

Supplemental Information

HTS by NMR of Combinatorial Libraries:

A Fragment-Based Approach to Ligand Discovery

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Inventory of Supplemental Information

Figure S1. Superposition of 2D [^{15}N , ^1H]-HSQC spectra of BIR3 in the absence and presence of the mixtures from tetra- and tri-peptide positional scanning library. Data associated related to those reported in Figure 2 of the manuscript.

Figure S2. Chemical shift mapping studies with compound 1. These data relates to the compounds and chemical shifts data listed in Figure 3.

Figure S3. 2D [^{15}N , ^1H]-HSQC spectra of EphA4 LBD with 3-methylindole, 4-cholorotulene, G-f16-G and G-G-f51. These data are associated with the data reported in Figure 4.

Figure S4. 2D [^{15}N , ^1H]-HSQC spectra of EphA4 LBD with compound 12 and 15. These data are supporting some of the data in Table 1 of the manuscript.

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Figure S6. ITC data for KYL, compounds 12, 15 and 22. These data are supporting the data reported in Table 1 of the manuscript.

Figure S7. Stability of compounds 15, 16, 22 and KYL in mouse serum or cell conditioned medium. These data support the statements in the manuscript that the compounds 15, 16 and 22 are much more stable than the reference peptide KYL.

Figure S8. Chemical shift mapping for two individual side chains of compound 6. These data support the binding of compound 6 side chains in different pockets of the EphA4 LBD.

Table S1. Summary of the nine mixtures applied in BIR3 studies. It supports the data reported in Figure 2.

Table S2. Summary of the components for the XXX library used against EphA4 LBD. It supports the data reported in Figure 4.

Table S3. Summary of the initial EphA4 LBD binding compounds resulting from the HTS by NMR approach. It supports the data reported in Figure 4.

Table S4. EphA4 LBD binding studies via ITC measurements. It supports the data reported in Table 1.

