Supporting Information

Structural Analysis of the Binding of Type I, $I_{1/2}$, and II Inhibitors to Eph Tyrosine Kinases

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2. Experimental Section

Protein expression and purification

A clone of the EphA3 kinase domain (residues: 606-947) was obtained from Prof. Sirano Dhe-Paganon's group [1] and expressed in *Escherichia coli* strain BL21 (DE3). Cells expressing EphA3 were induced with a 1 mM solution of isopropyl-*β*-D-thiogalactopyranoside (IPTG) for 12 h at 15 °C. Cell pellets were resuspended in buffer A (50 mM Tris, pH 8.0, and 100 mM NaCl, supplemented with protease inhibitors) and lysed by sonication. After centrifugation at 15,000 rpm for 1 h, the soluble fraction of EphA3 was purified using HisTrap FF crude and HiTrap Q HP columns (GE Healthcare), followed by gel filtration chromatography (Superdex75; GE Healthcare). The appropriate fractions were combined and concentrated to ~10 mg/mL using Amicon filter devices (10 kDa as cutoff) in a storage solution (100 mM sodium chloride and 10 mM Tris-HCl pH 8.0, 5% glycerol). The resulting solution was aliquoted and stored at -80 °C for further usage.

Crystallization, Data Collection, and Structure Determination

Apo crystals of the EphA3 kinase domain were grown at 20 °C using the hanging drop vapor diffusion method. Equal volumes of protein and reservoir solutions (0.1 M sodium cacodylate pH 6.5, 0.15 M ammonium sulfate, 22.5 % PEG 3350) were mixed and crystals appeared after 1 to 2 days. A 5 mM solution of inhibitor (in 100 % DMSO) was added into the hanging drop to reach a final DMSO concentration of 10 % (v/v). The crystals were soaked with compounds **7** for 1 to 24 h and flash-frozen in liquid nitrogen without extra cryoprotectant. Co-crystallization trials were performed for **Birb796**. EphA3 was first mixed with a 5 mM **Birb796** solution (in 100 % DMSO) to a final 10 % DMSO concentration and then incubated on ice for 30 minutes. EphA3- **Birb796** crystals were grown under the same conditions as described above for the EphA3 apo crystals. Data sets were collected on a Pilatus 6M detector at the Swiss Light Source beamline X06SA of the Paul Scherrer Institute (Villigen, Switzerland) and indexed, integrated and scaled with the XDS [2] and CCP4 programs.[3] The structures were solved by molecular replacement with PHASER [4] using the apo EphA3 kinase domain structure (PDB entry 2GSF) as a search model and refined with PHENIX.[5] The atomic coordinates and structure factors of EphA3 in complex with the inhibitors **7**, **Birb796** have been deposited with the Protein Data Bank as entries **4TWO** and **4TWN** respectively.

Table S1

Data collection and processing statistics of unpublished structures

	7	**Birb796**
Space group	P 1 21 1	P 1 21 1
Unit cell		
a (Å)	53.22	55.48
b (Å)	38.07	38.42
c (Å)	75.31	74.14
Resolution range (Å)	38.07-2.05	48.40-1.71
Unique reflections	18078(613)	33440(1134)
<I/σ(I)>	23.0(8.1)	17.6(2.1)
R merge	0.035 (0.137)	0.050(0.631)
Completeness (%)	95.5. (90.9)	99.1(95.4)
Multiplicity	2.7(2.7)	3.7(3.7)
Refinement		
Resolution range (Å)	30.77-2.05	48.40-1.71
R factor/R free	16.27/20.06	17.48/21.27
Mean B factors (A2)	25.80	25.10
RMS bonds (Å)	0.0072	0.0069
RMS angles (°)	0.990	1.247

*Values in parentheses are for the highest resolution shells.

