Supporting information for

Characterization of FGF401 as a reversible covalent inhibitor of fibroblast growth factor receptor 4

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Supplementary Methods

Protein Expression and Purification

The expression and purification procedure of the kinase domain of human FGFR4 was performed as previously described.[1] Briefly, the kinase domain of FGFR4 (residues 445–753) was cloned into modified pET28a (Sosoo Mix, TsingKe Co, Ltd., China) to construct the recombinant vector, which contains a PresCission protease-cleavable N-Terminal 6×His tag to aid in protein purification. The mutations (V550M, V550L, C552S, and C552A) were constructed using the QuikChange site-directed mutagenesis kit (Vazyme Biotech Co, Ltd., China). Co-expression with untagged YOPH provided the non-phosphorylated protein, $FGFR4^{WT}$, and other corresponding mutations were purified by Ni-NTA affinity chromatography (GE Healthcare), PresCission protease cleavage to remove the 6×His tag, anion exchange chromatography (Mono Q) and size exclusion chromatography (GE Healthcare). The target proteins were collected and concentrated to about 10-15 mg/mL before being snap frozen in liquid nitrogen and stored at -80°C for biophysical studies.

MALDI-TOF MS

The molecular weight of samples was assessed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using α-cyano-4-hydroxycinnamic acid as the matrix on Autoflex III (Bruker, USA). The operation mode was reflective mode with positive ion detection. The accelerating voltage was 19000 V and the delayed extraction time was 200 ns. Samples (0.2 mg/mL) were dissolved in Milli-Q™ water and then mixed with saturated matrix solution (CH_3CN:H_2O 1:1, v/v) at a volume ratio of 1:1.

Crystallization

Inhibitors of FGF401, BLU9931, Ponatinib, and LY2874455 were purchased from Selleckchem (Huston, TX, USA). To generate FGFR4/FGF401 co-crystals, FGFR4 and FGF401 were mixed at a molar ratio of 1:2 on ice for overnight to allow the formation of covalent bonds between FGF401 and cysteine 552 in the kinase before crystallization. The FGFR4/FGF401 crystals were obtained at 4 °C using the hanging drop vapor diffusion method in a buffer composed of 0.1 M Tris-HCl pH 7.5, 20% PEG4K, 0.15 M $(NH_4)_2SO_4$, and 4% (v/v) formamide. Crystals grew in about 3 days and were cryo protected in 0.1 M Tris-HCl pH 7.5, 20% PEG4K, 0.15 M $(NH_4)_2SO_4$, and 4% (v/v) formamide plus 20% glycerol, and then flash frozen in liquid nitrogen for data collection.

X-ray data collection, data processing and structures solution

Diffraction data were collected both in our lab and at Shanghai Synchrotron Radiation Facility (SSRF), beamline BL17U. The diffraction data was processed using the HKL3000. Crystal structure was solved by molecular replacement with Phaser from the CCP4 package, using the previously solved FGFR4/LY2874455 structure (PDB: 5JKG) as the search model. Model building was carried out using Coot, and refinements were operated using phenix.refine in the PHENIX package. Data collection and structure refinement statistics are listed in Table S2. Structural graphics were presented by PyMOL. Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data bank under accession number 6JPJ.

Kinase Inhibition Assay

All FGFR kinase inhibition assays were performed with optimized kinase assay buffer consisting of 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 20 mM NaCl, 0.1 mg/mL BSA, 1 mM TCEP, and 4% DMSO. Five microliter of FGFR4 proteins (0.1 μM) were treated with FGF401 and LY2874455 inhibitor (12 concentrations ranging from 25 μM to 0.01 nM, in duplicate) in 384-well plates. Then the kinase reactions were initiated by the addition of 5×ATP plus poly (4:1 Glu, Tyr) peptides (Abcam, UK) and incubated at room temperature for 30 min. All reactions were terminated by the addition of stop buffer ADP-Glo (Promega, USA) after 40 min of incubation. Inhibition data were collected on a multimode plate reader (Perkin Elmer) after the addition of detection reagent. IC50 values were fit with a four-parameter log[Inhibitor] versus response model using GraphPad Prism software.

Ba/F3 Cell-Viability Assay

Ba/F3 cell (Cell Resource Center, IBMS, CAMS, China) viability assays were performed as previously described.[2, 3] Viral supernatants were collected and used to infect Ba/F3 cells in the presence of IL-3. After infection, the infected Ba/F3 cells were washed twice with PBS to remove IL-3, and then screened by puromycin. After one week screening, the Ba/F3 cell transfectants were used to test the inhibitor potency. Ba/F3 cell transfectants were seeded in 96-cell plates and treated with the indicated concentrations of the FGF401 and LY2874455 (12 concentrations ranging from 1000 nM to 0.01 nM, in duplicate). After 72 h incubation, cell viability was assessed by MTS assay using cck-8 kit (Sigma-Aldrich, USA). The IC50 values were calculated using

GraphPad Prism software.

