

Supporting Information

A back-to-front fragment based drug design search strategy targeting the DFG-out pocket of protein tyrosine kinases

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Materials and Methods

Materials ATP, Bovine serum albumin (BSA), DL-Dithiothreitol (DTT) and poly-GT (4:1) were purchased from Sigma-Aldrich. Ni-NTA resin was purchased from Qiagen. ProfilerPro peptide 22 (5-FAM-EEPLYWSFPAKKK-CONH₂) was sourced from Caliper Life Sciences. The Kinase-Glo Luminescent Kinase Assay detection kit was purchased from Promega, Alexa Fluor 647 azide, 5 × Kinase Buffer A, and Eu-anti-His-tag antibody were obtained from Invitrogen.

Purification and phosphorylation of VEGFR2. The catalytic domain of human VEGFR2 (residues 806-1171) with deletion of the kinase insert domain (KID; residues 940-989) was expressed as an N-terminal His-tagged protein with the TEV protease site in a baculovirus expression system, as described previously¹. After digestion with TEV protease, 30 μM of purified VEGFR2 was auto-phosphorylated with 1 mM ATP and 1 M MgCl₂ on ice for 90 minutes. Then the reaction mixture was desalted by using a HiPrep desalting column.

VEGFR2 kinase inhibition assay. Inhibition of VEGFR2 kinase was assessed with the LabChip[®] mobility shift assay (Caliper Life Sciences). Reactions were performed in 50 mM Tris-HCl (pH 7.5), 5 mM MnCl₂, 5 mM MgCl₂, 0.01% Tween-20, and 2 mM DTT containing 20 μM ATP (K_m^{app} concentration), 1.5 μM ProfilerPro Peptide 22 (Caliper Life Sciences) and 8 nM of non-phosphorylated VEGFR2 in a final volume of 50 μl. In the case of phosphorylated VEGFR2, assay was performed as above with 20 μM ATP (K_m^{app}) and 3 nM of phosphorylated VEGFR2.

The reaction was quenched by adding 25 μL of 75 mM EDTA after 60 minutes and enzyme activity was monitored by detecting both the substrate peptide and the phosphorylated product peptide with a LabChip[®] EZreader II (Caliper Life Sciences). The concentration of inhibitor that caused 50% inhibition of kinase activity (IC₅₀) and the 95% confidence interval (95% CI) were analyzed for each assay using GraphPad Prism version 5.01 (GraphPad Software). Sigmoidal dose-response (variable slope) curves were fitted by non-linear regression analysis, with the top and bottom of each curve being constrained at 100 and 0, respectively. All compounds tested in this manuscript were soluble up to 100 μM in this condition.

For Yonetani-Theorell analysis², enzyme activity was monitored by using the LabChip[®] EZreader II without addition of EDTA. Obtained each data (% of conversion/min) was converted to reaction velocity (nM /min) followed by globally fitting to the equation below.³

$$1/v = 1/v_0(1 + [A]/K_{iA} + [B]/K_{iB} + [A][B]/\alpha K_{iA} K_{iB}) \quad \text{Equation 1}$$

v; velocity with various concentrations of two inhibitors

v₀; velocity without any inhibitors (control reaction)

[A]; concentration of inhibitor A

K_{iA}; dissociation constant of inhibitor A

[B]; concentration of inhibitor B

K_{iB}; dissociation constant of inhibitor B

α; parameter defining the interaction between the two compounds

VEGFR2 kinase inhibition assay for HTS. VEGFR2 kinase activity was detected by employing a Kinase-Glo Luminescent Kinase Assay detection kit (Promega, Fitchburg, WI). VEGFR2 kinase activity was detected by using a Kinase-Glo Luminescent Kinase Assay detection kit (Promega). Reactions were performed in 50 mM Tris-HCl (pH7.5), 5 mM MnCl₂, 0.01% Tween-20, 0.01% BSA, and 2 mM DTT containing 0.5 μM of ATP, 5 μg/ml of poly-GT (4:1) (Sigma), and 80 nM of the non-phosphorylated catalytic domain of VEGFR2 in a final volume of 25 μl. Prior to initiation of the reaction with ATP, the inhibitor and enzyme were incubated for 60 min at room temperature. After 40 min, the reaction was quenched by adding 25 μl of Kinase-Glo Luminescent Kinase Assay detection kit reagent. Then the plates were incubated for 10 min at room temperature and read on an EnVision 2102 multilabel reader (PerkinElmer). Inhibition of the phosphorylated catalytic domain of VEGFR2 was assessed as described above with 8 nM of the enzyme. Wells containing the substrates and the enzyme without inhibitor were used as the total reaction control, while wells containing the substrate without the enzyme were used as the basal control.

Thermal shift analysis. Thermal shift analysis was performed with Sypro Orange fluorescent dye (Invitrogen). Experiments were done with 50 mM Hepes (pH 7.5), 5 × Sypro Orange, and 0.6 μM of non-phosphorylated or phosphorylated VEGFR2 protein in a final volume of 15 μl. The plate was heated from 15°C to 80°C at a rate of 0.5°C/min on an Applied Biosystems 7900HT fast real-time PCR system (Applied Biosystems). Fluorescence data were obtained with excitation at 490 nm and emission 530 nm. The temperature at which the fluorescence reached 50% of maximum (T_m) was analyzed by GraphPad Prism software (version 5.01) using Boltzmann sigmoid curves. Then ΔT_m for each inhibitor was calculated as follows:

$$\Delta T_m = (T_m \text{ with the inhibitor}) - (T_m \text{ without the inhibitor}).$$

Ligand displacement analysis using the LanthaScreen™ Eu Kinase Binding Assay. The displacement assay with Alexa Fluor 647-labeled compound D was performed in 1 × Kinase Buffer A containing 2 mM DTT, 0.05% BSA, 200 nM (K_D value) of Alexa Fluor 647-labeled compound D, 5 nM of Eu-anti-His-tag antibody, various concentrations of inhibitor, and 5 nM of non-phosphorylated VEGFR2. Detection was done as described above. The reaction mixture was incubated for 60 minutes at room temperature and the maximal Lantha Screen™ time-resolved fluorescence resonance energy transfer (TR-FRET) signal was detected with an EnVision 2102 Multilabel Reader.