3. 2mFo – DFc Electron Density Map of Inhibitor 7

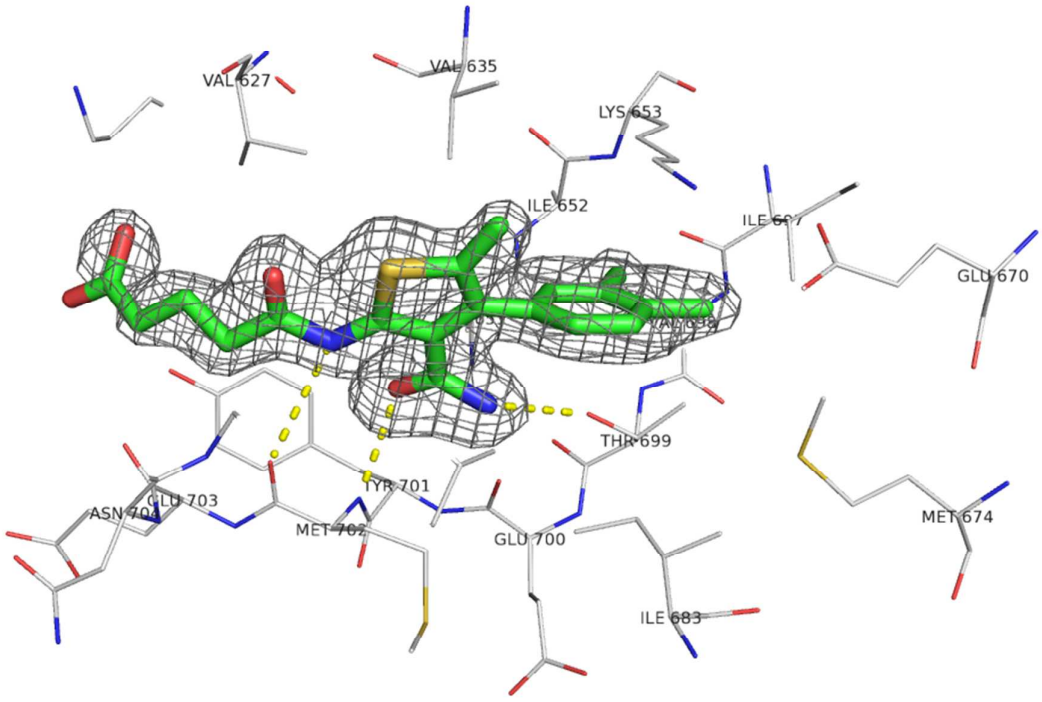


Figure S1. $2mF_o - DF_c$ electron density map contoured at 1σ (grey mesh) was generated in a region within 1.6 Å for compound **7** (pdb code **4TWO**) using PHENIX and Pymol.

