Distinct Binding Mode of Multi-kinase Inhibitor Lenvatinib Revealed by Biochemical Characterization

Supporting Information

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Compounds.
Lenvatinib and sunitinib were synthesized at Eisai Co., Ltd. (Tokyo, Japan). Sorafenib was extracted from Nexavar (sorafenib) tablets (Bayer HealthCare Pharmaceuticals, Inc).

Protein production and purification.
VEGFR2 (UniProt P35968, GenBank ID: NP_002244) kinase domain for crystallization was expressed in Sf9 insect cells. The construct comprises amino acids 814 to 1172 with the kinase insertion domain (aa 941 to 990) deleted and residue T940 substituted by Val[1]. The protein was initially captured by using a Q-Sepharose column (GE Healthcare, Freiburg, Germany). Subsequently, VEGFR2 was purified by hydroxyapartite and anion exchange chromatography on a Resource-Q column (GE Healthcare).
Autophosphorylation was then triggered by the addition of $MgCl_2$ and ATP. Activated protein was subjected to a final polishing step on a Superdex75 size-exclusion chromatography column (GE Healthcare) in buffer containing 10 mM HEPES 7.5, 20 mM NaCl, and 10 mM DTT.

GST-VEGFR2 (residues 807–1356) for assay was expressed in Sf9 insect cells. The protein was purified by glutathione-Sepharose affinity capture and subsequent size-exclusion chromatography on an Superdex 200 column (GE Healthcare) in a final buffer consisting of 10 mM HEPES pH 7.5, 100 mM NaCl, 5% (v/v) glycerol, and 5 mM DTT.

Crystallization and data collection.
Purified VEGFR2 was used at a concentration of 10 mg/mL. Single crystals of VEGFR2 in complex with sorafenib and lenvatinib were obtained by co-crystallization using the reservoir buffers comprising 10% to 25% PEG 3350, Na_3citrate, and 0.1 M Bis-Tris propane (pH 8.00) and 0.1M CHES (pH 9.5) and 5% to 20% PEG8000, respectively. The crystals were flash-frozen and measured at a temperature of 100 K. The X-ray diffraction data were collected from complexed crystals of VEGFR2 at the Swiss Light Source (SLS, Villigen, Switzerland) under cryogenic conditions. The crystals belonged to space group P21 (for lenvatinib) and C2 (for sorafenib). Both data sets were processed with XDS and XSCALE[2] (see Table S1).

Structure modeling and refinement.
The phase information needed to determine and analyze the structures was obtained by

molecular replacement. A previously solved structure of VEGFR2 was used as a search model. Subsequent model building and refinement were performed according to standard protocols with the software packages CCP4[3] (Version 6.2.0) and COOT[4] (Version 0.6). To calculate the free R-factor (a measure used to cross-validate the correctness of the final model, about 9.1% of measured reflections were excluded as part of the refinement procedure for the VEGFR2–lenvatinib complex and about 7.2% in the case of the VEGFR2–sorafenib complex (see Table S2). TLS refinement[5] using REFMAC5[6] from the CCP4 suite was performed and resulted in lower R-factors and higher quality of the electron density map. Ligand parameterization and generation of the corresponding library files were performed with the program CORINA (Molecular Networks GmbH Computerchemie, Erlangen, Germany).

The water model was built with the "Find Waters" algorithm of COOT by putting water molecules in peaks of the Fo-Fc map contoured at 3.0; this was followed by refinement with REFMAC5 and checking of all water molecules with the validation tool in COOT. The occupancy of side chains, which were in negative peaks in the Fo-Fc map (contoured at –3.0 σ), was set to zero and subsequently to 0.5 if a positive peak occurred after the next refinement cycle.

Proteros reporter displacement assay.

Experiments to determine the binding affinities and kinetic rate constants of the kinase inhibitors against VEGFR2 were performed as described previously.[7,8] Briefly, 20 nM VEGFR2 was preincubated with the reporter probe at a concentration equal to its binding affinity (K_d) in a reaction buffer consisting of 20 mM MOPS [pH 7.0], 1 mM DTT, and 0.01% Tween20. The final reaction volume was 10 µl in black NBS 384-well polypropylene plates. After transfer of serially diluted inhibitors, probe displacement was monitored for 60 min. K_d values were calculated by using the Cheng-Prusoff equation from the IC_{50} values obtained from the percentage displacement values at the last time point measured. Association rate constants of the inhibitors were calculated from the decay rate of probe displacement. Dissociation rate constants were determined as the product of K_d × association rate constant.