Crystallization of compound A and the phosphorylated catalytic domain of VEGFR2. Purified recombinant human VEGFR2 (residues 806-1171) without the kinase insertion domain (KID, residue 940-989) was phosphorylated and then crystallized as an apo form by the sitting-drop vapor diffusion method. Crystals were grown at 4°C from 1.0 μL drops that contained 0.5 μL of the protein solution (6-8 mg/mL VEGFR2, **A**, 10 mM HEPES (pH 7.5), 10 mM NaCl, 10% glycerol, and 1 mM DTT) and 0.5 μL of the reservoir solutions. The reservoir solutions contained 100 mM HEPES (pH 7.5) and 1.3 M trisodium citrate. Crystals obtained were soaked into solution containing 1 mM of **A** for 16 hours in a cold room, then harvested from a drop containing 23% ethylene glycol as a cryoprotectant and flash-cooled in liquid nitrogen prior to analysis. The X-ray diffraction data were collected using a RIGAKU MicroMax007 X-ray system at 100 K, after which data were processed and scaled with CrystalClear

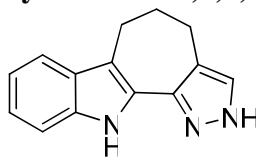
(Rigaku, 1999). The structure was solved by the molecular replacement method using the only protein structure of VEGFR2 from which ligand and water molecules were eliminated. The solved structure was modeled with Wincoot⁴, and the molecular replacement and refinement were carried out by AmoRe and Refmac5, respectively (CCP4 v6.0.0 Programs suits⁵). All structural figures were prepared by using PyMOL (DeLano Scientific LLC, 2002, <http://www.pymol.org/>). A summary of the crystallographic data is shown in Supplementary Table 4.

Crystallization of compound E and the non-phosphorylated catalytic domain of VEGFR2

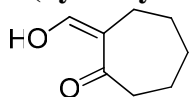
Purified recombinant human VEGFR2 (residues 806-1171) without the kinase insertion domain (KID, residue 940-989) was crystallized with **E** by the sitting-drop vapor diffusion method. Before crystallization, recombinant human VEGFR2 was incubated with 1 mM of **E** for one hour on ice, followed by centrifugation at 14,000 g for 30 min to remove precipitates. Crystals were grown at 20°C from 0.4 µL drops that contained 0.2 µL of the protein solution (8-12 mg/mL VEGFR2, **E**, 10 mM HEPES (pH 7.5), 10 mM NaCl, 10% glycerol, and 1 mM DTT) and 0.2 µL of the reservoir solutions. The reservoir solutions contained 100 mM HEPES (pH 7.0-8.0) and 880-1,440 mM tri-sodium citrate. Crystals were harvested from drops containing 25% ethylene glycol as a cryoprotectant and flash-cooled in liquid nitrogen prior to analysis. The X-ray diffraction data were collected at SPring-8 (Harima, Japan) on beam-line BL32B2 at 100 K, after which data were processed and scaled with CrystalClear (Rigaku, 1999). The structure was solved by the molecular replacement method using the only protein structure of VEGFR2 from which ligand and water molecules were eliminated. The solved structure was modeled with Wincoot⁴, and the molecular replacement and refinement were carried out by MolRep and Refmac5, respectively (CCP4 v6.0.0 Programs suits⁵). All structural figures were prepared by using PyMOL (DeLano Scientific LLC, 2002, <http://www.pymol.org/>). A summary of the crystallographic data is shown in Supplementary Table 5.

Synthesis and chemical characterization of compounds

Synthesis of 4,5,6,11-tetrahydro-2*H*-pyrazolo[4',3':6,7]cyclohepta[1,2-*b*]indole (A)



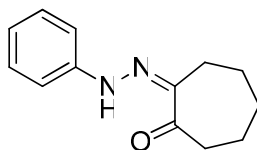
2-(hydroxymethylidene)cycloheptanone (A1)



To a stirred solution of cycloheptanone (1.18 ml, 9.98 mmol) in diethyl ether (10 ml) was added sodium methoxide (1.08 g, 20 mmol) followed by ethyl formate (1.09 ml), and the mixture was stirred at room temperature for 18 h. The mixture was treated with 1 N hydrochloric and extracted with EtOAc. The organic layer was washed with saturated brine, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to give the titled compound (1.32 g, 94%) as a colorless liquid.

$^1\text{H-NMR}$ (CDCl_3) δ 1.50-1.85 (6H, m), 2.20-2.30 (2H, m), 2.50-2.60 (2H, m), 7.62 (1H, d, $J = 8.7$ Hz).

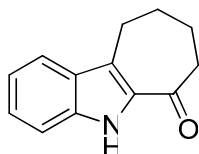
2-[(2-phenylhydrazino)methylidene]cycloheptanone (A2)



A mixture of aniline (0.913 ml, 10.0 mmol), concentrated hydrochloric acid (2.06 g, 56.5 mmol), water (6 ml), and sodium nitrite (690 mg, 14.6 mmol) was stirred at 0° C for 20 min. The mixture was added to a mixed solution of **A1** (1.40 g, 9.99 mmol) in ethanol (16 ml) and potassium hydroxide (561 mg, 10 mmol) in water (0.6 ml) at 0° C, and the mixture was stirred at 0° C for 10 min and at room temperature for 1 h and added to water. The precipitate was collected by filtration and washed with water to give the title compound (2.03 g, 94%) as a yellow solid.

$^1\text{H-NMR}$ (CDCl_3) δ 1.65-1.90 (6H, m), 2.50-2.75 (4H, m), 6.90-7.05 (1H, m), 7.15-7.40 (4H, m).

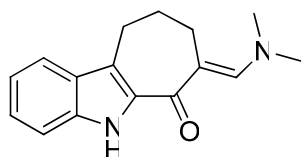
7,8,9,10-tetrahydrocyclohepta[*b*]indol-6(5*H*)-one (A3)



A mixture of **A2** (1.90 g, 8.83 mmol) and formic acid (20 ml) was stirred at 100 °C for 30 min, and the mixture was allowed to cool to room temperature. Water was added to the reaction mixture, and the mixture was extracted with EtOAc. The organic layer was washed with water, saturated aqueous sodium hydrogen carbonate solution, and saturated brine, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was triturated with a mixed solution of diethyl ether and n-hexane to give the title compound (0.98 g, 56%) as a colorless solid.