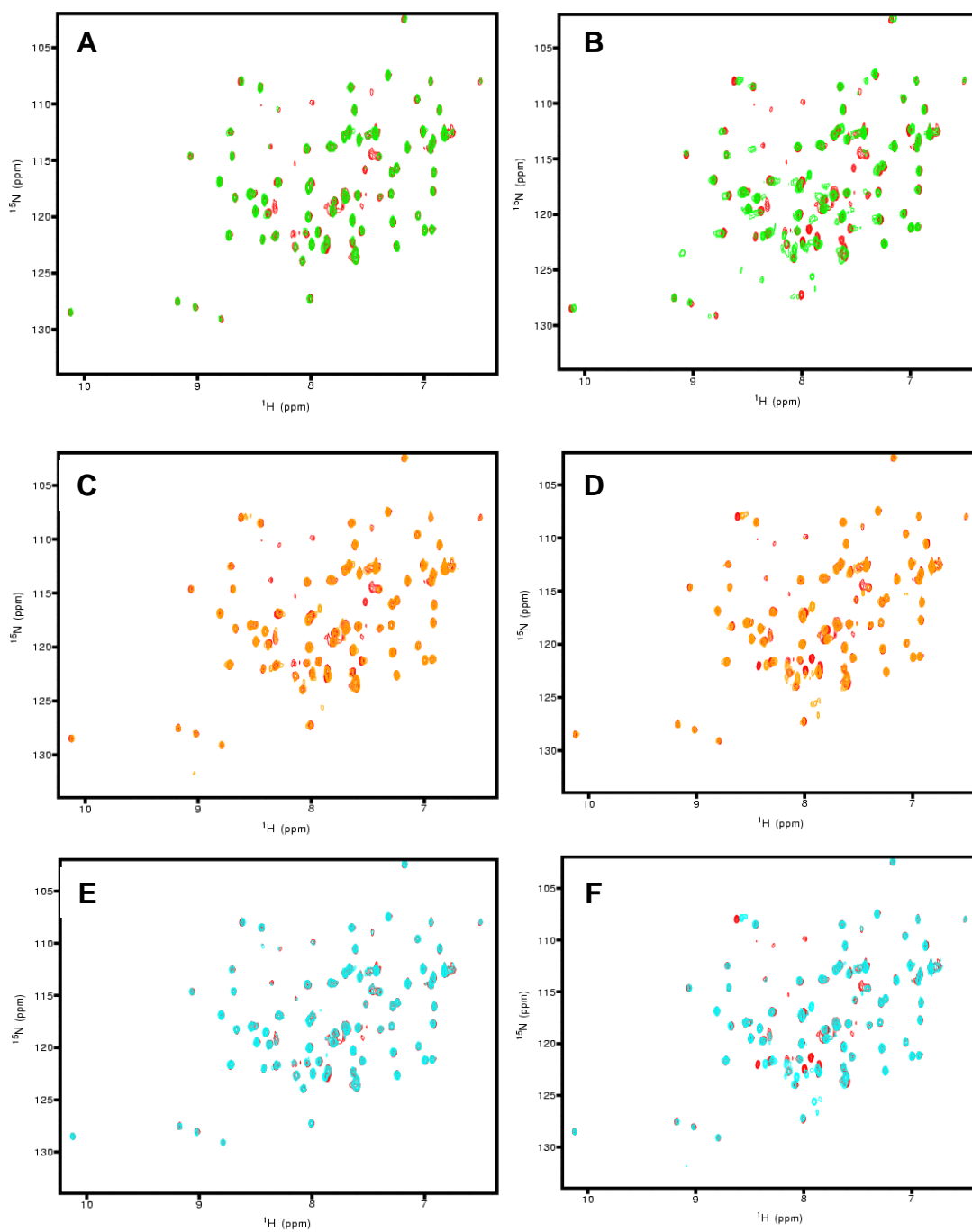


Figure S1. Superposition of 2D [^{15}N , ^1H]-HSQC spectra of BIR3 in the absence and presence of the mixtures from tetra- and tri-peptide positional scanning library. The spectra of apo BIR3 are colored in red. The spectra are colored in green for the presence of AXX (A) and AXXX (B), orange for the presence of XXP (C) and XXPX (D), cyan for the presence of GXX (E) and GXXX (F). X represents the mixture of all fragments, while A, P and G represent alanine, proline and glycine respectively.

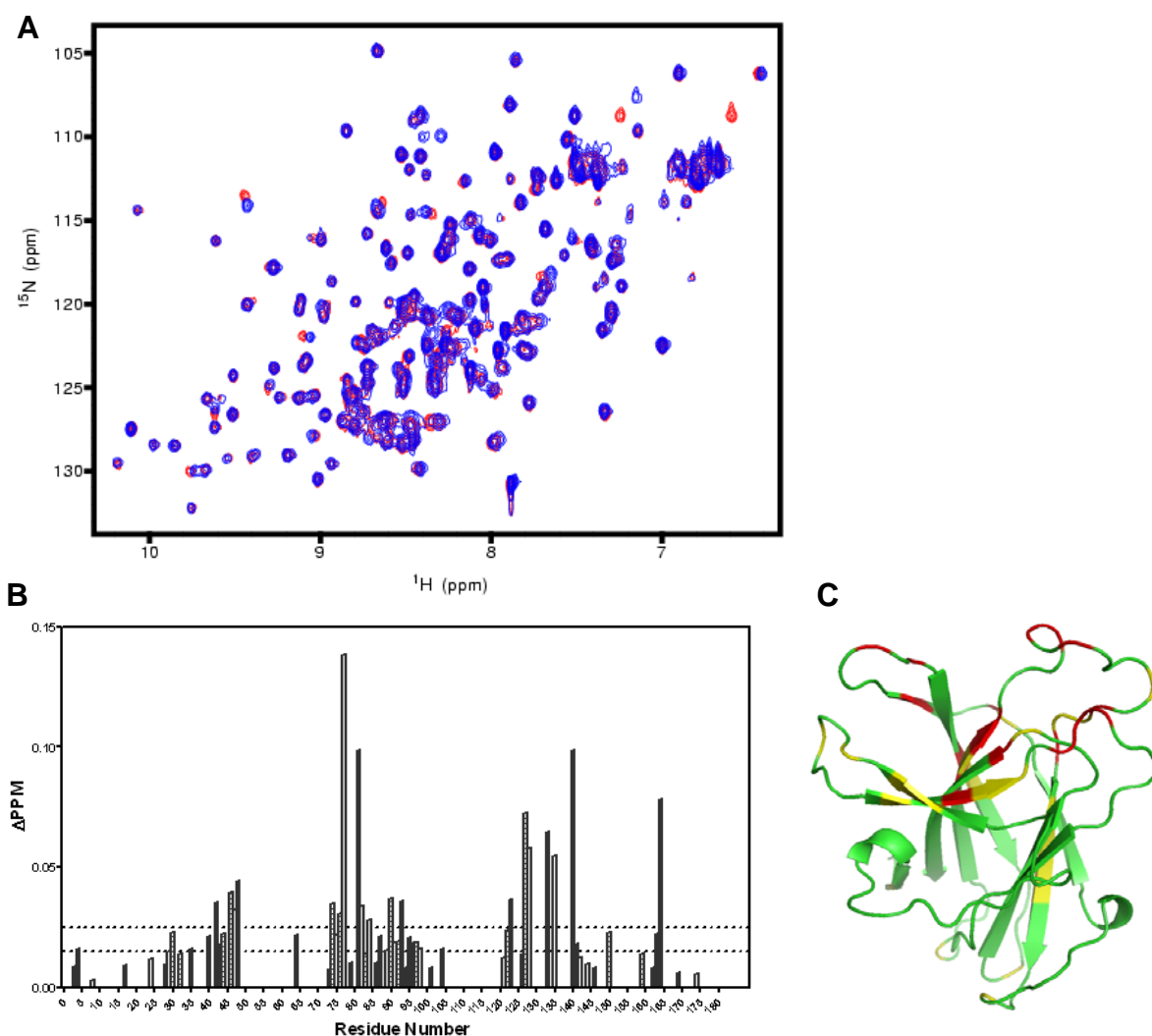


Figure S2. A) Superposition of 2D [^{15}N , ^1H]-HSQC spectra of $50\ \mu\text{M}$ EphA4 LBD in the absence (red) and presence (blue) of $100\ \mu\text{M}$ compound 1. B) Summary of chemical shift perturbations resulting from the binding of $100\ \mu\text{M}$ compound 1 to the EphA4 LBD. The average chemical shift changes of each residue are calculated based on equation (1) in “MATERIALS AND METHODS”. C) Chemical shift mapping for the binding of compound 6 on the ribbon representation of the EphA4 LBD. Residues with averaged $\Delta\delta$ above $0.025\ \text{ppm}$ are colored in red and those with $\Delta\delta$ between 0.015 and $0.025\ \text{ppm}$ are colored in yellow.