Dialysis of FGFR4 after treatment with FGF401

FGFR4 (200 nM) was first activated in the kinase assay buffer consisting of 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 20 mM NaCl, 0.1 mg/mL BSA, 1 mM TCEP, and 4% DMSO, and then treated with 4 μM inhibitors for 60 min at room temperature. The reactions were transferred to a dialysis cassette (1 mL, 3 kDa MWCO) and dialysis in 1 L of kinase assay buffer at 4 ˚C. Buffer was removed every 24 h during the experiment time of 4 days. Fifty milliliter aliquots were removed each day for kinase assays and FGFR4 activity was determined using the kinase assay as described above.

References

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2. D. Wu, M. Guo, X. Min, S. Dai, M. Li, S. Tan, G. Li, X. Chen, Y. Ma, J. Li, L. Jiang, L. Qu, Z. Zhou, Z. Chen, L. Chen, G. Xu and Y. Chen, *Chem Commun (Camb)*, 2018, **54**, 12089-12092.
3. J. S. Melnick, J. Janes, S. Kim, J. Y. Chang, D. G. Sipes, D. Gunderson, L. Jarnes, J. T. Matzen, M. E. Garcia, T. L. Hood, R. Beigi, G. Xia, R. A. Harig, H. Asatryan, S. F. Yan, Y. Zhou, X. J. Gu, A. Saadat, V. Zhou, F. J. King, C. M. Shaw, A. I. Su, R. Downs, N. S. Gray, P. G. Schultz, M. Warmuth and J. S. Caldwell, *Proceedings of the National Academy of Sciences of the United States of America*, 2006, **103**, 3153-3158.

Supplementary Tables and Figures

Table S1. Selectivity and potency of LY2874455 and FGF401 against wild-type FGFR1-4 in kinase assay and Ba/F3 assay.

Kinase/inhibito	Kinase assay (nM)		Ba/F3 assay (nM)	
r	LY2874455	FGF401	LY2874455	FGF401
FGFR1	0.6	>10000	18.9	>1000
FGFR2	0.6	>10000	N.A	N.A
FGFR3	2.3	>10000	N.A	N.A
FGFR4	5.6	6.2	24.6	37.0

Table S2. Data collection and refinement statistics.

	FGFR4/FGF401 Complex
Wavelength (Å)	1.54
Resolution range (Å)	41.8 - 2.64 (2.73 - 2.64)
Space group	$P2_1$
Unit cell	42.28, 60.93, 61.15
	90.00, 98.56, 90.00
Total reflections	53200 (2433)
Unique reflections	8724 (589)
Multiplicity	6.1 (4.1)
Completeness (%)	95.25 (67.78)
Mean I/sigma(I)	14.36 (5.82)
Wilson B-factor	33.05
R-merge	0.08 (0.18)
R-meas	0.088
CC1/2	0.997 (0.984)
CC*	0.999 (0.996)
R-work	0.195 (0.275)
R-free	0.243 (0.301)
Number of non-hydrogen atoms	2444
macromolecules	2389
ligands	10
Protein residues	299
RMS (bonds)	0.008
RMS (angles)	0.84
Ramachandran favored (%)	97
Ramachandran outliers (%)	0
Clashscore	3.58
Average B-factor	38.30
macromolecules	38.40
ligands	52.90

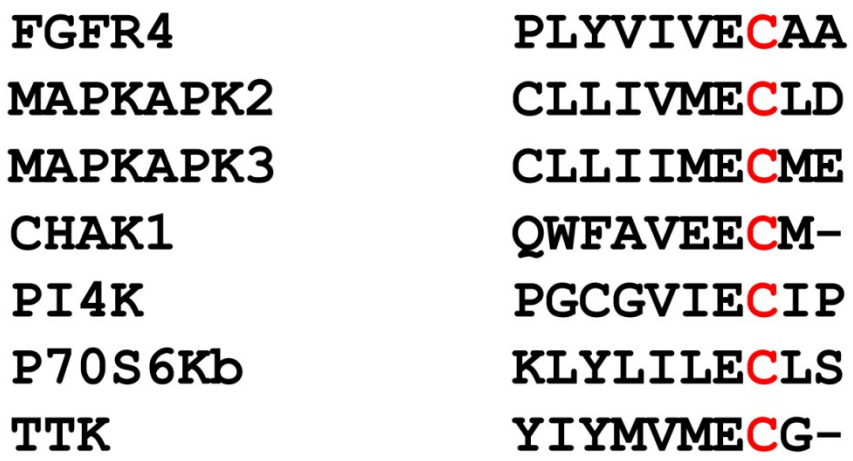

Figure S1. Kinases with a cysteine at the equivalent position of Cys552 in FGFR4 within the human kinome.