$^1\text{H-NMR}$ (CDCl_3) δ 1.95-2.20 (4H, m), 2.84 (2H, t, $J = 5.7$ Hz), 3.16 (2H, t, $J = 6.3$ Hz), 7.05-7.70 (4H, m), 8.87 (1H, brs).

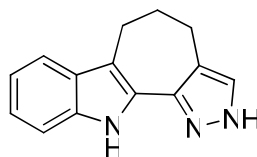
7-[(dimethylamino)methylidene]-7,8,9,10-tetrahydrocyclohepta[b]indol-6(5H)-one (A4)



A mixture of **A3** (200 mg, 1.01 mmol), tris(dimethylamino)methane (436 mg, 3.00 mmol) and *N,N*-dimethylformamide (2 ml) was stirred at 70° C for 3 h. After cooling to room temperature, the reaction mixture was treated with water and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to give the title compound (195 mg, 76%) as a yellow solid.

¹H-NMR (CDCl₃) δ 2.00-2.15 (2H, m), 2.70-2.85 (2H, m), 3.00-3.20 (2H, m), 3.13 (6H, s), 7.00-7.65 (4H, m), 3.16 (2H, t, *J* = 6.3 Hz), 7.05-7.70 (4H, m), 7.66 (1H, s), 9.17 (1H, brs).

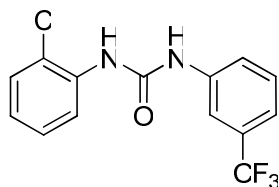
4,5,6,11-tetrahydro-2H-pyrazolo[4',3':6,7]cyclohepta[1,2-*b*]indole (A)



A mixture of **A4** (195 mg, 0.77 mmol), hydrazine monohydrate (0.15 ml, 4.82 mmol), and ethanol (5 ml) was heated under reflux for 1 h. After cooling to room temperature, the reaction mixture was treated with water and extracted with EtOAc. The organic layer was washed with saturated brine, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was triturated with *n*-hexane to give the title compound (152 mg, 88%) as a colorless solid.

¹H-NMR (CDCl₃) δ 2.00-2.20 (2H, m), 2.92 (2H, t, *J* = 5.6 Hz), 3.15 (2H, t, *J* = 5.8 Hz), 7.00-7.60 (5H, m), 8.84 (1H, brs).

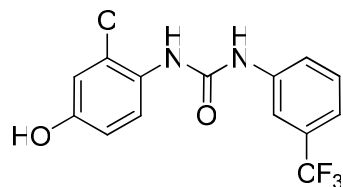
Synthesis of 1-(2-chlorophenyl)-3-[3-(trifluoromethyl)phenyl]urea (B)



A mixture of 2-chloroaniline (1.28 g, 10 mmol), tetrahydrofuran (10 ml), triethylamine (4.18 ml, 30 mmol) and 3-(trifluoromethyl)phenyl isocyanate (1.65 ml, 12 mmol) was stirred at room temperature for 16 h, concentrated, and washed with diethyl ether (20 ml, twice) to give title compound (1.57 g, 50%) as a colorless powder.

¹H-NMR (DMSO-*d*₆) δ 7.00-7.65 (6H, m), 8.00-8.20 (2H, m), 8.34 (1H, s), 9.15 (1H, s).

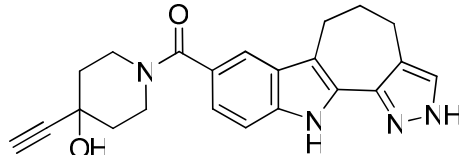
Synthesis of 1-(2-chloro-4-hydroxyphenyl)-3-[3-(trifluoromethyl)phenyl]urea (C)



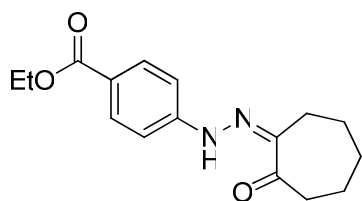
A mixture of 4-amino-3-chlorophenol (1.44 g, 10 mmol), tetrahydrofuran (10 ml), triethylamine (4.18 ml, 30 mmol) and 3-(trifluoromethyl)phenyl isocyanate (1.65 ml, 12 mmol) was stirred at room temperature for 18 h. The reaction was quenched with 4 N hydrochloric acid (50 ml), followed by extraction with EtOAc (100 ml). The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/n-hexane from 20:80 to 33:67) to give title compound (613 mg, 19%).

$^1\text{H-NMR}$ (DMSO- d_6) δ 6.73 (1H, dd, $J = 2.6, 8.8$ Hz), 6.85 (1H, d, $J = 2.6$ Hz), 7.25-7.60 (3H, m), 7.69 (1H, d, $J = 8.8$ Hz), 7.95-8.15 (2H, m), 9.45 (1H, s), 9.67 (1H, s).

Synthesis of 4-ethynyl-1-(4,5,6,11-tetrahydro-2H-pyrazolo[4',3':6,7]cyclohepta[1,2-b]indol-8-ylcarbonyl)piperidin-4-ol (D)



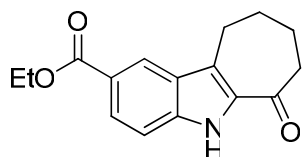
ethyl 4-[2-(2-oxocycloheptylidene)hydrazino]benzoate (D1)



The title compound was obtained by a method similar to that described for compound **A**₂ using ethyl 4-aminobenzoate instead of aniline.

$^1\text{H-NMR}$ (CDCl₃) δ 1.38 (3H, t, $J = 7.2$ Hz), 1.70-1.90 (6H, m), 2.50-2.80 (4H, m), 4.34 (2H, q, $J = 7.2$ Hz), 7.15-7.30 (2H, m), 7.90 (1H, brs), 7.90-8.00 (2H, m).