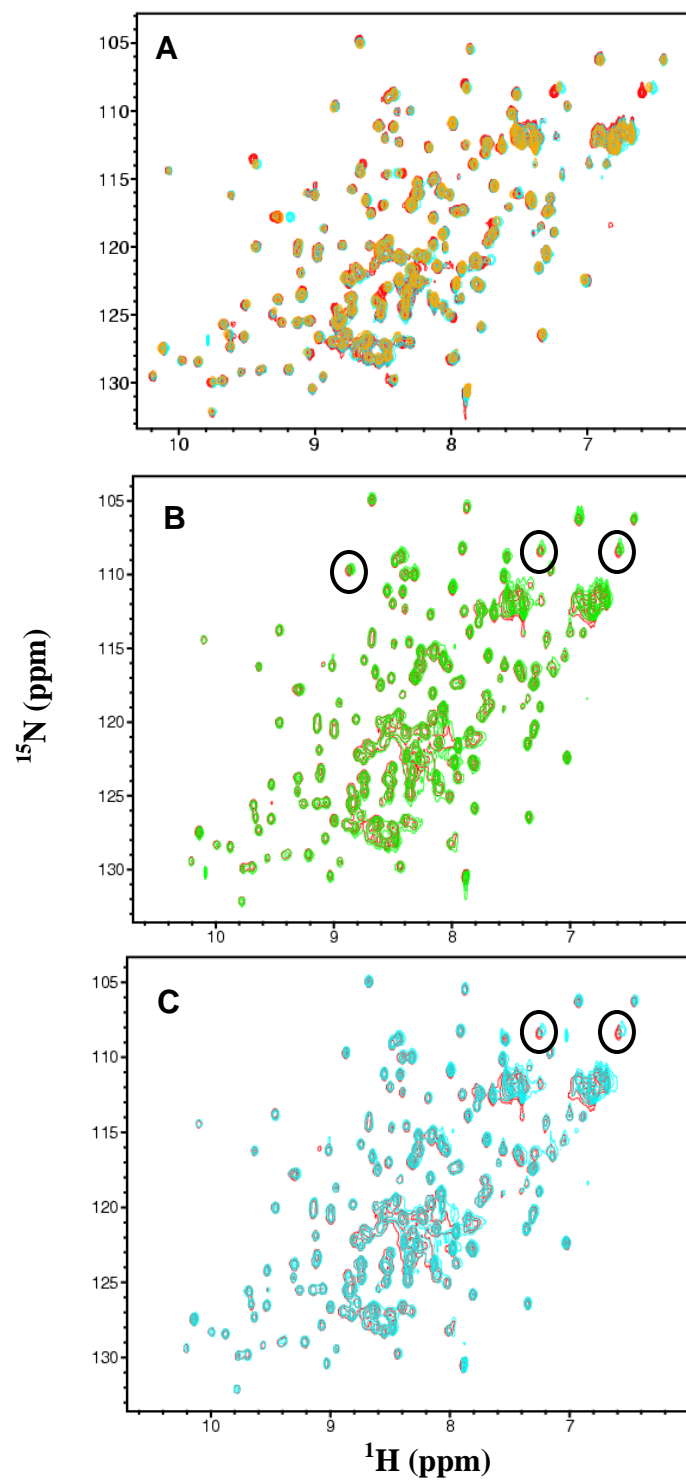


Figure S3. A) Superposition of 2D ^{15}N , ^1H -HSQC spectra of 50 μM EphA4 LBD in the absence (red) and presence of 3 $m\text{M}$ 3-methylindole (cyan) and 4-chlorotoluene (orange). B) and C) Superposition of 2D ^{15}N , ^1H -HSQC spectra of 50 μM EphA4 LBD in the absence (red) and presence of 2 $m\text{M}$ G-f16-G (green) and G-G-f51 (cyan). f16 and f51 are fragments listed in Table S2. The characteristic movements of site chain amide from Gln-43 have been circled in black.