4. Structural Analysis Section

4.1 Xanthine inhibitors

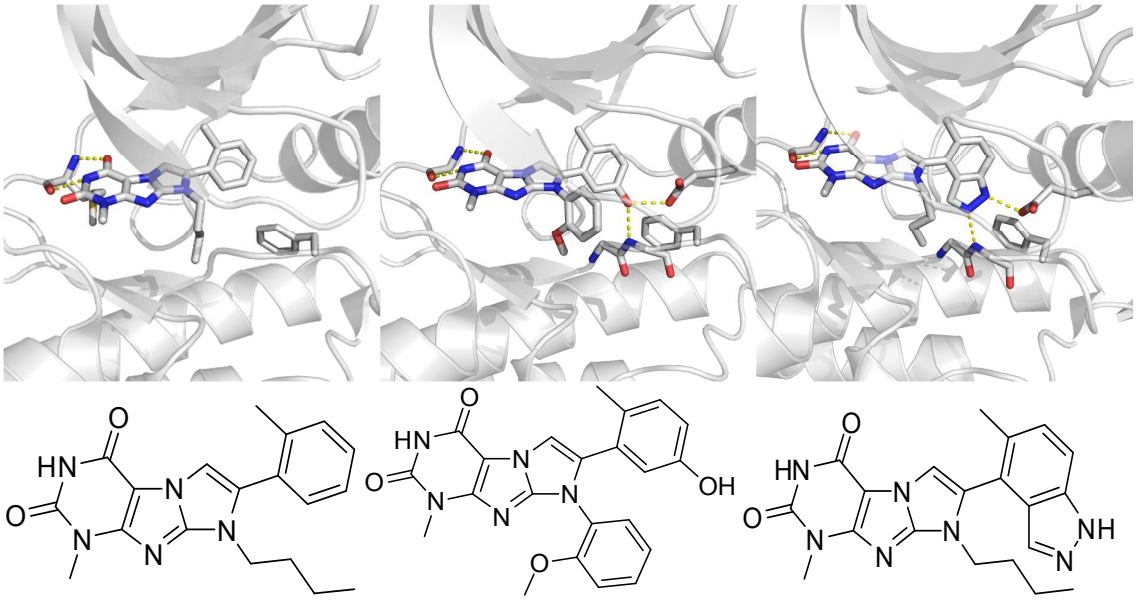


Figure S2. Crystal structures of the catalytic domain of the tyrosine kinase EphA3 in complex with the inhibitors **1** (left, pdb code **4GK3**), **2** (middle, **4GK2**) and **3** (right, **4GK4**). The ATP binding site of the EphA3 kinase is shown by ribbons while the side chains of the DFG Phe and C-helix Glu are shown by sticks.

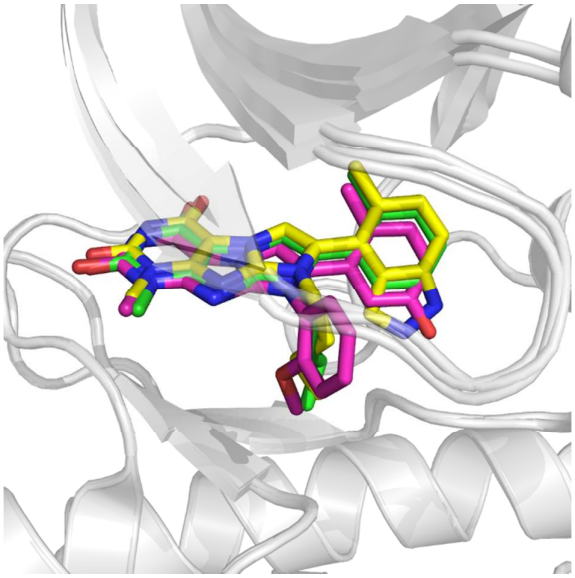


Figure S3. Superposition of X-ray structures of the xanthine inhibitors **1**, **2,** and **3** in complex with EphA3.

