Optimized NMR-based Fragment Screening Rapidly Reveals Several Specific Low Molecular Weight Binders for the Ligand-binding Domain of EphA2 Receptor

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Supplementary information

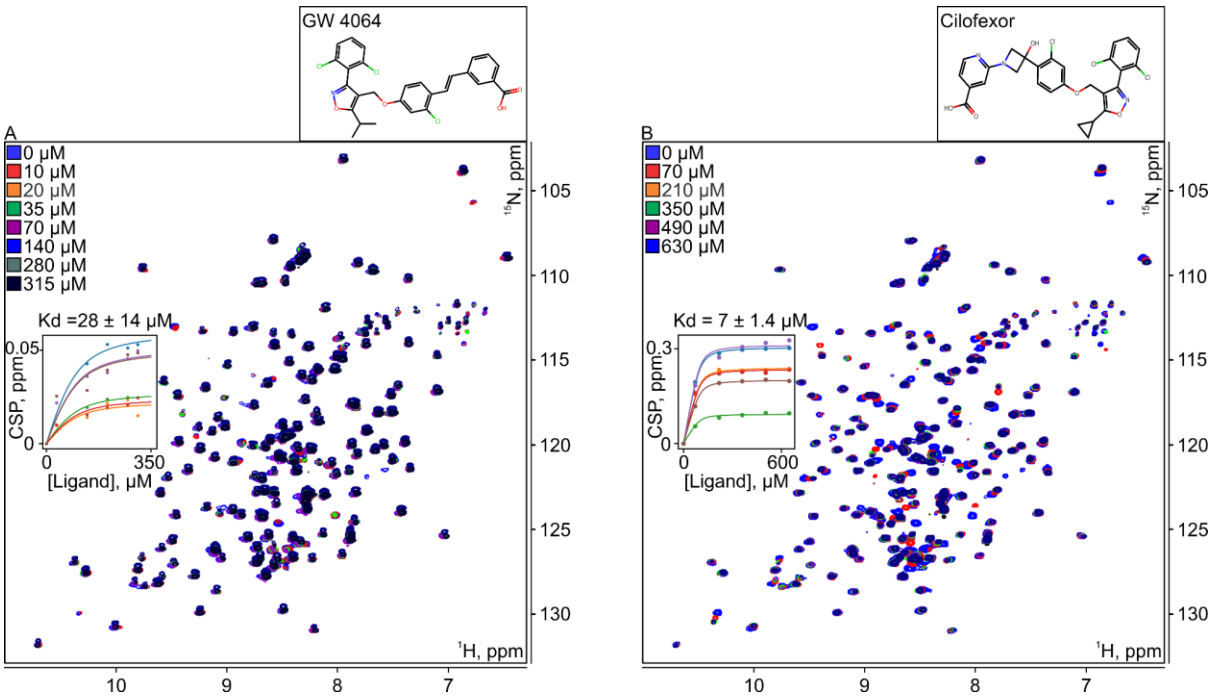

Figure S1. **Binding of EphA2 LBD to the known ligands**. **A**) Overlay of ^{1}H,^{15}N-HSQC spectra of EphA2 LBD recorded in the presence of GW4064 at different concentrations: 0, 10, 20, 35, 70, 140, 280, 315 μM. The structure of GW 4064 is shown on the top of the panel. **B**) Overlay of ^{1}H,^{15}N-HSQC spectra of EphA2 LBD recorded in the presence of Cilofexor at different concentrations: 0, 70, 210, 350, 490, 630 μM. The structures of GW 4064 and cilofexor are shown on the top of the panels. The CSP dependence on the ligand concentration is shown for 6 residues to estimate the K_D in **A** and **B**. Solid lines show the result of approximation of experimental data with the theoretical dependence (eq. (4))