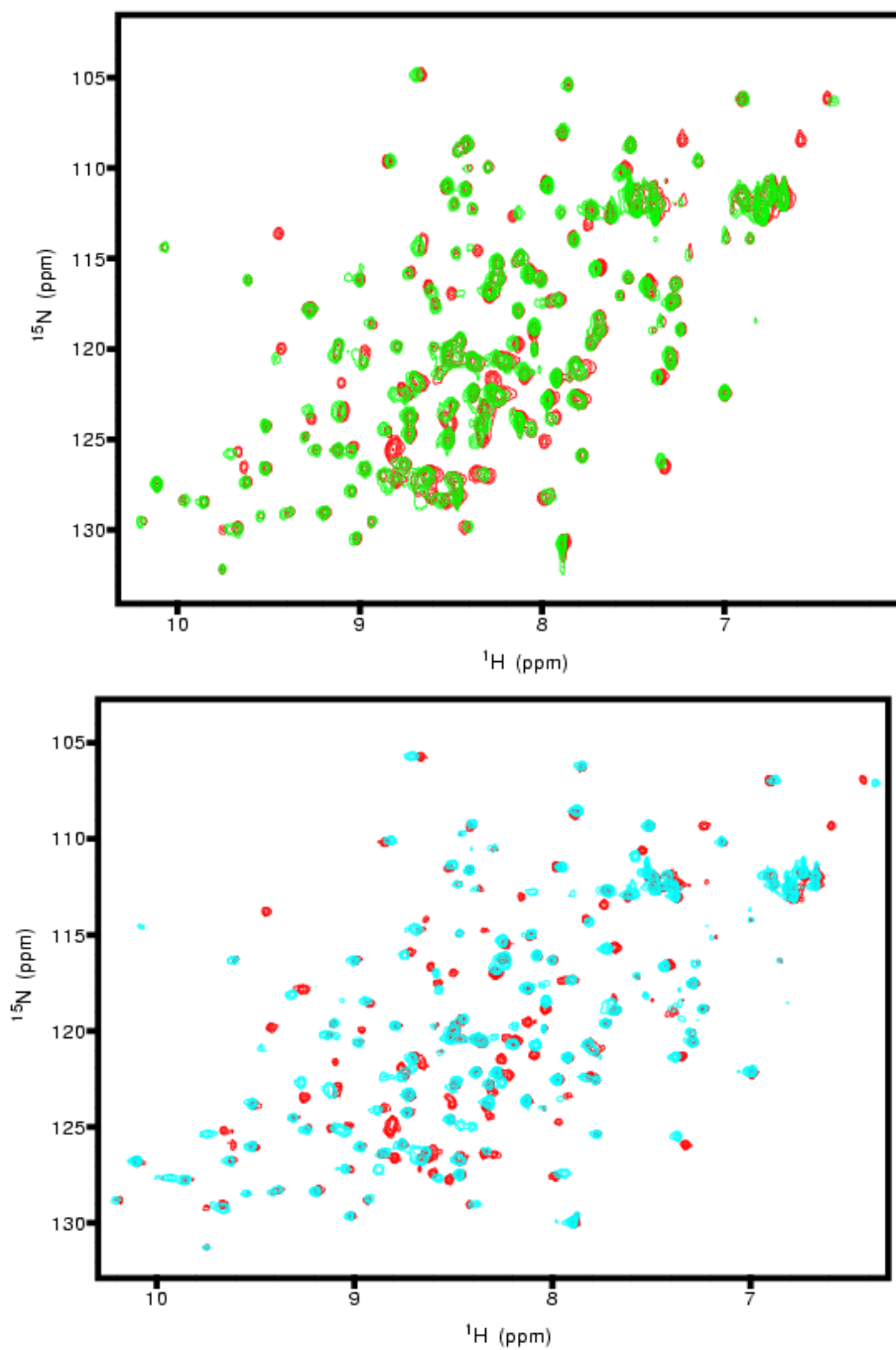


Figure S4. Superposition of 2D [^{15}N , ^1H]-HSQC spectra of 50 μM EphA4 LBD in the absence (red) and presence (green) of 100 μM compound 12 or presence (cyan) of 100 μM compound 15.