Supplemental References

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Table S1. Data collection and processing statistics[1]

Ligand	Lenvatinib	Sorafenib
X-ray source	PXI/X06SA (SLS[2])	PXI/X06SA (SLS[2])
Wavelength [Å]	1.00003	0.99985
Detector	PILATUS 6M	PILATUS 6M
Temperature [K]	100	100
Space group	P 2_1	C 2
Cell: a; b; c; [Å]	42.33; 84.23; 46.89	135.92; 57.36; 52.17
α; β; γ; [°]	90.0; 100.2; 90.0	90.0; 94.2; 90.0
Resolution [Å]	1.57 (1.82 – 1.57)	1.90 (2.15 –1.90)
Unique reflections	44907 (16031)	30435 (9512)
Multiplicity	4.2 (4.2)	2.7 (2.6)
Completeness [%]	99.5 (99.7)	95.9 (97.6)
R_{sym} [%][3]	6.0 (44.4)	3.1 (44.8)
R_{meas} [%][4]	6.9 (50.9)	3.8 (55.7)
Mean(I)/sd[5]	11.36 (3.01)	19.40 (2.56)

[1] Values in parenthesis refer to the highest resolution bin.

[2] SWISS LIGHT SOURCE (SLS, Villigen, Switzerland)

Table S2. Refinement statistics[1]

Ligand	Lenvatinib	Sorafenib
Resolution [Å]	46.15 – 1.57	67.78 – 1.90
Number of reflections (working /test)	40823 / 4082	28256 / 2178
R_{cryst} [%]	17.8	18.2
R_{free} [%][2]	20.7	22.0
Total number of atoms:		
Protein	2252	2427
Water	216	110
Ligand	30	32
Sulphate	5	0
DTT	32	8
Glycerol	6	0
1,2-Ethanediol	16	0
Acetate	0	4
Deviation from ideal geometry: [2]		
Bond lengths [Å]	0.014	0.012
Bond angles [°]	1.55	1.45
Bonded B's [Å^2] [3]	3.1	6.7
Ramachandran plot: [4]		
Most favoured regions [%]	92.9	93.0
Additional allowed regions [%]	6.6	6.6
Generously allowed regions [%]	0.4	0.4
Disallowed regions [%]	0.0	0.0

[1] Values as defined in REFMAC5, without sigma cut-off

[2] Root mean square deviations from geometric target values

[3] Calculated with MOLEMAN

[4] Calculated with PROCHECK

Table S3. VEGFR2 Amino Acid Residues Located Less than 3.9 Å from Lenvatinib or Sorafenib

Site/Region	Amino Acid Residues	
	Lenvatinib	Sorafenib
ATP-binding site	Leu840, Gly841, Ala866 , Glu885, Val899, Glu917, Phe918, Cys919, Lys920, Gly922, Leu1035, Cys1045, Asp1046	Leu840, Val848, Ala866 , Glu885, Val899, Glu917, Phe918, Cys919, Lys920, Gly922, Leu1035, Cys1045, Phe1047
Gatekeeper	Val916	Val916
Neighboring region	Lys868, Ile888, Leu889, Phe1047, Leu1049	Lys868, Ile892, Leu889, Val898, Leu1019, His1026, Ile1044, Asp1046

Each amino acid residue is classified according to the work of Traxlera et al.[1] Numbers for each amino acid residue of VEGFR2 are based on Universal Protein Resource Knowledgebase P35968. Amino acid residues specific to lenvatinib or sorafenib are underlined. Among the amino acid residues belonging to the DFG motif, Asp1046 and Phe1047 were respectively located at the ATP-binding site and in the neighboring region in the VEGFR2–lenvatinib complex; in contrast, in the VEGFR2–sorafenib complex they were respectively located in the neighboring region and at the ATP binding site.

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