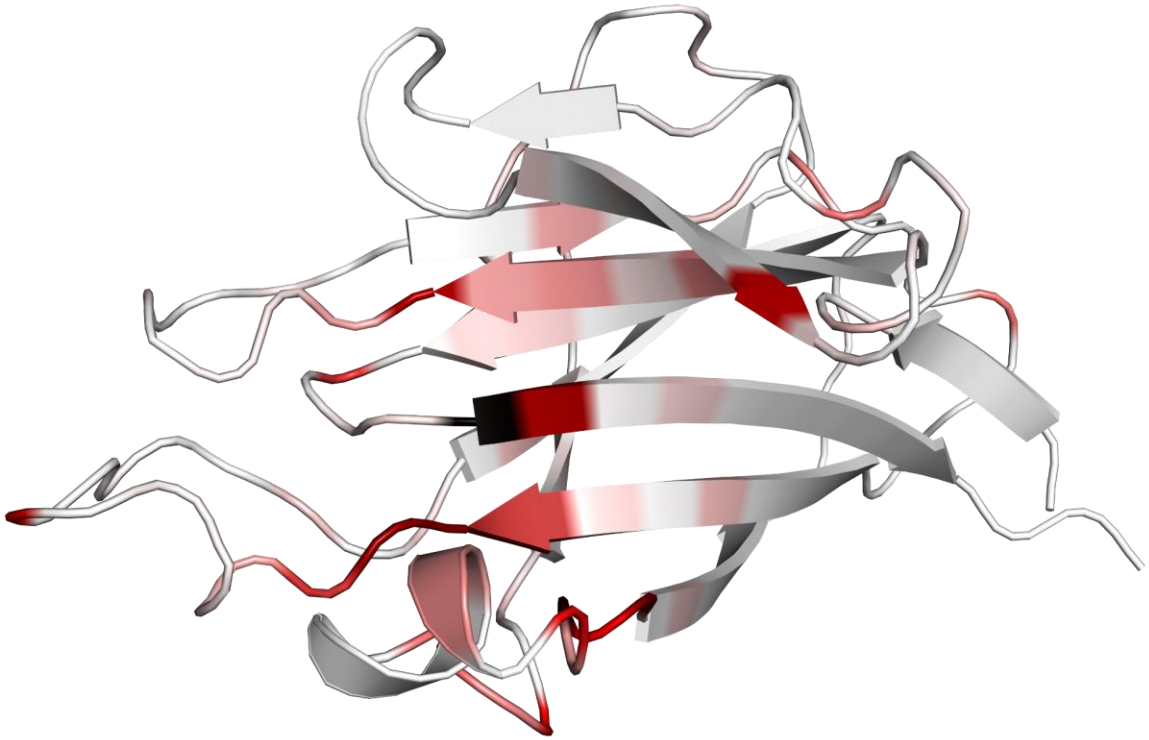

Figure S2. **Binding mode of the FBS hits**. EphA2 LBD is painted according to the average value of normalized CSPs in all titrations. Residues with dark red and black colors have higher CSPs in all the titrations.

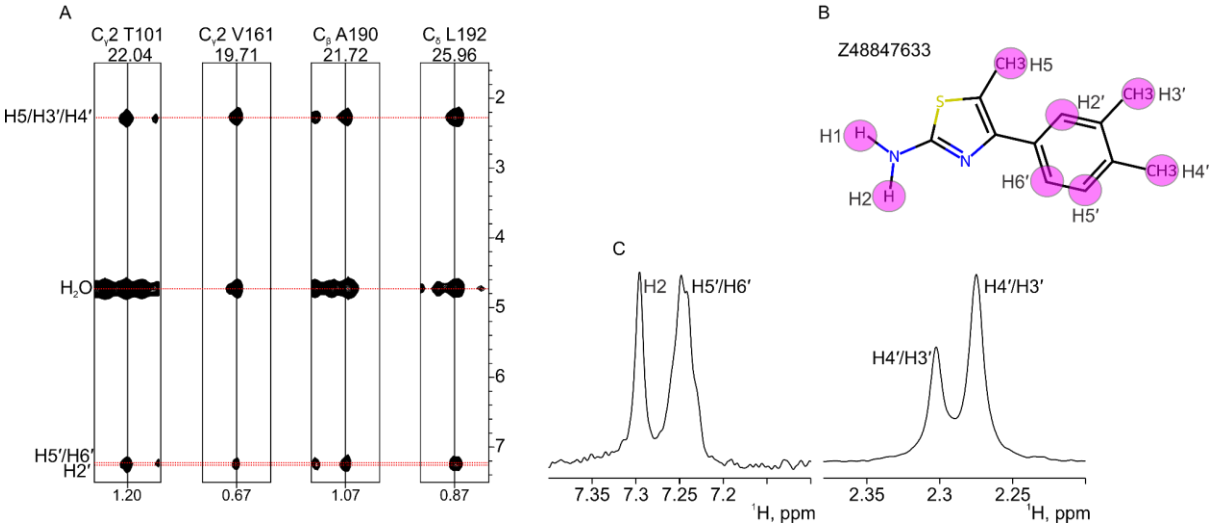

Figure S3. **NMR data, characterizing the interaction between Z48847633 and EphA2 LBD**. **A**) 2D slices of a 3D $^{15}N/^{13}C$-filtered,^{13}C-edited-NOESY-HSQC are indicated for the methyl group resonances of T101, V161, A190, and L192. Assignments of the intermolecular cross-peaks are indicated. **B**) Structure of the compound with the chemical shift nomenclature. **C**) Fragments of 1D $^{13}C/^{15}N$-filtered-NOESY spectrum, containing the signals of Z48847633 with the indication of the assignments.