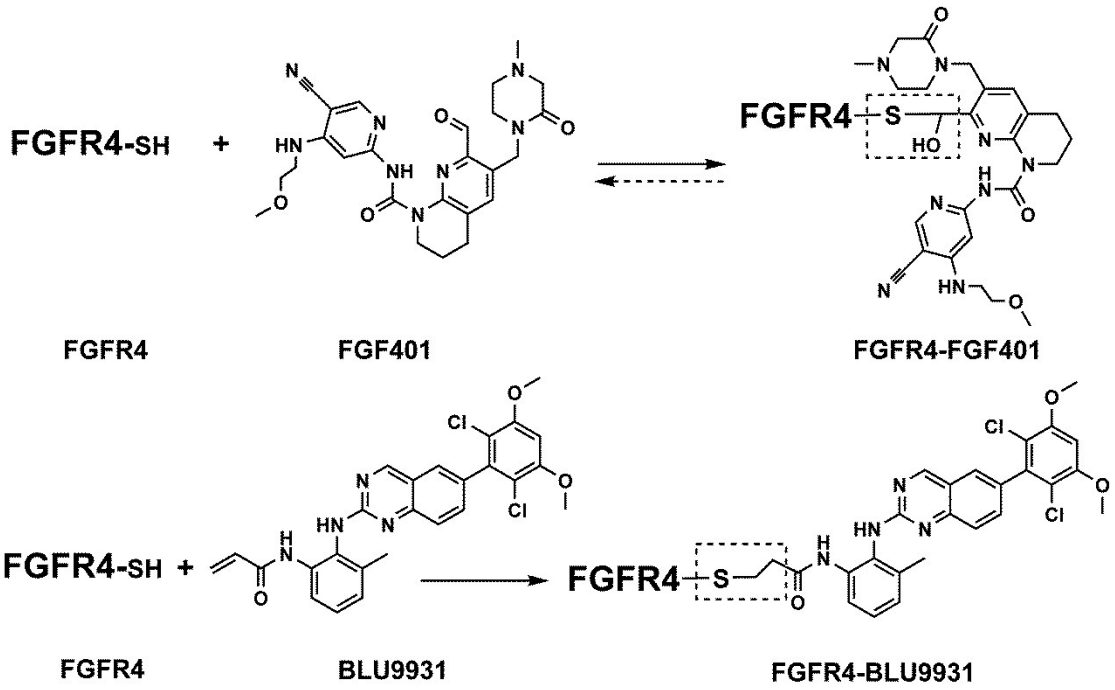

Figure S2. The covalent coupling reaction of FGF401 and BLU9931 to FGFR4. The putative electrophile aldehyde group of FGF401 binds to the free thiol group of FGFR4 Cys552 in a reversible-covalent manner through the formation of a hemithioacetal, whereas the conjugate double bond of BLU9931 reacts with the free thiol group of FGFR4 Cys552 by forming an irreversible covalent linkage through a Michael addition reaction.

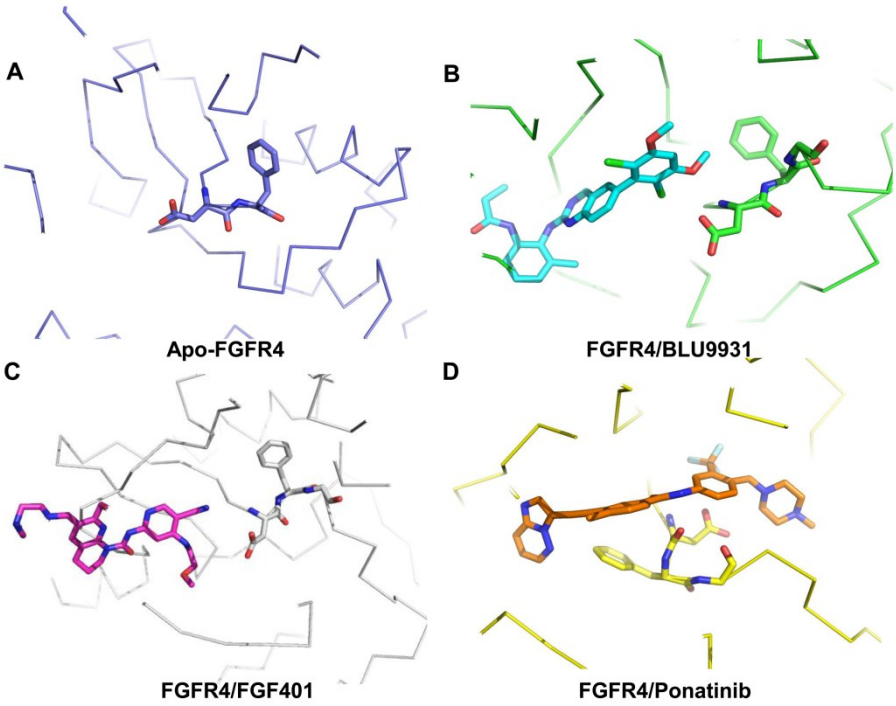

Figure S3. The DFG motif conformation of FGFR4. (A) Active Apo-FGFR4 DFG-in conformation (PDB: 4QQT). (B) Active FGFR4 DFG-in conformation in FGFR4/BLU9931 complex (PDB: 4XCU). (C) Active FGFR4 DFG-in conformation in FGFR4/FGF401 complex (this work, PDB: 6JPJ). (D) Inactive FGFR4 DFG-out conformation in FGFR4/Ponatinib complex (PDB: 4UXT).