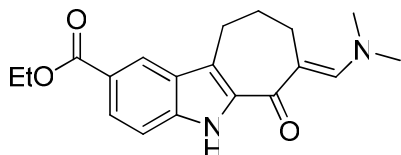
ethyl 6-oxo-5,6,7,8,9,10-hexahydrocyclohepta[b]indole-2-carboxylate (D2)



The title compound was obtained by a method similar to that described for compound **A**₃ using compound **D1** instead of compound **A2**.

$^1\text{H-NMR}$ (CDCl₃) δ 1.43 (3H, t, $J = 7.0$ Hz), 1.90-2.20 (4H, m), 2.86 (2H, t, $J = 5.2$ Hz), 3.20 (2H, t, $J = 6.6$ Hz), 4.41 (2H, q, $J = 7.0$ Hz), 7.30-7.45(1H, m), 8.00-8.10(1H, m), 8.46(1H, m), 9.13 (1H, brs).

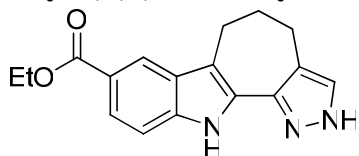
ethyl 7-[(dimethylamino)methylidene]-6-oxo-5,6,7,8,9,10-hexahydrocyclohepta[b]indole-2-carboxylate (D3)



The title compound was obtained by a method similar to that described for compound **A**₄ using compound **D**₂ instead of compound **A**₃.

¹H-NMR (CDCl₃) δ 1.42 (3H, t, *J* = 7.2 Hz), 2.00-2.20 (2H, m), 2.70-2.85 (2H, m), 3.10-3.20 (2H, m), 3.16 (6H, s), 4.40 (2H, q, *J* = 7.2 Hz), 7.37 (1H, d, *J* = 8.1 Hz), 7.70 (1H, s), 7.97 (1H, dd, *J* = 8.1, 1.5 Hz), 8.40 (1H, d, *J* = 1.5 Hz), 9.35(1H, brs).

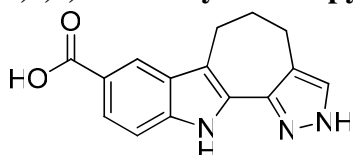
ethyl 4,5,6,11-tetrahydro-2H-pyrazolo[4',3':6,7]cyclohepta[1,2-*b*]indole-8-carboxylate (D4)



The title compound was obtained by a method similar to that described for compound **A** using **D**₃ instead of **A**₄.

¹H-NMR (CDCl₃) δ 1.42 (3H, t, *J* = 6.9 Hz), 2.05-2.20 (2H, m), 2.93(2H, t, *J* = 5.7 Hz), 3.18 (2H, t, *J* = 5.4 Hz), 4.40 (2H, q, *J* = 6.9 Hz), 7.29 (1H, d, *J* = 7.8 Hz), 7.35 (1H, s), 7.88 (1H, dd, *J* = 7.8, 1.2 Hz), 8.29 (1H, d, *J* = 1.2 Hz), 9.12(1H, s).

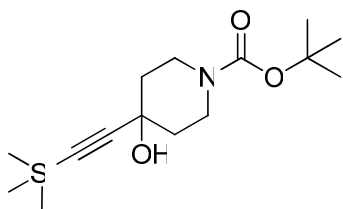
4,5,6,11-tetrahydro-2H-pyrazolo[4',3':6,7]cyclohepta[1,2-*b*]indole-8-carboxylic acid (D5)



To a solution of **D**₄ (1.47 g, 5.00 mmol) in MeOH (20ml) and THF (10 ml) was added 3 N aqueous sodium hydroxide solution (15 ml), and the mixture was stirred at room temperature for 90 h. To the reaction mixture was added 1 N hydrochloric acid solution (100ml), and the mixture was extracted with EtOAc. The organic layer was washed with saturated brine, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was triturated with a mixed solution of diethyl ether and n-hexane to give the title compound (1.26 g, 95%) as a colorless solid.

¹H-NMR (CD₃OD/CDCl₃) δ 2.00-2.20 (2H, m), 2.93 (2H, t, *J* = 6.0 Hz), 3.18 (2H, t, *J* = 5.7 Hz), 7.34 (1H, s), 7.30-7.45 (2H, m), 7.85-7.95 (1H, m), 8.33 (1H, s).

tert-Butyl 4-Hydroxy-4-[(trimethylsilyl)ethynyl]piperidine-1-carboxylate (D6)

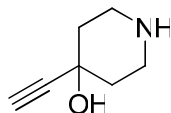


To a stirred solution of trimethylsilylacetylene (14.8 g, 151 mmol) in tetrahydrofuran (300 mL) was added n-BuLi (1.6 M in n-hexane, 94 mL, 150 mmol) dropwise at -40 °C, and the mixture was stirred at

−40 °C for 1 h. To the mixture was added a solution of tert-butyl 4-oxopiperidine-1-carboxylate (25.0 g, 125 mmol) in tetrahydrofuran (200 mL) dropwise at −60 °C. After stirring at −60 °C for 30 min, the mixture was allowed to warm to room temperature and stirred for 18 h. The mixture was treated with 10% aqueous NH₄Cl (500 mL) and extracted with EtOAc (500 mL). The organic layer was washed with brine and evaporated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/n-hexane = 0/100 to 30/70) to give **D6** (31.8 g, 85%) as a white solid.

¹H NMR (CDCl₃) δ 0.17 (9H, s), 1.46 (9H, s), 1.67 (2H, ddd, *J* = 3.8, 9.6, 13.2 Hz), 1.80-1.95 (2H, m), 3.22 (2H, ddd, *J* = 3.6, 9.7, 13.5 Hz), 3.70-3.90 (2H, m).

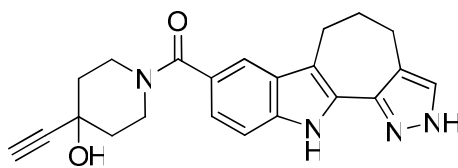
4-Ethynylpiperidin-4-ol (**D7**)



To a stirred solution of **D6** (2.97 g, 10 mmol) in MeOH (10 mL) was added a solution of 4N HCl in EtOAc (20 mL), and the mixture was stirred at room temperature for 18 h. The mixture was treated with 1N aqueous NaOH (80 mL) at 0 °C and evaporated in vacuo. The residue was suspended in EtOAc, and the insoluble material was removed by filtration. The filtrate was evaporated in vacuo, and the residue was dissolved in MeOH (50 mL). To the mixture was added K₂CO₃ (4.15 g, 30 mmol), and the mixture was stirred at room temperature for 18 h and evaporated in vacuo. The residue was suspended in EtOAc, and the insoluble material was removed by filtration. The filtrate was evaporated in vacuo, and the residue was recrystallized from EtOAc to give **D7** (954 mg, 76%) as a white solid.