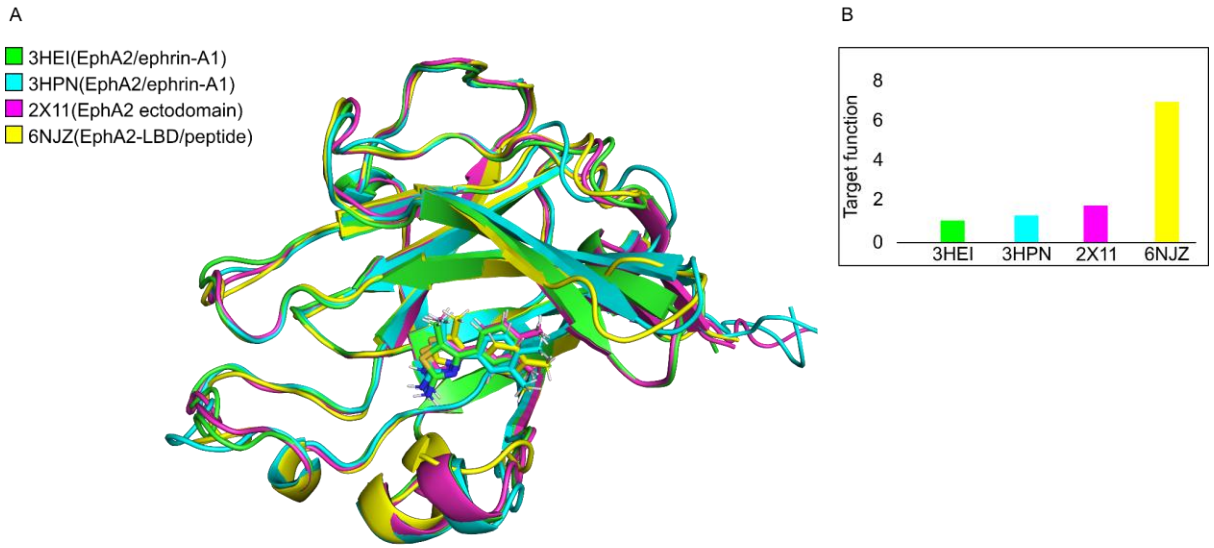

Figure S4. Selection of X-ray templates for NMR structure determination of EphA2 LBD in complex with Z48847633. A) Overlay of EphA2 LBD/Z48847633 complex conformations obtained based on several X-ray structures (PDB IDs: 3HEI, 3HPN, 2X11, and 6NJZ). **B**) CYANA target function for selected templates.

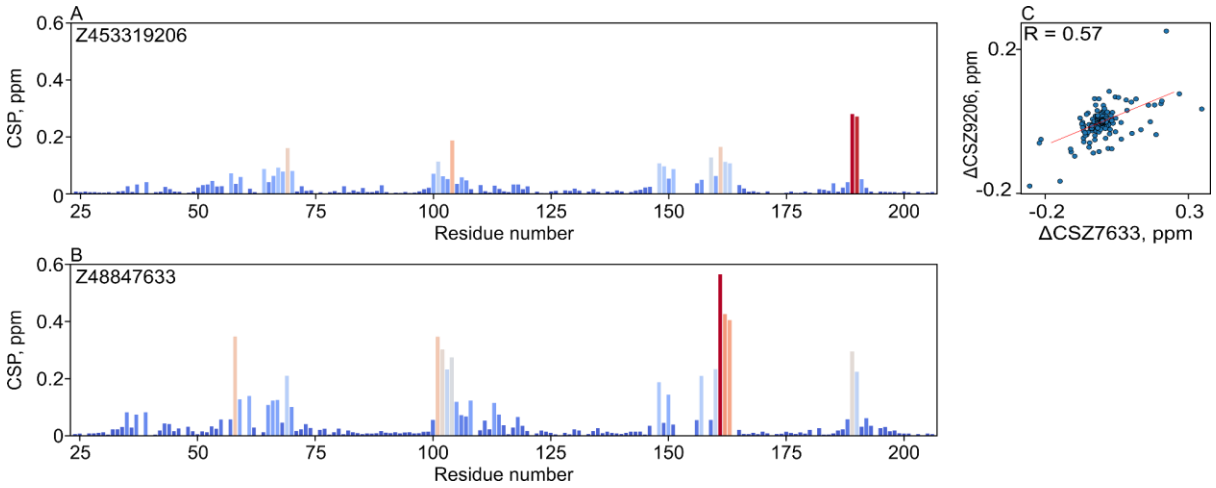

Figure S5. **Binding modes of Z453319206 and Z48847633 according to the CSP data**. **A**) ligand-induced CSPs in EphA2-LBD are plotted versus the residue number for the Z453319206 (**A**) and Z48847633 (**B**). Residues with high CSP have warm colors. **C**) Correlation between the CSPs induced by Z48847633 and by Z453319206. Pearson coefficient (R) is indicated.