4.2 Pyrrolo[3,2-b]quinoxaline inhibitors

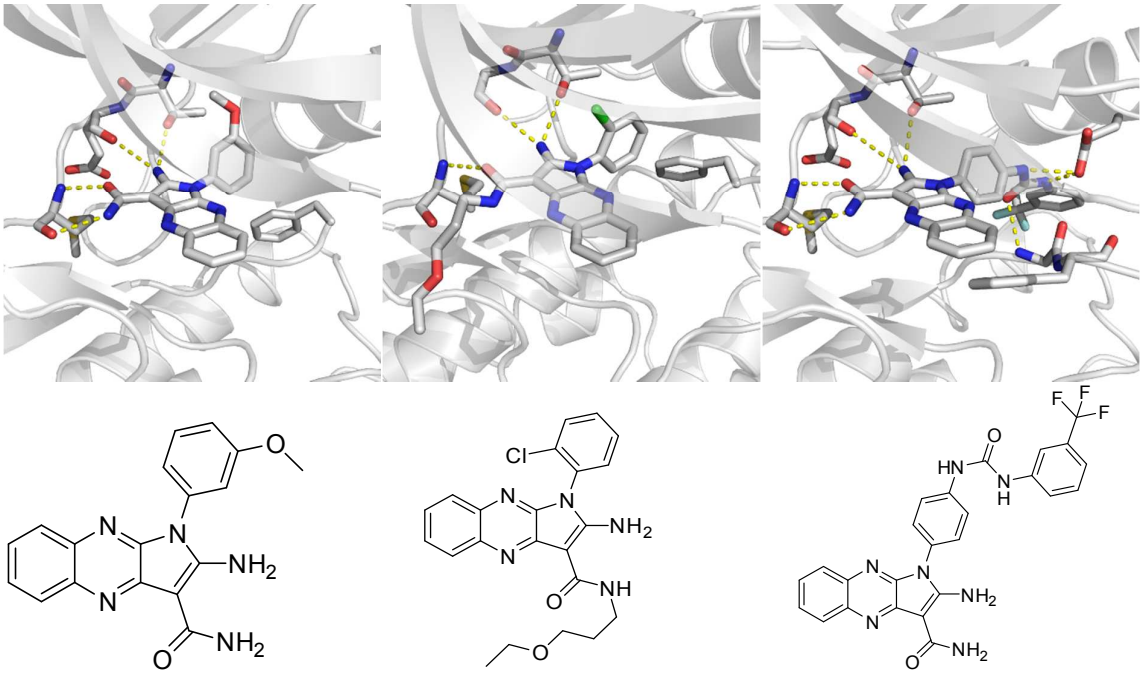


Figure S4. Crystal structures of the catalytic domain of the tyrosine kinase EphA3 in complex with the inhibitors **4** (left, pdb code **4P4C**), **5** (middle, **4P5Q**), and **6** (right, **4P5Z**). The ATP binding site of the EphA3 kinase is shown by ribbons while the side chains of the DFG Phe and C-helix Glu are shown by sticks.

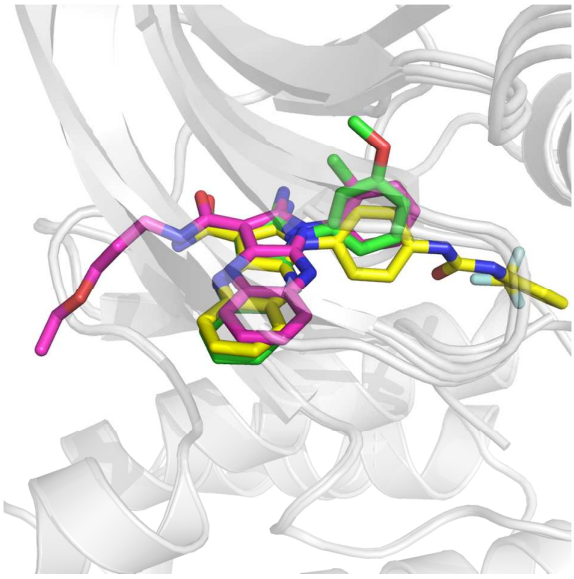
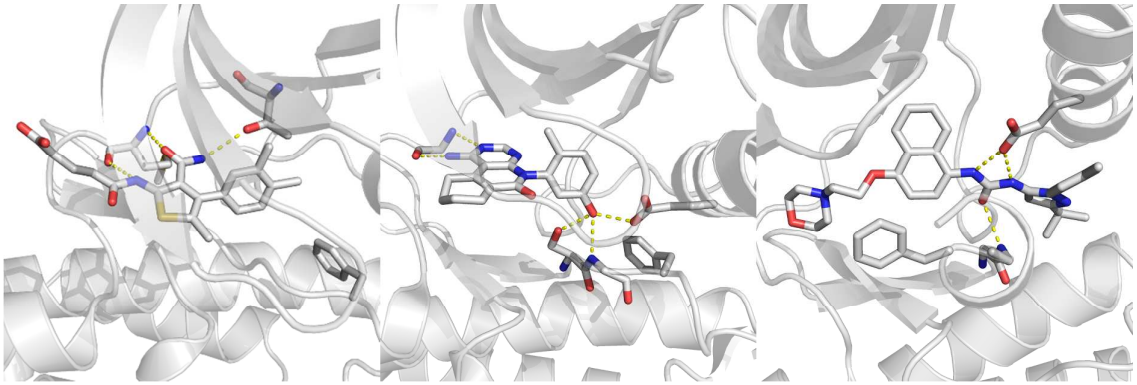