¹H NMR (CDCl₃) δ 1.68 (2H, ddd, *J* = 3.6, 9.6, 12.9 Hz), 1.85-2.00 (2H, m), 2.52 (1H, s), 2.86 (2H, ddd, *J* = 3.1, 9.7, 12.8 Hz), 3.05 (2H, td, *J* = 4.7, 13.0 Hz).

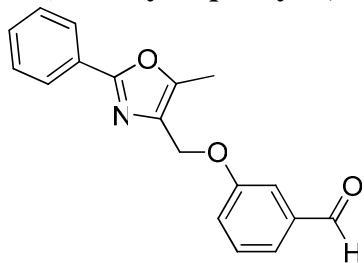
4-Ethynyl-1-(4,5,6,11-tetrahydro-2H-pyrazolo[4',3':6,7]cyclohepta[1,2-b]indol-8-ylcarbonyl)piperidin-4-ol (**D**)



To a mixture of **D6** (876 mg, 7.0 mmol), **D7** (1871 mg, 7.0 mmol), 1-hydroxybenzotriazole (946 mg, 7.0 mmol), and triethylamine (2.93 mL, 21 mmol) in *N,N*-dimethylformamide (20 mL) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.01 g, 10.5 mmol), and the mixture was stirred at room temperature for 18 h. The mixture was evaporated in vacuo, and the residue was partitioned between EtOAc and 1N aqueous NaOH. The organic layer was separated, and the aqueous layer was extracted with EtOAc. The combined organic layer was evaporated in vacuo. The residue was purified by column chromatography (NH silica gel, MeOH/EtOAc = 0/100 to 20/80; then silica gel, MeOH/EtOAc = 0/100 to 20/80) followed by recrystallization (EtOAc) to give **D** (1719 mg, 66%) as a slightly yellow solid.

¹H NMR (DMSO-*d*₆) δ 1.55-1.70 (2H, m), 1.70-1.85 (2H, m), 1.90-2.05 (2H, m), 2.80-2.90 (2H, m), 3.00-3.10 (2H, m), 3.30-3.50 (2H, m), 3.42 (1H, s), 3.60-3.90 (2H, m), 5.68 (1H, s), 7.09 (1H, d, *J* = 8.3 Hz), 7.34 (1H, d, *J* = 8.3 Hz), 7.48 (1H, s), 7.55 (1H, s), 11.20 (1H, s), 12.70 (1H, s).

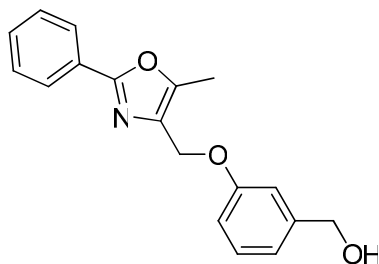
3-[(5-methyl-2-phenyl-1,3-oxazol-4-yl)methoxy]benzaldehyde (**E1**)



To a solution of 3-hydroxybenzaldehyde (6.14 g, 50.3 mmol) in tetrahydrofuran (100 ml) / *N,N*-dimethylformamide (20 ml) was added NaH (60% in oil, 2.01 g, 50.3 mmol) at room temperature. After stirring for 0.5 h, the mixture was treated with 4-(chloromethyl)-5-methyl-2-phenyl-1,3-oxazole (11.5 g, 55.3 mmol) and stirred at room temperature for 2 h and at reflux for an additional 2 h. The reaction mixture was diluted with H₂O and extracted with EtOAc. The organic layer was dried (MgSO₄) and concentrated in vacuo. The residue was purified by silica gel column chromatography (n-hexane/EtOAc 6:1 to 3:1) to give **E1** (12.16 g, 83%) as a yellow oil.

¹H-NMR (CDCl₃, 200 MHz) δ 2.46 (3H, s), 5.05 (2H, s), 7.27-7.33 (1H, m), 7.41-7.55 (6H, m), 7.98-8.05 (2H, m), 9.99 (1H, s); IR (neat) 1695, 1590, 1485, 1450, 1255, 1020, 775 cm⁻¹

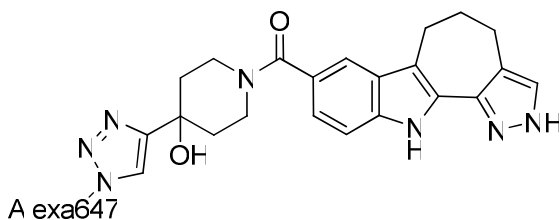
{3-[(5-methyl-2-phenyl-1,3-oxazol-4-yl)methoxy]phenyl}methanol (**E**)



To a solution of compound **E1** (12.2 g, 41.5 mmol) in MeOH (100 ml) was added NaBH₄ (2.35 g, 62.2 mmol) while cooling in an ice-water bath, and the mixture was stirred at 0 °C for 0.5 h. The reaction mixture was poured into H₂O and extracted with EtOAc. The organic layer was washed with H₂O and brine, dried (MgSO₄), and concentrated in vacuo to give **E** (12.1 g, 99 %) as a yellow solid. Recrystallization from CHCl₃-Et₂O gave white crystals.

mp 100-101 °C. ¹H-NMR (CDCl₃, 200 MHz) δ 2.38 (1H, br s), 2.43 (3H, s), 4.67 (2H, s), 4.96 (2H, s), 6.89-6.98 (2H, m), 7.03 (1H, s), 7.27 (1H, t, *J* = 7.9 Hz), 7.40-7.46 (3H, m), 7.97-8.04 (2H, m). IR (KBr) 3310, 1595, 1555, 1490, 1440, 1250, 1050, 1010, 980, 790, 720 cm⁻¹. Anal. Calcd for C₁₈H₁₇NO₃: C, 73.20; H, 5.80; N, 4.74. Found: C, 73.03; H, 5.77; N, 4.73.