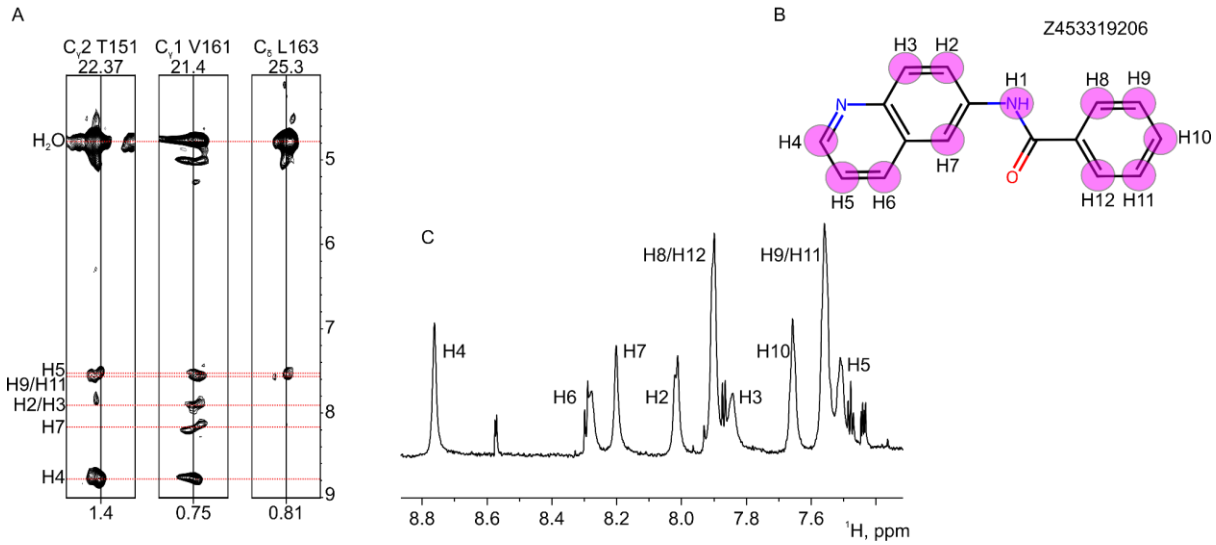

Figure S6. NMR data, reporting on the interaction between Z453319206 and EphA2 LBD. **A**) 2D strips of a 3D $^{15}N/^{13}C$-filtered,^{13}C-edited-NOESY-HSQC are indicated for the methyl group resonances of T151, V161, and L163. Assignments of the intermolecular cross-peaks are indicated. **B**) Structure of the compound with the chemical shift nomenclature. **C**) Fragment of a 1D $^{13}C/^{15}N$-filtered-NOESY spectrum, containing the signals of Z453319206 with the indication of the assignments.

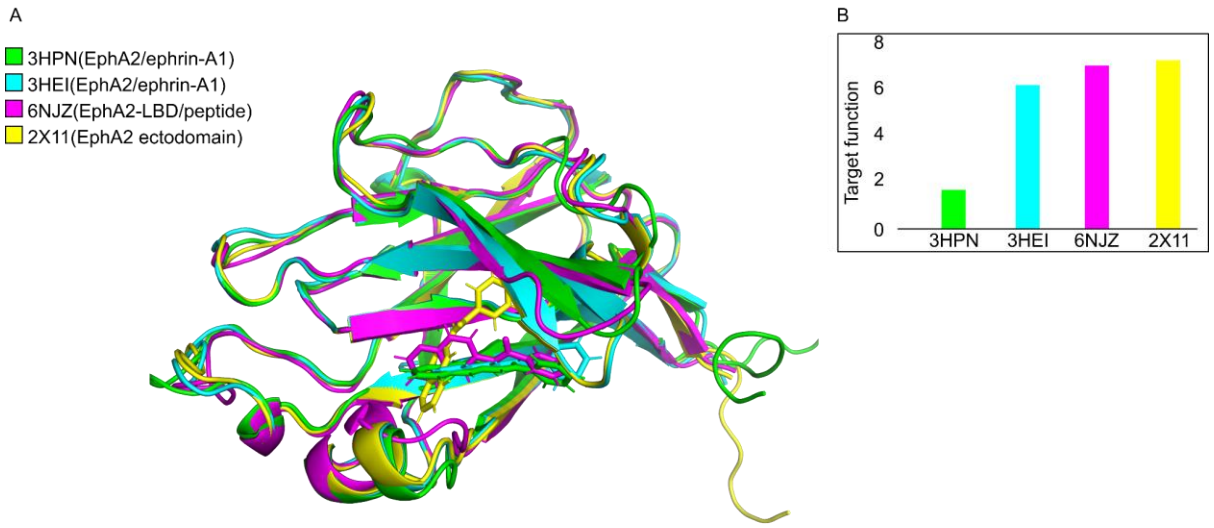

Figure S7. **Selection of X-ray templates for NMR structure determination of EphA2 LBD in complex with Z453319206**. **A**) Overlay of EphA2 LBD/Z453319206 structures obtained based on several X-ray templates (PDB IDs: 3HEI, 3HPN, 2X11, and 6NJZ). **B**) CYANA target function for selected templates.