Figure S5. Superposition of the X-ray structures of pyrrolo[3,2-*b*]quinoxaline inhibitors **4**, **5,** and **6** in complex with EphA3.

4.3 Inhibitors 7, 8, and Birb796



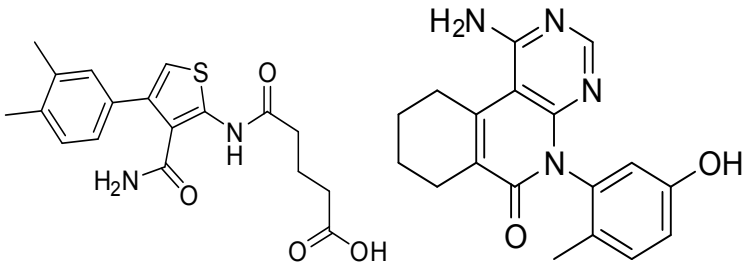


Figure S6. Crystal structures of the catalytic domain of the tyrosine kinase EphA3 in complex with the inhibitors **7** (left, **4TWO**), **8** (middle, **4G2F**), and **Birb796** (right, **4TWN**). The ATP binding site of the EphA3 kinase is shown by ribbons while the side chains of the DFG Phe and C-helix Glu are shown by sticks.

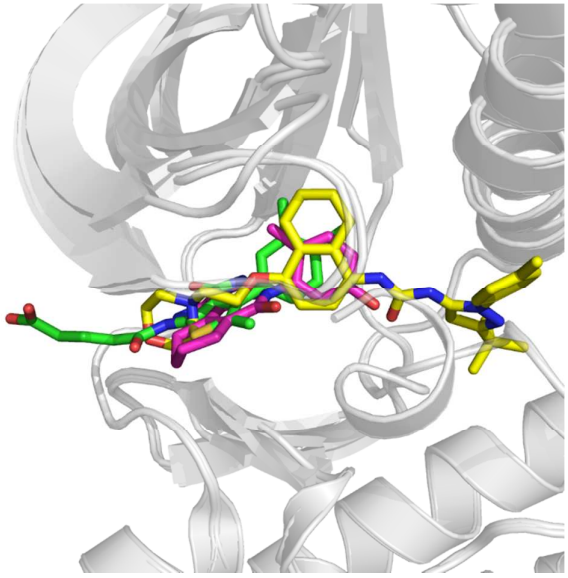


Figure S7. Superposition of the X-ray structures of inhibitors **7**, **8,** and **Birb796** in complex with EphA3.

References

1. Choi, Y.; Syeda, F.; Walker, J. R.; Finerty, P. J.; Cuerrier, D.; Wojciechowski, A.; Liu, Q. S.; Dhe-Paganon, S.; Gray, N. S. Discovery and structural analysis of Eph receptor tyrosine kinase inhibitors. *Bioorganic & Medicinal Chemistry Letters* **2009,** 19, 4467-4470.

2. Kabsch, W. Automatic Processing of Rotation Diffraction Data from Crystals of Initially Unknown Symmetry and Cell Constants. *Journal of Applied Crystallography* **1993,** 26, 795-800.

3. Bailey, S. The Ccp4 Suite - Programs for Protein Crystallography. *Acta Crystallographica Section D-Biological Crystallography* **1994,** 50, 760-763.

4. Mccoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *Journal of Applied Crystallography* **2007,** 40, 658-674.

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