Click reaction^{6,7} of compound **D** with Alexa Fluor 647 azide

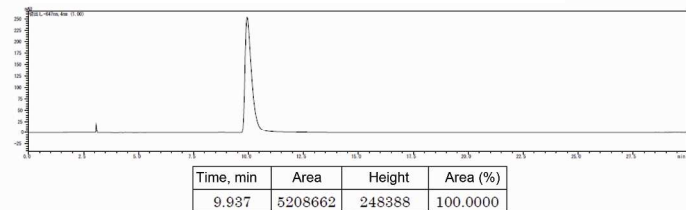


Before use, **D** and Alexa Fluor 647 azide (Invitrogen) were dissolved in DMF at a concentration 10 mM. Then 500 μM of **D** and the same concentration of Alexa Fluor 647 azide were mixed in an aqueous

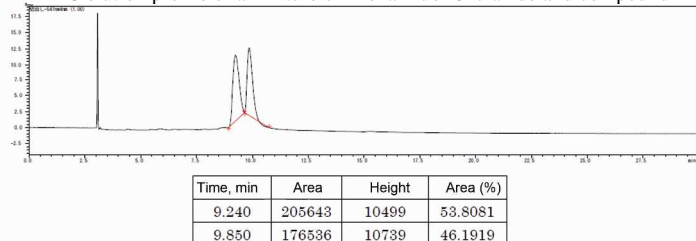
solution containing 10 mM CuSO₄ and 100 mM ascorbic acid. The coupling reaction was performed at room temperature for over 12 hours in the dark, after which the product was purified by HPLC and lyophilized. The fluorescent tracer was reconstituted with DMSO at 1mM of the concentration.

HPLC elution profiles and mass spectrum of Alexa Fluor 647-labeled compound D

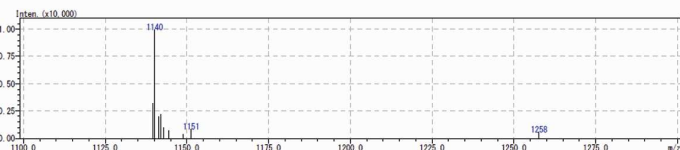
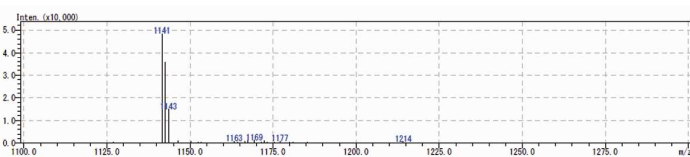
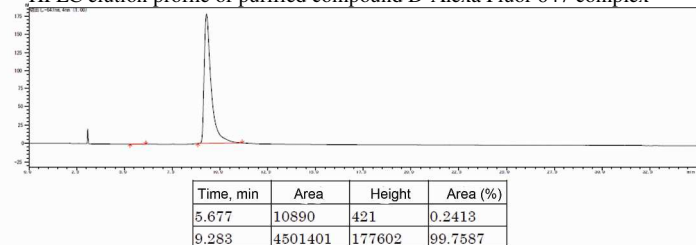
HPLC elution profile of Alexa Fluor 647 azide



HPLC elution profile of a mixture of Alexa Fluor 647 azide and compound D



HPLC elution profile of purified compound D-Alexa Fluor 647 complex



VEGFR2 sequences used in this study

Catalytic domain (806-1171) without the kinase insert domain (940-989)

GAMDPDELPLDEHCERLPYDASKWEFPRDRLKLGKPLGRGAFGQVIEADAFGIDKTATCRTVA
VKMLKEGATHSEHRALMSELKILIHIGHHLNVNLLGACTKPGGPLMVIVEFCKFGNLSTYLRS
KRNEFVPYKEAPEDLYKDFTLEHLICYSFQVAKGMEFLASRKCIHRDLAARNILLSEKNVVKIC
DFGLARDIYKDPDYVRKGDARLPLKWMAPETIFDRVYTIQSDVWSFGVLLWEIFSLGASPYPGV
KIDEEFCRRLKEGTRMRAPDYTTPEMYQTMLDCWHGEPQRSRPTFSELVEHLGNLLQANAQQD

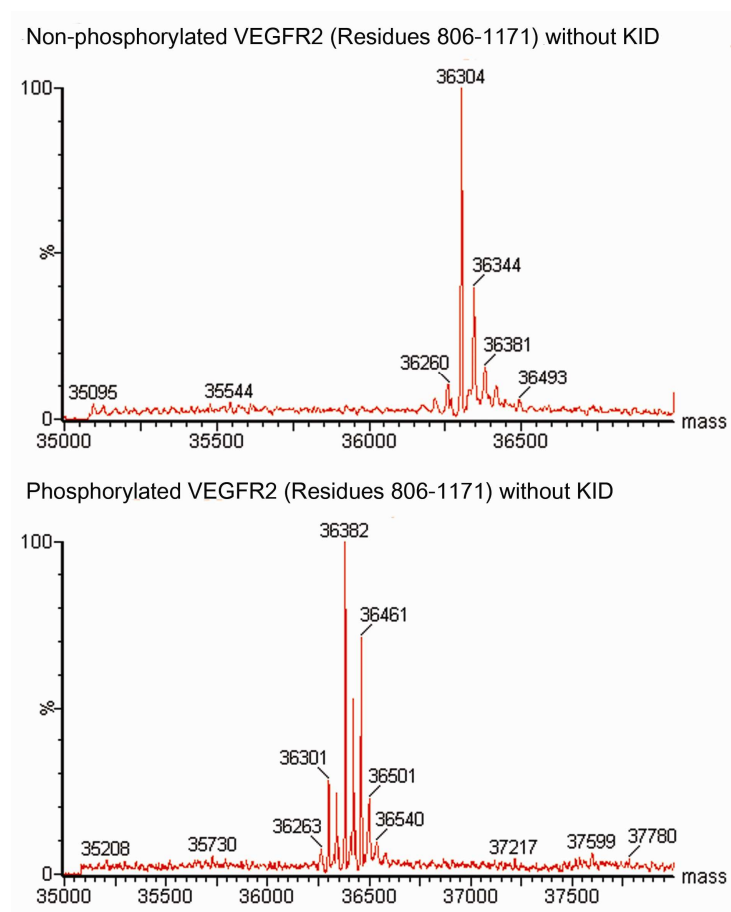
Catalytic domain (806-1171) without the kinase insert domain (940-989) and N-terminal 6 × His-tag