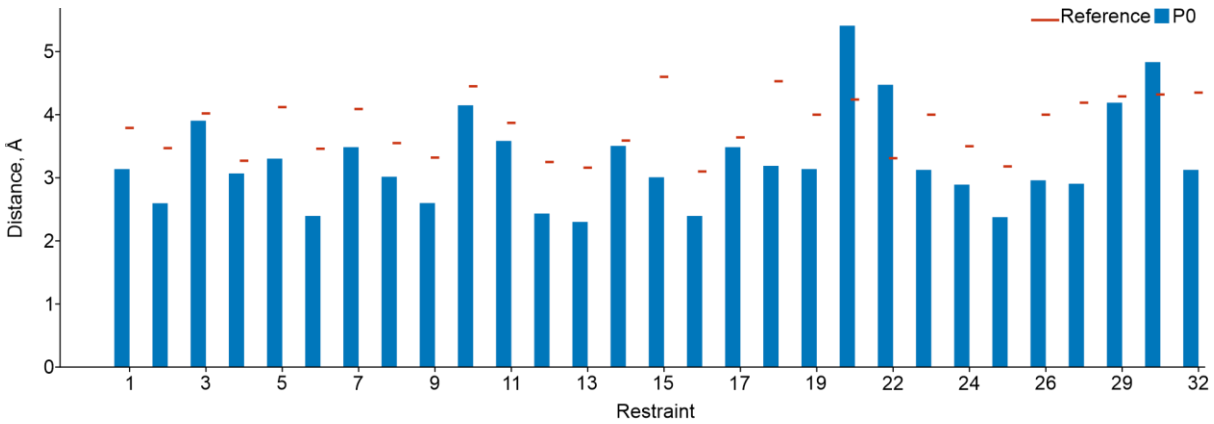

Figure S8. Comparison of MD simulation distances with the NMR restraints. Bars indicate the distances, corresponding to the each of the 32 peaks observed in the 3D ^{15}N,^{13}C-filtered-NOESY-HSQC spectrum of Z48847633/EphA2 LBD complex. The distances were averaged over all MD trajectories. Red lines denote the NMR-derived distances for the peaks. Ambiguity of assignment is taken into account by averaging the sum of $1/r^6$ of possible distances that can contribute to the respective NOE peak.

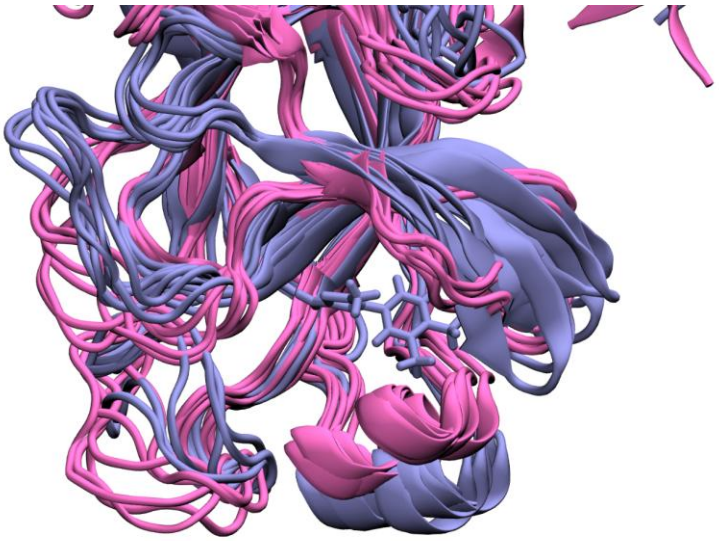

Figure S9. Changes in the EphA2 LBD structure upon binding to Z48847633 . EphA2 LBD pocket with ligand (iceblue) and without ligand (malve) between 500 and 550 ns of MD simulation.