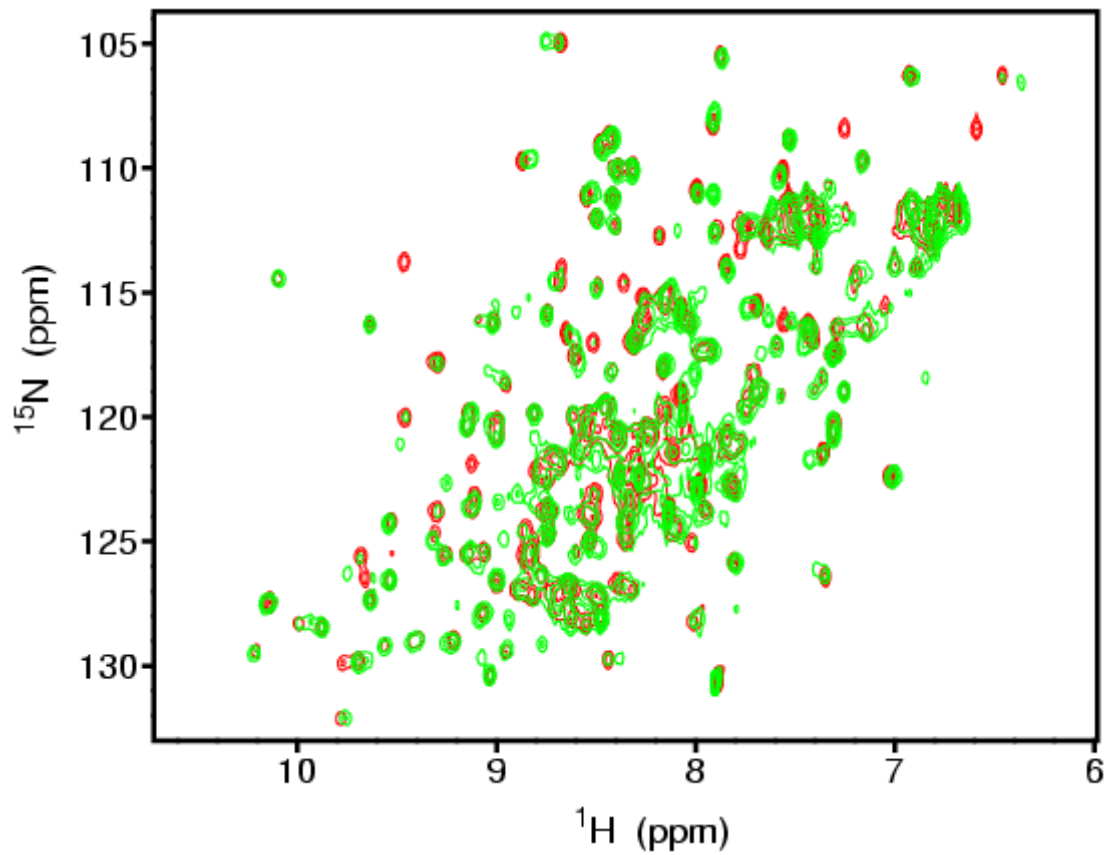


Figure S5. Superposition of 2D [^{15}N , ^1H]-HSQC spectra of 50 μM EphA4 LBD in the absence (red) and presence (green) of 50 μM compound **22**.

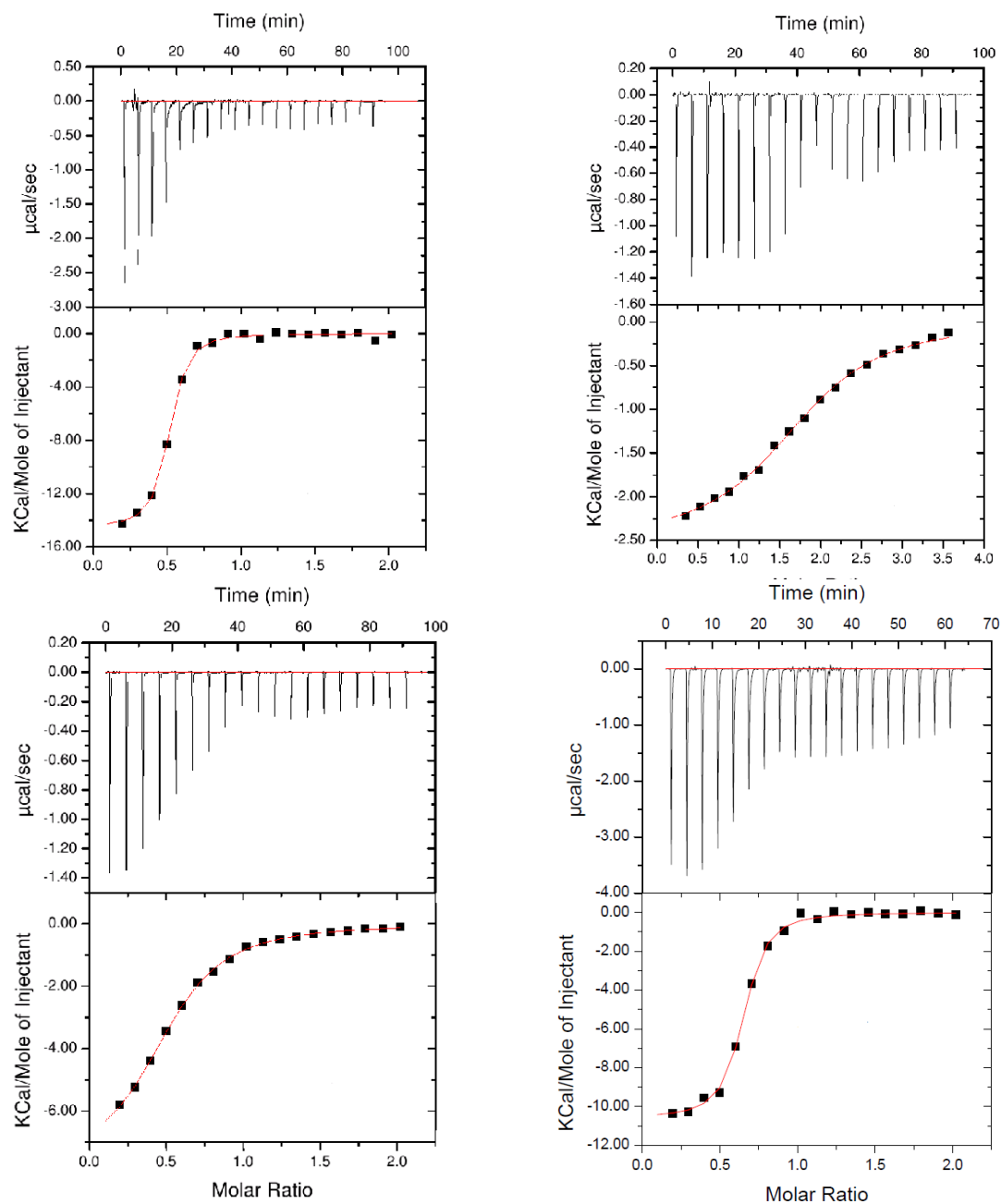


Figure S6. ITC data for interaction between EphA4 LBD and KYL peptide (top left), compound 12 (top right), compound 15 (bottom left) and compound 22 (bottom right).

