

Supplemental Material

Structure-based Design of Novel Class II c-Met Inhibitors: 1. Identification of Pyrazolone-based Derivatives

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Experimental Procedures

Crystallization of c-Met complexes. The kinase domain of c-Met (residues 1048–1350) was expressed, purified, and crystallized as described previously (1). Briefly, crystals grew in space group C222₁ at room temperature from solutions containing 12% (w/v) PEG 6000, 1.0 M lithium chloride, and 0.1 M sodium citrate, pH 5.0. Inhibitors were added to the protein solution at a concentration of 0.8 mM prior to crystallization. Microseeding was used to obtain large, single crystals suitable for data collection. Diffraction data were collected on a FRE rotating anode X-ray source equipped with an RAXIS IV++ detector and images were processed using DENZO/SCALEPACK (2). The structures were solved by molecular replacement with AMORE using 2RFN as a search model and they were refined by using CNX and REFMAC (3, 4). Model building was performed with COOT (5).

Table S1. Data collection and refinement statistics

cMet / Compound 26	
Data Collection	
Space group	C2221
Cell dimensions	
a, b, c (Å)	71.6, 82.0, 127.0
α, β, γ (°)	90, 90, 90
Resolution (Å)	20–2.00 (2.07–2.00)
Total reflections	116897
Unique reflections	23515
Completeness (%)	91.8 (57.5)
R_{merge}	0.054 (0.260)
$I/\sigma(I)$	35.8 (4.6)
Refinement	
Reflections used	21192
$R_{\text{work}}/R_{\text{free}}$	0.215/0.274
Average B-value (Å ²)	31.4
Number of atoms	
Protein	2234
Ligand	39
Water	257
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.20
PDB ID code	3U6H

c-Met Enzyme Assay. K_i values for specific compounds were derived from IC_{50} measurements versus the c-Met kinase determined using homogenous time-resolved fluorescence (HTRF) assays as previously described (6). Molecules were tested in a 10-point serial dilution using an ATP concentration of two thirds K_m value that was determined experimentally and calculated using the Eadie-Hofstee and Lineweaver-Burke methods.

Cell-based Assays (7). PC3 cells were obtained from American Type Culture Collection (ATCC, Rockville, Maryland). Cells were grown as monolayers using standard cell culture conditions. IC_{50} measurements of compound activities on HGF-mediated c-Met autophosphorylation were determined in serum-starved PC3 (human) using a quantitative electrochemiluminescent immunoassay. Cells were plated at a density of 20,000 cells/well in 96 well-plates. Twenty-four hours after plating, cells were starved in media containing 0.1% BSA for 18 to 20 hours. Cells were then treated with a 10-point serial dilution of each tested compound for one hour at 37 °C followed by stimulation with optimal concentrations of recombinant human HGF for 10 minutes at 37 °C. Cells were washed once with PBS and lysed (1% Triton X-100, 50 mM Tris pH 8.0, 100 mM NaCl, 300 μ M Na_3VO_4 and protease inhibitors). Cell lysates were incubated with a biotin-labeled goat-anti-c-Met antibody (BAF358 – for human c-Met -, BAF527 – for mouse c-Met -, R&D Systems, Minneapolis, MN) for capture followed by a mouse anti-phosphotyrosine antibody 4G10 (Upstate, Charlottesville, VA) and a BV-tagTM labeled anti-mouse IgG (BioVeris Inc., Gaithersburg, MD) as the detection antibody. Levels of c-Met phosphorylation were then measured on a BioVeris M-Series instrument. The IC_{50} values are calculated using Xlfit4-parameter equation.

Pharmacodynamic Assay (7). The effect of compound **26** on HGF-mediated c-Met phosphorylation *in vivo* was evaluated in the liver of female Balb/c mice. Compound **26** was

administered by oral gavage at 10, 30 or 100 mg/kg. Two hours after a single dose, 12 µg of human HGF was injected intravenously to phosphorylate c-Met in the liver. Five minutes after HGF injection, the livers were harvested and levels of phosphorylated c-Met were determined by a quantitative electrochemiluminescent assay. c-Met phosphorylation measured in livers from mice injected with BSA and treated with vehicle served as a negative control and indicated the background level. Livers from mice injected with HGF and treated with vehicle established the maximal level of c-Met phosphorylation in the liver. Statistical significance was evaluated by Analysis of Variance (ANOVA) followed by Bonferroni/Dunn post hoc test using STatView software v5.0.1. Data represent the mean (n=3) ± standard deviation.

Kinase selectivity panel for 26.

Table S2. Kinase selectivity profile of **26**

Kinase	IC50, nM ^a	Kinase	IC50, nM
RON	1.0	AURORA2	5760.8
c-MET	3.2	JAK3	10687.6
LCK	4.7	MSK1	14253.0
VEGFR-2	11.1	PKBA	14600.0
ABL	11.9	PKBa	18102.7
BTK	29.0	ALK	19550.6
RET	30.2	JAK1	20335.2
IRK	35.8	ERK1	32534.4
SRC	47.4	CDK5P25	>100000
IGFRK	56.7	JNK2	>100000
CKIT	66.9	PKAA	>100000
ABL_Me15I	84.4	AKT1	>125000
TIE2	294.8	CDK2E2	>25000
AURORA1	645.6	PKBB	>25000
FGFRK	1573.0		
CKIT_V654A	2559.9		
P38A	2693.6		
P70S6K	2709.6		
JAK2	2893.9		

^a Data from internal assay as well as Upstate kinase panel

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