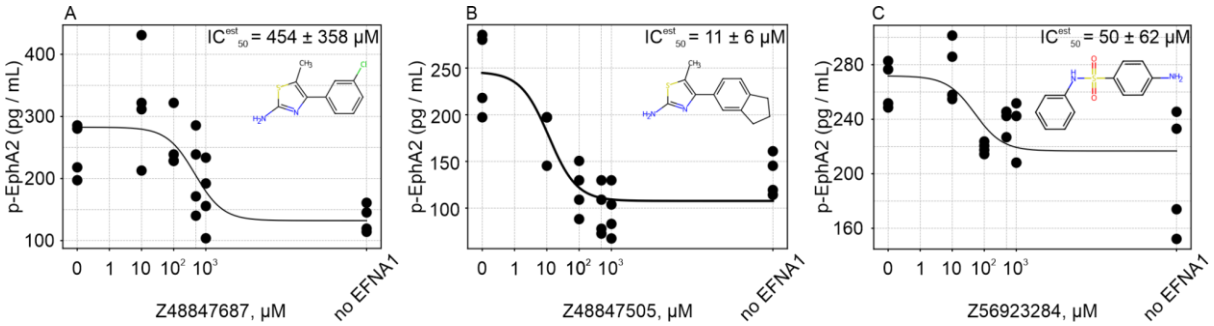

Figure S10. Quantitative analysis of the inhibitory activities of FBS hits and their derivatives. A, B, C) Dependencies of ELSA readout on ligand concentration in Figure 7 were approximated with eq (6) to determine the IC_{50} of the Z48847505, Z48847687, and Z56923284 compounds. (+) and (-) represent the positive (no inhibitor added) and negative (no ephrin A1 added) controls, respectively. The data is representative of 4 independent experiments (n = 4).