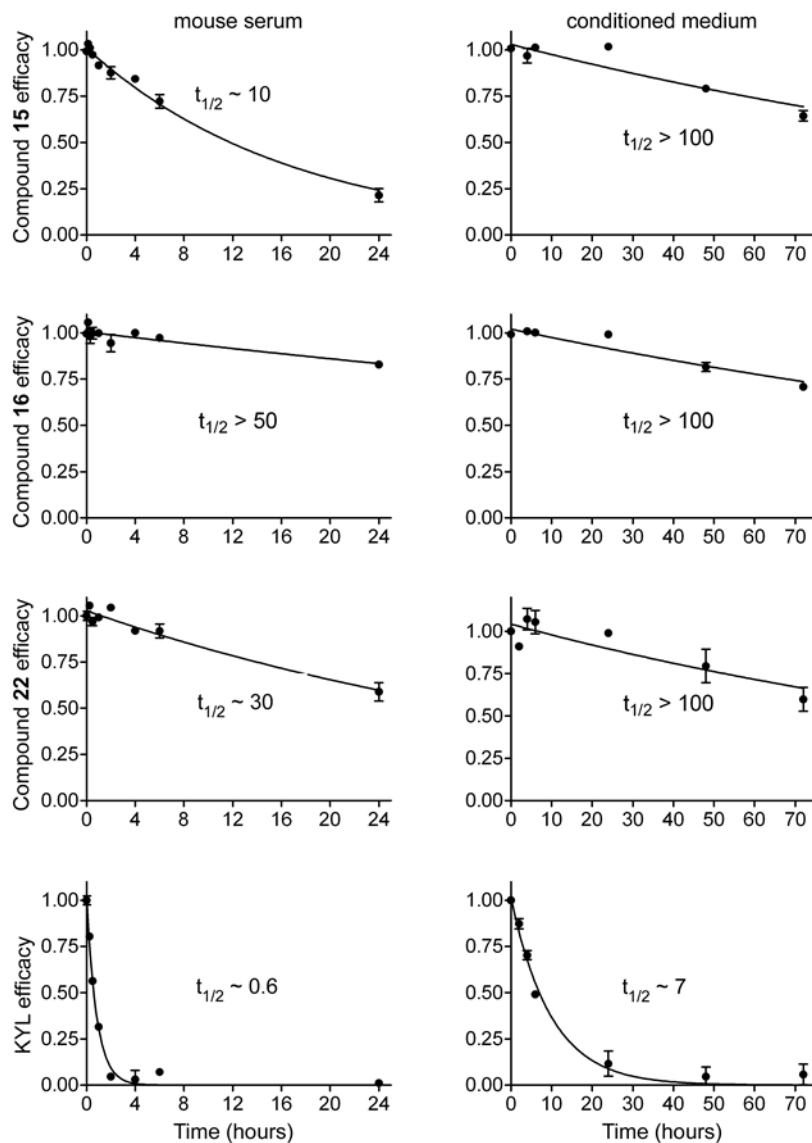


Figure S7. Stability of compounds **15**, **16**, **22** and KYL peptide in mouse serum or cell conditioned medium. The compounds were incubated in mouse serum or PC3 prostate cancer cell conditioned medium for the indicated times at 37°C and then tested for inhibition of ephrin-A5 AP binding to immobilized EphA4 Fc. In the absence of proteolytic degradation, the compounds at the concentration used (150 *mM*) inhibit ephrin-A5 AP-EphA4 Fc interaction by ~80%. Efficacy represents the relative ability of the compounds to inhibit EphA4-ephrin-A5 interaction, with efficacy = 1 for the inhibition observed with the intact peptides.

