Supplementary Information for

LY2874455 potently inhibits FGFR gatekeeper mutant and overcomes mutation-based resistance

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Supplementary Methods

Protein expression and purification

The recombinant expression vectors of the kinase domain of human FGFR1 (residues 458-765), FGFR2 (residues 458-768), FGFR3 (residues 450-758) and FGFR4 (residues 445-753) were constructed in pET28a via homologous recombination (Sosoo Mix, TsingKe Co, Ltd., China). And their gatekeeper mutations (FGFR1^{V561M}, FGFR2^{V564F}, FGFR3^{V555M}, FGFR4^{V550L} and FGFR4^{V550M}) were introduced using the QuikChange site-directed mutagenesis (Vazyme Biotech Co, Ltd., China). The expression and purification procedures of these kinase domains were performed as previously described [1]. The proteins were finally concentrated to about 10 mg/ml and flash frozen for storage at -80 °C.

Kinase assay

The ADP-Glo kinase assay (Promega, USA) was used to calculate the IC50 values of FGFR inhibitors following the manufacturer's instructions. Each kinase assay contained the kinase buffer (40 mM Tris-HCl pH 7.5, 20 mM NaCl, 20 mM $MgCl_2$, 2 mM DTT, 2 mM $MnCl_2$, 0.1 mM Na_3VO_4, 0.1 mg/mL BSA, 4 % DMSO), 10 μM ATP, 0.05 mg/mL poly(Glu_4Tyr) substrate (abcam, UK), 0.08 μg/ml kinase and gradient inhibitors [2, 3]. The luminescence values were recorded by Microplate Reader (PerkinElmer, USA). Curve fitting was performed using GraphPad Prism sigmoidal dose-response (variable slope) software. The results were obtained from more than 3 repeated experiments.

Cell viability assay

Ba/F3 cells transfectants (expressing wild-type FGFR4 or FGFR4^{V550L}) were seeded in a 96-well plate and were treated with gradient concentration of LY2874455 (0-1000 nM). After 72 h, the cell viability was measured using MTS assay. The IC50 values were calculated using GraphPad Prism sigmoidal dose-response (variable slope) software. To generate FGFR4^{V550L} expressing BaF3 cell line, the V550L mutation was introduced into the FGFR4^{WT}, which had been sub-cloned into the retroviral expression vector, using site-directed mutagenesis and was tranduced into BaF3 cell line using retroviral infection [4]. The results were obtained from 6 repeated experiments.

Crystallization, data collection and structure determination

Inhibitor LY2874455 was purchased from Selleckchem (Huston, TX, USA). Before crystallization, LY2874455 was mixed with FGFR4^{V550L} or FGFR4^{V550M} on ice at a molar ratio of 2:1 for 30 min. The crystals were harvested in 0.7 M $NH_4H_2PO_4$ with 20 % v/v glycerol as cryoprotectant.

Data were collected at the Shanghai Synchrotron Radiation Facility (SSRF), Beamline BL17U and reduced using HKL3000. Structure determination was carried out as described previously [5-9]. Initial phase determination was performed by molecular replacement with Phaser from the CCP4 package, using the previously solved FGFR4/ponatinib structure (PDB code: 4TYJ) as the search model. The structure was refined using Phenix.refine and Coot from the Phenix package. The statistics of the crystallographic analysis are presented in Table 1. Graphical representations of structure were prepared using PyMol, and the structure superimpositions were aligned with the Cα atoms of the whole kinase domain. The diagrams of protein-ligand interaction were generated using PDBsum. The refined and validated structures of LY2874455/FGFR4^{V550L} and LY2874455/FGFR4^{V550M} have been deposited in the Protein Data Bank (PDB). The PDB codes are 5XFF and 5XFJ, respectively.