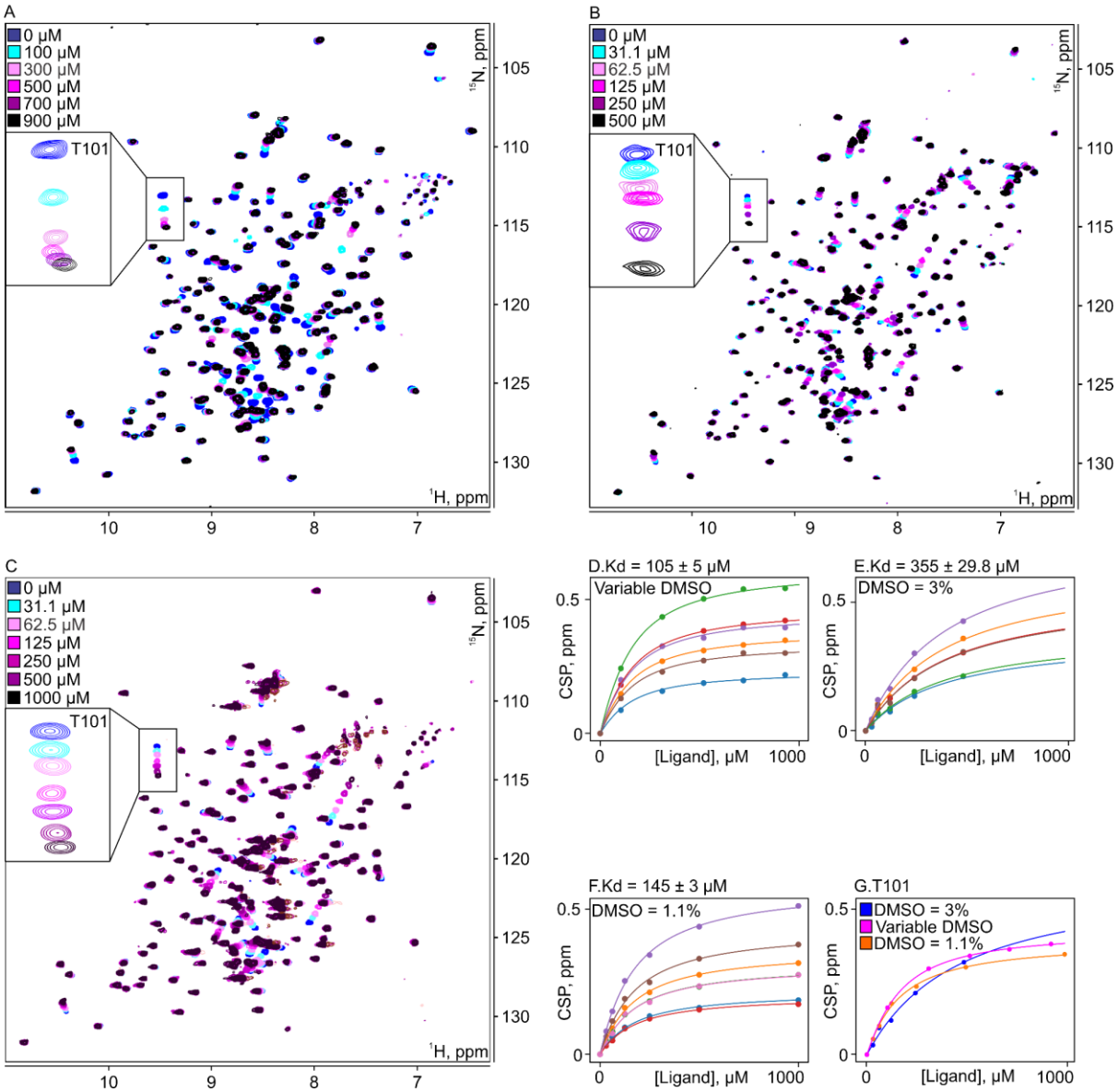

Figure S11. Effect of DMSO concentration on the affinity of Z48847633. **A**, **B**, **C**) Overlay of 2D ^{1}H,^{15}N-HSQC spectra of EphA2 LBD recorded in the presence of Z48847633 at variable (**A**), 3% (**B**), 1.1% DMSO concentration (**D**). The concentrations of Z48847633 are indicated. The spectra were obtained at 100 μM of the protein in **A** and 50 μM in **B** and **C**, 298 K, pH 6.7. The close-up view of the T101 cross-peak is shown in a rectangle. **D**, **E**, **F**) The CSP dependence on the ligand concentration is shown for 6 residues to estimate the K_D in **A**, **B**, and **C**, respectively**.** Solid lines show the result of the approximation of experimental data with the theoretical dependence (eq. (4)). **G**) The overlay of CSP dependencies on the ligand concentration is shown for T101 in three different set-ups.

Table S1. Binding of FBS hits to the ligand-binding domain of EphA2, as found in target-observed NMR titrations.

Name	Smiles	K_D(μM)
Z26548228	N#Cc1cccc(NC(=O)CCC2CCCC2)c1	49.2 ± 23.6
Z48847633	Cc1ccc(cc1(C))c2nc(N)sc2(C)	108.1 ± 4.8
Z56923284	Nc1ccc(S(=O)(=O)Nc2ccccc2)cc1	130.5 ± 53
Z198195774	NC(=O)Nc1ccc(-c2ccccc2)cc1	187.6 ± 28.4
Z19750454	Cc1cc(NC(=O)CSc2ccccc2)no1	264.9 ± 88.6
Z1623890017	C1CCN(CC1)c2noc(n2)c3cccs3	298.7 ± 62.5
Z363993198	Nc1nccc(-c2ccc(Cl)cc2)n1	318.3 ± 47.2
Z53860899	CCS(=O)(=O)NCc1ccc2c(c1)OCO2	337.7 ± 125.7
Z413792090	O=C(NCC1CC1)Nc2ccc3OCCc3c2	354.1 ± 80.7
Z28429411	Cc1ccc(CNC(=O)Cc2cccs2)cc1	369.5 ± 74.4
Z735557654	CCOC(=O)c1sc(nc1C)c2cnccn2	372.6 ± 78.8
Z1230032143	CCOC(=O)c1sc(N(C)C(C)=O)nc1C	380.4 ± 89.4
Z453319206	O=C(Nc1ccc2ncccc2c1)c1ccccc1	452.6 ± 41.1
Z19735192	O=C(COc1ccccc1)Nc1ccccc1O	459.9 ± 109.2
Z68195082	O=C(COc1ccccc1)Nc1ccccc1O	540.4 ± 81.8
Z27678561	COc1ccc2nc(NC(=O)C3CC3)sc2c1	550.2 ± 84.1
Z57299529	c1ccc(CCNc2nc3ccccc3[nH]2)cc1	644.8 ± 251.1
Z1461982627	CC(C)c1nnc(NCC(C)(C)C)s1	705.7 ± 300
Z933326822	Nc1cccc(OCc2ccccc2)c1	737.5 ± 47.6
Z26333448	Fc1ccc(Cn2cnc3ccccc32)cc1	808.6 ± 50.6
Z28290384	COc1cccc(c1)C(=O)Nc2ccccc2(F)	830 ± 52.4
Z2204875953	c1cc(-c2ccc3c(c2)OCO3)n[nH]1	874.5 ± 137.3
Z1741964527	OC1CN(C(c2ccccc2)c2ccccc2)C1	887.1 ± 364.3
Z89385775	CCc2ccccc2(NC(=O)c1c(C)nns1)	935 ± 273.1
Z2856434805	NCc1ccc(-c2cccs2)cc1	954.7 ± 237.7
Z136583524	Cc1occc1C(=O)Nc1ccncc1	989.2 ± 381.5
Z30917949	CCc1ccc(NC(=O)c2cnccn2)cc1	1003.6 ± 256.7
Z1954800564	COc1ccc2sc(N)nc2c1	1021.6 ± 263.3
Z26769872	CC1CCN(CC1)C(=O)c2cc3ccccc3(o2)	1079.7 ± 362.6
Z57515803	O=C(OCc1ccc(Cl)cc1)c1cccnc1	1393.9 ± 168.8
Z57101343	Cc1ccc(Nc2ncnc3[nH]ncc23)cc1	1487.6 ± 215.3
Z419884046	CC1CCN(C(=O)CCCc2ccccc2)CC1	1491.9 ± 524.6
Z57190020	COC(=O)Nc1ccc(Cc2ccncc2)cc1	1515.3 ± 367
Z1217741507	CC(C)(C)C1CCN(CC1)C(=O)c2ccccc2	1584.2 ± 301.9
Z56837087	N#CCC(=O)Nc1ccc(Cl)cc1	1698.2 ± 566.2
Z30891796	Cc1ccc(C)c(c1)OC(=O)c2ccncc2	2161.5 ± 329.9
Z45516134	CCN(CC)S(=O)(=O)c1ccc(C)cc1	2165.6 ± 522
Z48847594	CC(=O)Nc1ccc(-c2csc(N)n2)cc1	2665.6 ± 939
Z1429867185	COc1cccc2[nH]ccc12	4554.3 ± 2820.4
Z1891776064	OCc1cn(-c2ccc(Cl)c(Cl)c2)nn1	9242.6 ± 8685
Z425449682	NCc1cc(-c2ccccc2)no1	nd[a]
Z2856434898	c1ccc(CN2CCC(n3cccc3)CC2)cc1	nd
Z2856434806	N#CCN1CCN(c2cccc(Cl)c2)CC1	nd