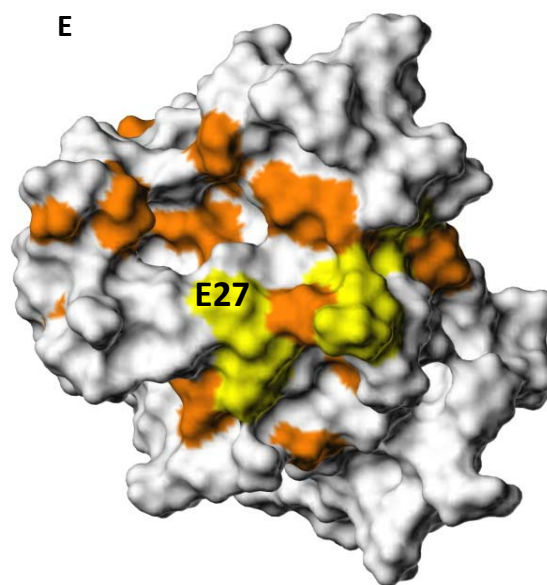
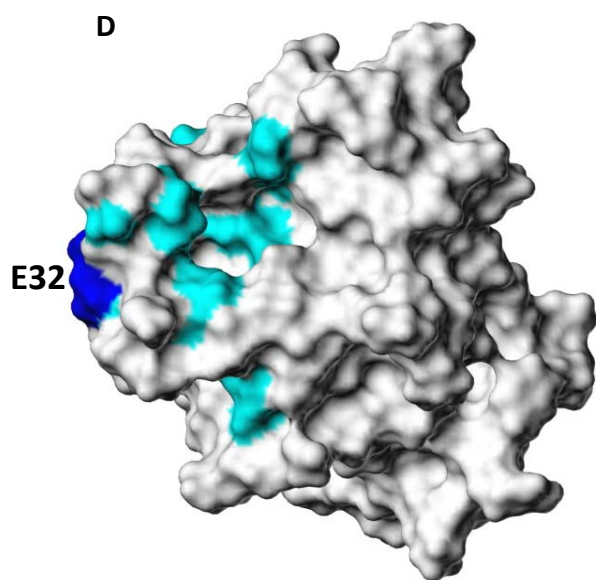
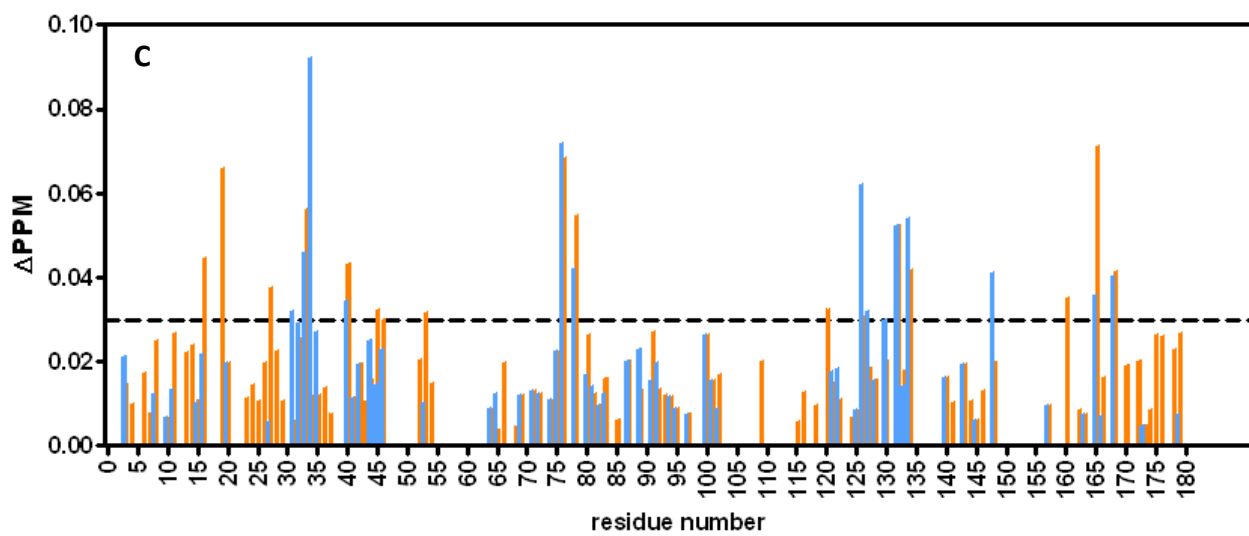
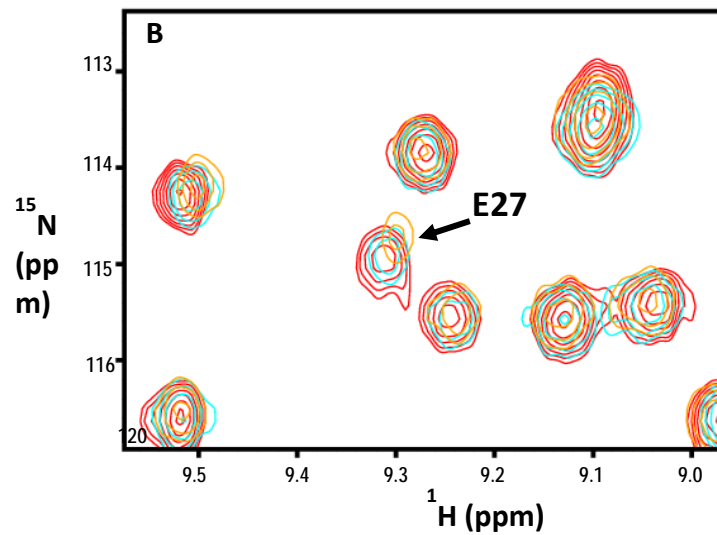
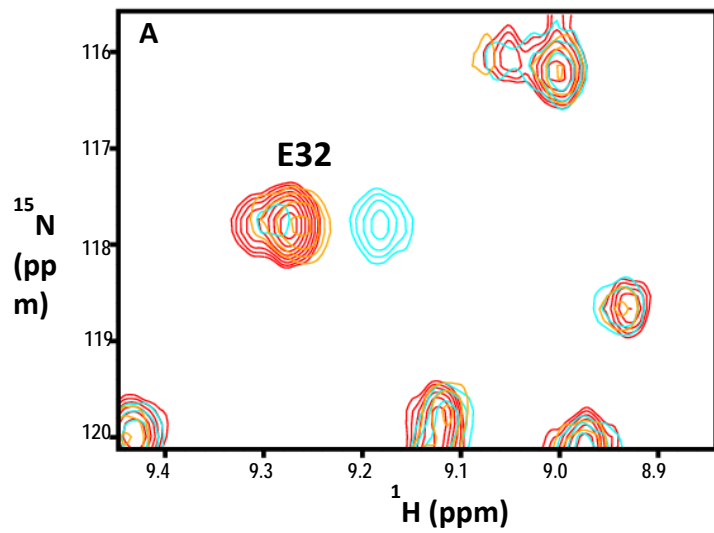