MSYYHHHHHDYDIPTTENLYFQGAMDPDELPLDEHCERLPYDASKWEFPRDRLKLGKPLGR
GAFGQVIEADAFGIDKTATCRTVAVKMLKEGATHSEHRALMSELKILIHIGHHLNVNLLGACT
KPGGPLMVIVEFCKFGNLSTYLRSKRNEFVPYKEAPEDLYKDFTLEHLICYSFQVAKGMEFLA
SRKCIHRDLAARNILLSEKNVVKICDFGLARDIYKDPDYVRKGDARLPLKWMAPETIFDRVYTI
QSDVWSFGVLLWEIFSLGASPYPGVKIDEEFCRRLKEGTRMRAPDYTTPEMYQTMLDCWHGEP
SQRPTFSELVEHLGNLLQANAQQD

Assessment of phosphorylation of recombinant VEGFR2 by LC/MS

LC/MS analysis was performed with an LCT Premier (Waters, USA). Protein samples were dissolved at a concentration of 0.2 mg/ml with 0.025% of trifluoroacetic acid. Each sample was loaded onto a Symmetry 300 C4 column (Waters) and eluted with a gradient of acetonitrile. The mass spectrum was monitored in the positive ion mode and data were processed with MaxEnt1 software (Waters).

LC/MS results of non phosphorylated and phosphorylated catalytic domain (806- 1171) without the kinase insert domain (940 989) and N terminal 6 × His tag have been already displayed.⁸



Supplementary Table 1. Inhibitory activity for non-phosphorylated and phosphorylated VEGFR2

	Non-phosphorylated	Phosphorylated
A	0.62 (0.56 – 0.69)	0.86 (0.78 – 0.94)
B	4.4 (3.6 – 5.3)	> 100
C	22 (19 – 26)	> 100
D	0.033 (0.031 – 0.036)	0.044 (0.041 – 0.047)
E	49 (42 – 57)	> 100

Estimated IC₅₀ values with 95% CI in parentheses.

Supplementary Table 2. Results of the thermal shift assay

	Concentration (μ M)	Non-phosphorylated ΔT_m ($^{\circ}$ C)	Phosphorylated ΔT_m ($^{\circ}$ C)
A	30	6.1 (0.2)	7.4 (0.4)
B	100	3.8 (0.5)	1.5 (0.9)
C	100	2.5 (0.4)	0.5 (0.3)
D	30	6.7 (0.4)	7.6 (0.5)
E	300	0.9 (0.1)	-0.1 (0.0)
Staurosporine	3	6.3 (0.3)	8.5 (0.6)

^aEstimated ΔT_m ($^{\circ}$ C) values with SD in parentheses. Data are from three independent analyses.

Supplementary Table 3. Results of the Yonetani-Thorell Plots

	Binding site	Yonetani-Thorell analysis with			
		compound A	α	compound B	α
A	Hinge	NA ^b	-	Independent ^c	0.21 ^c
B	BP ^a	Independent ^c	0.21 ^c	NA ^b	-
C	BP ^a	Not independent	1.6 ^d	Not independent	2.4
D	Hinge	Exclusive	>10	Independent	0.47
E	BP ^a	Not independent	4.9	Exclusive	>10
Staurosporine	Hinge	Exclusive	>10	Exclusive	>10

^aBP; Back pocket. ^bNA; Not applicable. ^c the same result. ^dFrom tracer displacement analysis

Supplementary Table 4. Summary of crystallographic parameters and diffraction data and refinement statistics of compound **A** and phosphorylated VEGFR2

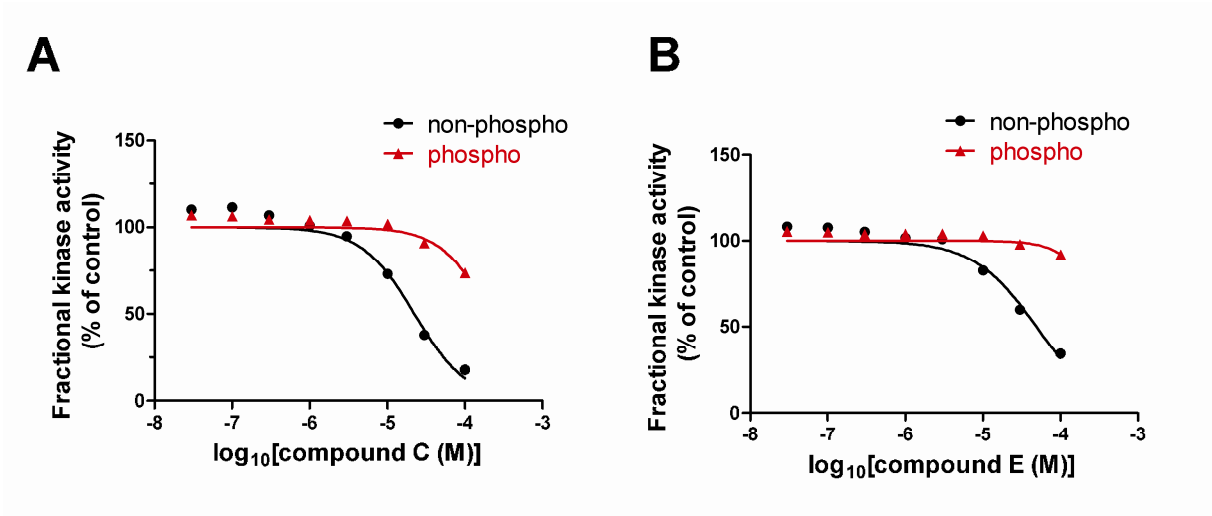
Crystallographic data	
Space group/asymmetric unit	$P2_12_12_1$
Unit Cell dimensions (Å)	$a = 38.2, b = 94.6, c = 96.3$
Data Statistics	
Resolution (Å)	67.52 – 2.30
Completeness (%)	97.0
R_{sym} (%)	7.7
Average $I/\sigma(I)$	10.1
Redundancy	3.0
Refinement data	
R (R_{free}) (%)	24.5 (28.1)
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.238
¹ Completeness, R_{sym} and $I/\sigma(I)$ are given for all data. $R_{\text{sym}} = \sum I - \langle I \rangle / \sum I$, where I is the intensity for individual reflections and $\langle I \rangle$ is the mean intensity of that reflection. $R = \sum F_o - F_c / \sum F_o $, where F_o and F_c are the observed and calculated structure factors, respectively. R_{free} was calculated using a test set of 5% of the data.	