[a]K_D is weaker than 10 mM and could not be determined in the used set-up.

Table S2. The relative free energy of dissociation (ΔΔG) of the second-generation SAR compounds, Z48847633 is taken as a reference compound

Name	Smiles	ΔΔG(Kcal/mol)
Z48847631	CCCC=1C=CC(=CC1)C=2N=C(N)SC2C	-0.40
Z48847639	CCC=1C=CC(=CC1)C=2N=C(N)SC2C	-0.34
Z275127546	CC=1SC(N)=NC1C=2C=CC(=CC2)C(C)C	-0.23
Z48847687	CC=1SC(N)=NC1C=2C=CC=C(Cl)C2	-0.17
Z48847505	CC=1SC(N)=NC1C=2C=CC=3CCCC3C2	-0.15
Z48847688	CC=1SC(N)=NC1C=2C=CC(Cl)=CC2	-0.03
Z3241472499	Cl.Cl.CC=1SC(N)=NC1C=2C=CC=NC2	0.10
Z48847551	CC=1C=CC(=CC1C)C2=CSC(N)=N2	0.25
Z48847640	CC=1SC(N)=NC1C2=CC(C)=C(C)C=C2C	0.27
Z48847651	CCC=1C=CC(=CC1)C2=CSC(N)=N2	0.35
Z48847632	CC=1SC(N)=NC1C=2C=CC(C)=CC2	0.36
Z48847630	CC=1SC(N)=NC1C2=CC(C)=CC=C2C	0.43
Z48847564	CC1=CC(C)=C(C)C=C1C2=CSC(N)=N2	0.55
Z48847498	COC=1C=CC(=CC1)C=2N=C(N)SC2C	0.56
Z53054008	CC=1C=C(C2=CSC(N)=N2)C(C)=C(C)C1C	0.57
Z48847685	CC=1SC(N)=NC1C=2C=CC=CC2	0.62
Z48847690	CCC=1SC(N)=NC1C=2C=CC=CC2	0.64
Z2733095325	CCC=1SC(N)=NC1C2=CC=CC=C2C	0.72
Z48847572	CC=1C=CC(C)=C(C1)C2=CSC(N)=N2	0.79
Z48847515	CC=1SC(N)=NC1C=2C=CC(F)=CC2	0.82
Z1575082020	CC=1C=CC=C(C1)C2=CSC(N)=N2	0.85
Z275128488	Cl.CC=1SC(=NC1C=2C=CC=CC2)NN	0.85
Z381785752	CC=1C=CC=CC1C2=CSC(N)=N2	0.89
Z48847645	CC1=C(N=C(N)S1)C1=CC=C(F)C(F)=C1	0.90
Z406680124	CC(C)C=1SC(N)=NC1C=2C=CC=CC2	0.94
Z1347869217	CC=1C=C(C=CC1F)C2=CSC(N)=N2	1.00
Z227972890	CC=1SC(N)=NC1C=2C=CC(F)=CC2F	1.00
Z48847560	CC=1C=CC(=CC1)C2=CSC(N)=N2	1.01
Z48847553	CC=1C=CC(C2=CSC(N)=N2)=C(C)C1	1.16
Z48847565	CC1=CC(C)=C(C2=CSC(N)=N2)C(C)=C1	1.56