Figure S8

Figure S8. Chemical shift mapping studies for two individual side chains of compound **6**. A) and B) report close-up views of superposition of 2D [¹⁵N, ¹H]-HSQC spectra for 50 μM EphA4 LBD in the absence (red) and presence of 3 mM 3-methylindole (cyan) or 4-chlorotoluene (yellow), respectively. C) Chemical shift perturbations caused by 3 mM 3-methylindole and 4-chlorotoluene to the EphA4 LBD. D) Chemical shift mapping relative to the binding of 3-methylindole on the surface of the EphA4 LBD. Residues with averaged $\Delta\delta$ above 0.03 ppm are colored in cyan and residue E34 experiencing intermediate exchange is colored in blue. E) Chemical shift mapping relative to the binding of 4-chlorotoluene on the surface of the EphA4 LBD. Residues with averaged $\Delta\delta$ above 0.03 ppm are colored in orange; residues exhibiting differential perturbations when comparing the spectra of bound receptor in presence of each ligand are colored in yellow.

Table S1. Summary of the nine mixtures applied in BIR3 inhibition studies.

Sequence	Mixture composition	#compounds/mix	DMSO stock conc. for overall mixture (mM)	DMSO stock conc. for individual compound (μM)
AXX	X=58 (L, D, unnatural)	3,384	141	4.2
GXX	X=58 (L, D, unnatural)	3,384	164	4.8
XXP	X=58 (L, D, unnatural)	3,384	141	4.1
AXXX	X=52 (L, D, unnatural)	140,608	155	1.1
GXXX	X=52 (L, D, unnatural)	140,608	172	1.2
XXPX	X=52 (L, D, unnatural)	140,608	146	1.0
AXXXXX	X=19 (L amino acids, no Cys)	2.5E+06	158	0.063
GXXXXX	X=19 (L amino acids, no Cys)	2.5E+06	139	0.056
XXPXXX	X=19 (L amino acids, no Cys)	2.5E+06	139	0.056

Table S2. List of components used to build the library.

Components	position	Components	position	Components	position
L-Ala	1	D-Phe	21	α N-CBZ-L-Ornithine(Boc)	40
L-Asp(Bzl)	2	D-Ile	22	L-Phenylglycine	41
L-Glu(Bzl)	3	D-Lys(CBZ)	23	D-Phenylglycine	42
L-Phe	4	D-Leu	24	L-Naphthylalanine	43
Gly	5	D-Asn	25	D-Naphthylalanine	44
L-Ile	6	D-Pro	26	β -Alanine	45
L-Lys(CBZ)	7	D-Gln	27	L-Hydroxyproline(Bzl)	46
L-Leu	8	D-Arg(Tos)	28	L-cyclohexylAlanine	47
L-Asn	9	D-Ser(Bzl)	29	D-cyclohexylAlanine	48
L-Pro	10	D-Thr(Bzl)	30	L-Aspartic acid(aBzl)	49
L-Gln	11	D-Val	31	L-Glutamic acid(aBzl)	50
L-Arg(Tos)	12	D-Trp(CHO)	32	L-4-Chlorophenylalanine	51
L-Ser(Bzl)	13	D-Tyr(BrZ)	33	D-4-Chlorophenylalanine	52
L-Thr(Bzl)	14	L-Norvaline	34	L-4-Fluorophenylalanine	53
L-Val	15	D-Norvaline	35	D-4-Fluorophenylalanine	54
L-Trp(CHO)	16	L-Norleucine	36	L- α -tButylglycine	55
L-Tyr(BrZ)	17	D-Norleucine	37	L-Homoproline	56
D-Ala	18	α N-CBZ-L-Lysine(Boc)	38	O-ethyl-L-Tyrosine	57
D-Asp(Bzl)	19	L-Ornithine(CBZ)	39	O-ethyl-D-Tyrosine	58
D-Glu(Bzl)	20				

Table S3. Summary of the initial EphA4 LBD binding compounds resulting from the HTS by NMR approach.

compd.	Position combination	NMR $\Delta\delta$ (ppm)	IC ₅₀ by FPA (μ M)
1	45-44-51	0.086	> 1000
2	32-44-51	N.B.	-
3	45-47-51	0.034	-
4	45-51-51	0.075	-
5	45-53-51	0.036	-
6	45-16-51	0.234	398

Table S4. EphA4 LBD binding studies via ITC measurements.

compound	K_d (μ M)	ΔH (Kcal/mol)	ΔS (Kcal/mol)
6	50	-844	17.1
9	41	-5874	0.258
12*	20	-2442	13.3
15	12	-7686	-3.43
16	14.9	-7021	-1.62
22	1.2	-10600	-8.65
KYL	1.3	-14520	-22

* ITC experiment was performed by titrating compound into protein solution.