction was initiated by dispensing 2 µL of 10 nM WT or D1228V protein and 2 µL of substrate-mix (ATP and Poly(L-glutamic acid-L-tyrosine)) for a total kinase reaction volume of 4 µL/well. Following 60 minutes of incubation at room temperature, 2 µL ADP-Glo Reagent 1 was added. This was incubated for 45 minutes at room temperature to deplete the remaining ATP and to stop the kinase reaction. 4 µL of ADP-Glo Reagent 2 was added and the reaction was incubated at room temperature for 30 minutes to convert the ADP product to ATP, before luminescence measurement using an Envision plate reader. Genedata software was used to fit concentration-response data to calculate IC_{50} values.

Surface plasmon resonance

SPR experiments were performed using a Biacore 8K instrument (GE Healthcare) at 20 °C. A Series S Sensor Chip NTA (GE Healthcare) was docked into the system in 20 mM Bicine-Na pH 7.5, 100 mM NaCl, 0.1 mM TCEP, 0.05% (w/w) Tween-20 and 1% (v/v) DMSO. The Sensor Chip surface was conditioned with 3 x 60 s injections of 50 mM NaOH/1 M NaCl at 30 µL/min and 1 x 60 s injection of 500 mM EDTA. Proteins were diluted in running buffer to 15 µg/mL and immobilised onto the surface using a His-capture/couple approach. Briefly, the surface was activated using a 30 s injection of 0.2 M EDC/0.05 M NHS, followed by charging of the NTA group with a 60 s injection of 500 µM $NiCl_2$. The proteins were then injected for 120 s, followed by 3 x 60 s injections of 0.5 M ethanolamine/0.25 M EDTA to block remaining reactive groups and remove non-covalently bound protein. Reference surfaces were prepared identically but without the protein injection. All steps were performed at 20 °C, with a flow rate of 10 µL/min. This resulted on an average of 1500 ± 120 RU of protein on the active flow cells.

Single-cycle kinetic experiments were performed as 8-point 2-fold dilution series from the top concentrations listed below. Each concentration of ligand was injected for 120 s followed by a single dissociation phase of 3600 s at 30 µL/min.

	D1228V	**WT**
Crizotinib (nM)	200	200
Savolitinib (nM)	10000	100
Foretinib (nM)	2000	2000
BMS-777607 (nM)	1000	1000

Crystallography

The purified dephosphorylated kinase domains (residues 1038-1346) of wild-type and D1228V cMET were used at concentrations of approximately 10 mg/ml for crystallisation trials. Prior to crystallisation, compounds were added to protein samples to final concentrations of 1 mM from 100 mM stocks in 100 % DMSO, equivalent to a 3-fold molar excess of compound over protein. Co-crystals were grown at 20 °C in sitting drops composed of 100 nL of protein-inhibitor complex plus an equal volume of crystallisation reagent. Details of crystallisation conditions can be found in Table S3. Prior to data collection, if needed, crystals were cryo-protected in solutions composed of 75 % of the crystallisation reagent supplemented with 25 % glycerol. X-ray diffraction data were collected at the Diamond Light Source and SOLEIL synchrotrons. Data were processed using XDS[4], DIALS[5], xia2[6] and CCP4[7], and structures solved by molecular replacement using publicly available cMET structures[1, 8] using Phaser[9] from the CCP4 package[7]. Model building and refinement were performed using Coot[10] and Buster (Global Phasing Ltd.), respectively, with geometry restraints for small molecule inhibitors generated using Grade (Global Phasing Ltd.). Data collection and refinement statistics plus PDB accession codes can be found in Table S3.

4. Supporting information references

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