Table S1 Data collection and refinement statistics

	FGFR4^{V550L}/LY2874455	**FGFR4^{V550M}/LY2874455**
Wavelength (Å)	0.9789	0.9789
Resolution range (Å)	39.75-2.70 (2.80-2.70)	39.73-3.25 (3.36-3.25)
Space group	$P\,4_32_12$	$P\,4_32_12$
Unit cell	62.29, 62.29, 184.59, 90, 90, 90	62.22, 62.22, 184.96 90, 90, 90
Total reflections	282551 (27850)	137563 (15230)
Unique reflections	10635 (1014)	6209 (590)
Multiplicity	26.6 (27.5)	22.2 (25.8)
Completeness (%)	99.9 (98.9)	99.3 (98.5)
Mean I/sigma(I)	29.63 (2.54)	19.18 (4.58)
Wilson B-factor	79.07	97.28
R-merge	0.116 (2.1)	0.134 (0.852)
R-meas	0.1183	0.1372
CC1/2	1.000 (0.912)	0.997 (0.986)
CC*	1.000 (0.977)	0.999 (0.997)
***R*-work**	0.265 (0.327)	0.278 (0.325)
***R*-free**	0.319 (0.379)	0.336 (0.404)
Number of non-hydrogen atoms	2158	2134
macromolecules	2123	2104
ligands	30	30
Protein residues	269	266
RMS deviation (bonds)	0.006	0.007
RMS deviation (angles)	1.56	1.53
Ramachandran favored (%)	94.0	90
Ramachandran outliers (%)	1.5	3.5

Molprobity Clashscore	13.03	14.32
Average *B*-factor	97.7	100.1
macromolecules	97.90	100.00
ligands	86.80	100.80

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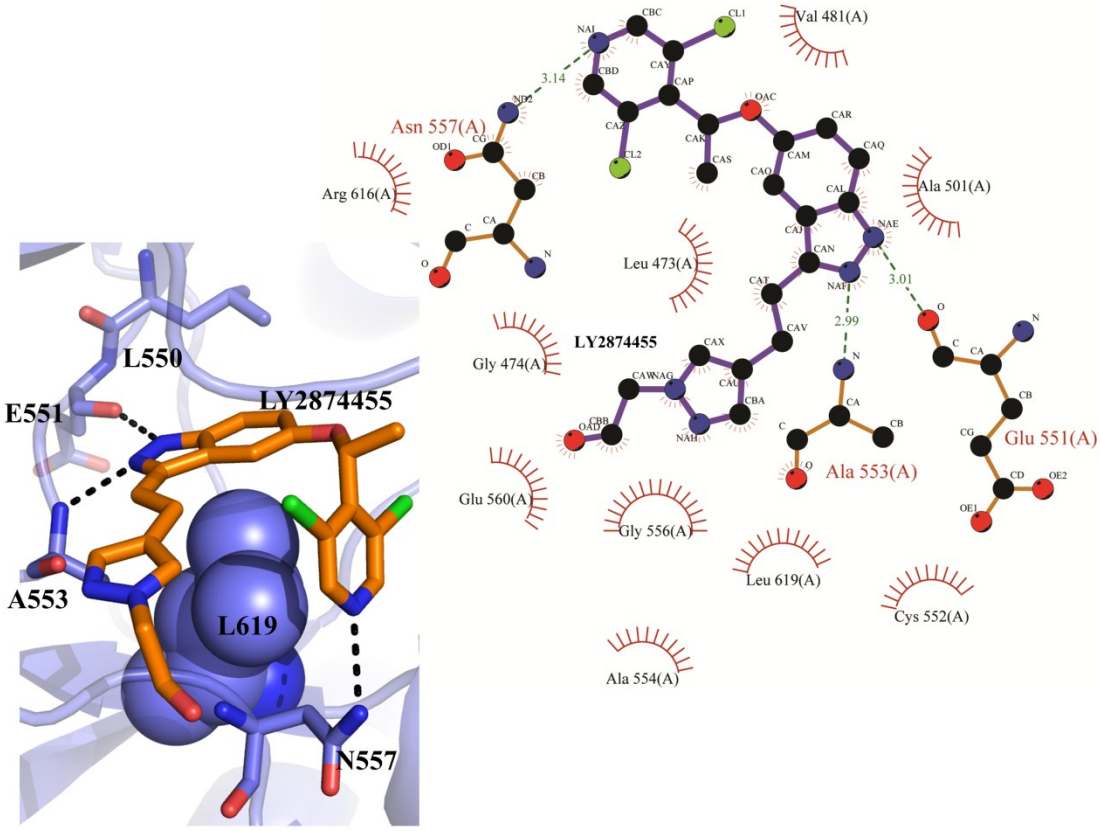