Supplementary Table 5. Summary of crystallographic parameters and diffraction data and refinement statistics¹ of compound **E** and non-phosphorylated VEGFR2

Crystallographic data	
Space group/asymmetric unit	<i>C</i> 2
Unit Cell dimensions (Å)	<i>a</i> = 135.7, <i>b</i> = 56.4, <i>c</i> = 51.9
Data Statistics	
Resolution (Å)	37.35 – 2.49
Completeness (%)	98.8
<i>R</i> _{sym} (%)	6.9
Average <i>I</i> /σ(<i>I</i>)	11.4
Redundancy	3.73
Refinement data	
<i>R</i> (<i>R</i> _{free}) (%)	21.0 (26.2)
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	0.923
¹ Completeness, <i>R</i> _{sym} and <i>I</i> /σ(<i>I</i>) are given for all data. $R_{\text{sym}} = \sum I - \langle I \rangle / \sum I$, where <i>I</i> is the intensity for individual reflections and $\langle I \rangle$ is the mean intensity of that reflection. $R = \sum F_o - F_c / \sum F_o $, where <i>F</i> _o and <i>F</i> _c are the observed and calculated structure factors, respectively. <i>R</i> _{free} was calculated using a test set of 5% of the data.	

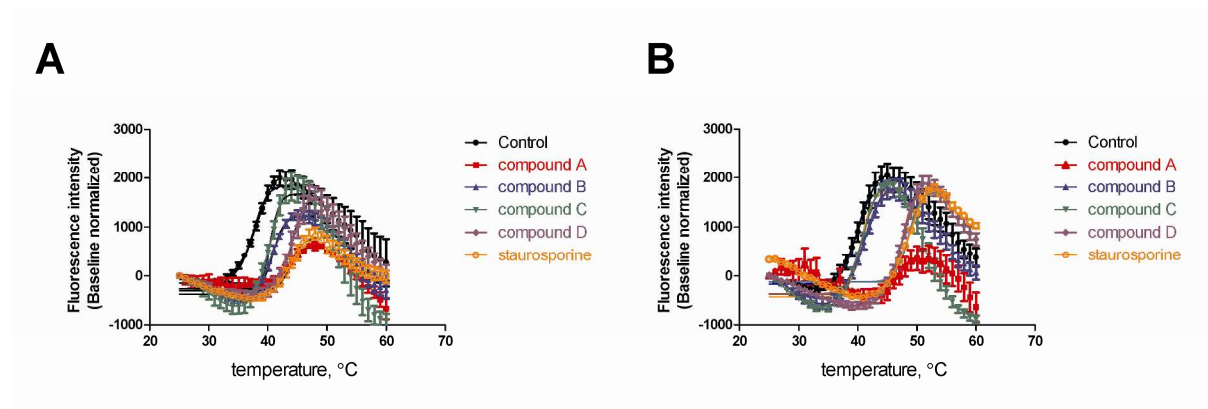
Supplementary Figure 1

Inhibitory activity of compound C (A) and compound E (B) for non-phosphorylated (black) and phosphorylated (red) VEGFR2



Supplementary Figure 2

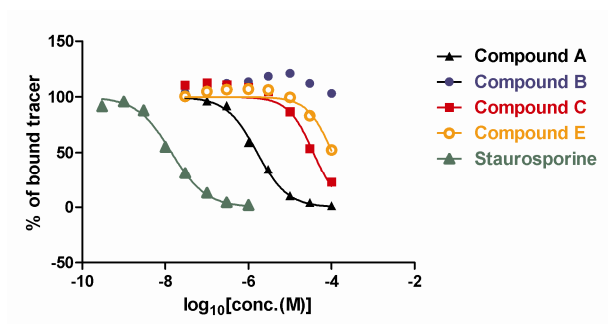
Thermal shift assay with the non-phosphorylated catalytic domain of VEGFR2 (A) and the phosphorylated catalytic domain of VEGFR2 (B). Data represent the mean \pm SD (n=3). Representative results from three independent experiments are shown.



Supplementary Figure 3

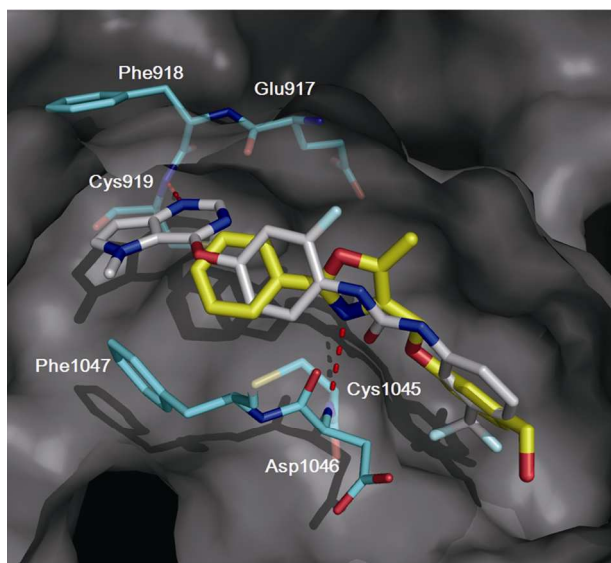
Tracer displacement assay from the non-phosphorylated catalytic domain of VEGFR2

Results obtained by use of fluorescent labeled compound **D**



Supplementary Figure 4

Overlay comparison of E (Yellow) and 20d (white) in complex with non-phosphorylated VEGFR2. H-bonds are depicted as red dashes.



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