Fig. S1 The binding mode of LY2874455 in FGFR4^{V500L} mutant. LY2874455 adopted a chair-like conformation and folded up on the hydrophobic residue Leu619 in FGFR4^{V550L}, and formed three hydrogen bonds (E551, A553 and N557) and a number of van der Waals contacts with FGFR4 mutants. The left panel was prepared by Pymol and the right diagram was generated by PDBsum. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes.

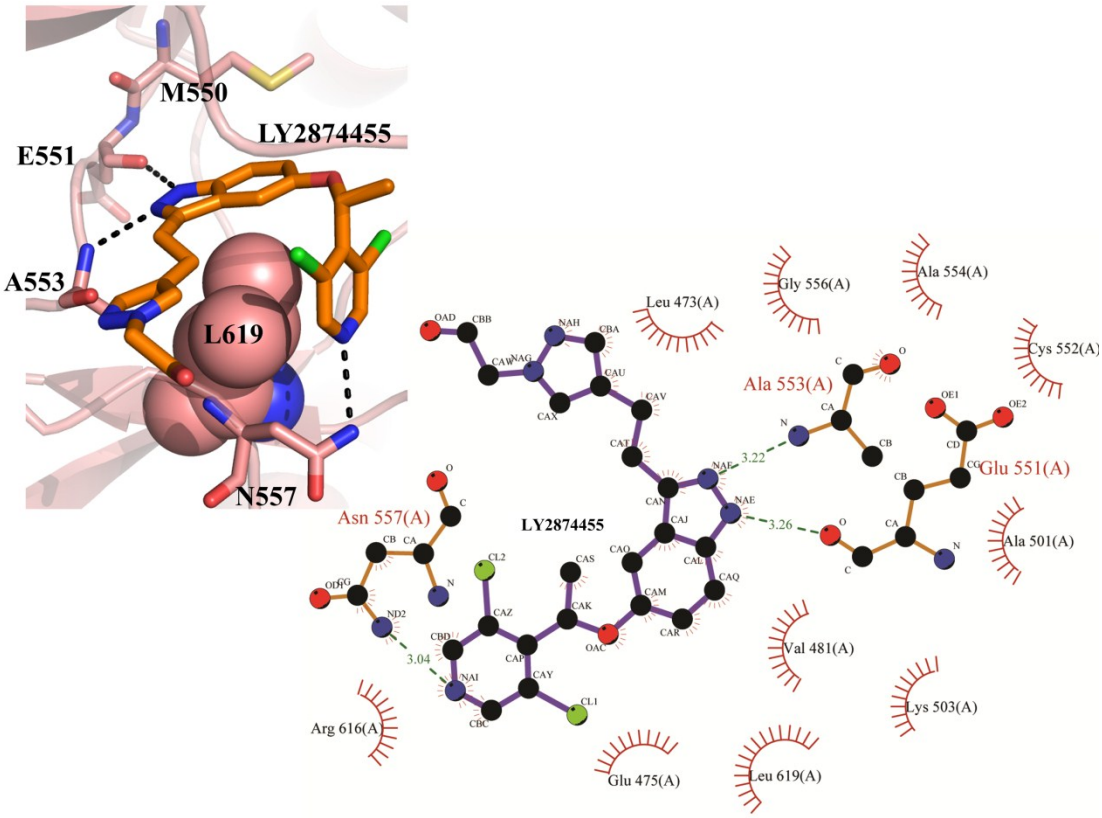

Fig. S2 The binding mode of LY2874455 in FGFR4^{V500M} mutant. LY2874455 adopted a chair-like conformation and folded up on the hydrophobic residue Leu619 in FGFR4^{V550M}, and formed three hydrogen bonds (E551, A553 and N557) and a number of van der Waals contacts with FGFR4 mutants. The left panel was prepared by Pymol and the right diagram was generated by PDBsum. Hydrogen bonds are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes.