Supplementary information

Deciphering binding interactions of IL-23R with HDX-MS: Mapping protein and macrocyclic dodecapeptide ligands

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Expression and purification

The nucleotide sequences encoding extracellular domain (residue 1-353) of human IL-23R (NP_653302.2) followed by Flag and HIS tags were inserted into a mammalian expression vector containing CMV promoter. Protein expression was done through transient transfection in HEK293F cells cultured in serum-free media, and culture media were harvested 5 days post transfection and stored at 4°C for subsequent protein purification. Protein purification was conducted at 4°C. 2L culture media supplemented with 1M Tris-HCl (pH 8.0) and NaCl to final concentration of 25 mM and 150 mM, respectively. The media were then incubated with 25 ml of Ni-NTA resin (Qiagen) for 3.5 hours. The resin was then packed into a column and washed with buffer A (50 mM Tris-HCl, pH 8.0, 0.3 M NaCl) containing 5 mM Imidazole. The IL-23R protein was eluted with 5-500 mM Imidazole gradient in buffer A. Pooled IL-23R containing fractions were concentrated, loaded onto a HiLoad Superdex 200 column (GE Healthcare Biosciences), and eluted with storage buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl). Fractions containing IL-23R were pooled, concentrated and protein concentration determined with UV280. The protein was aliquoted and stored at -80°C.

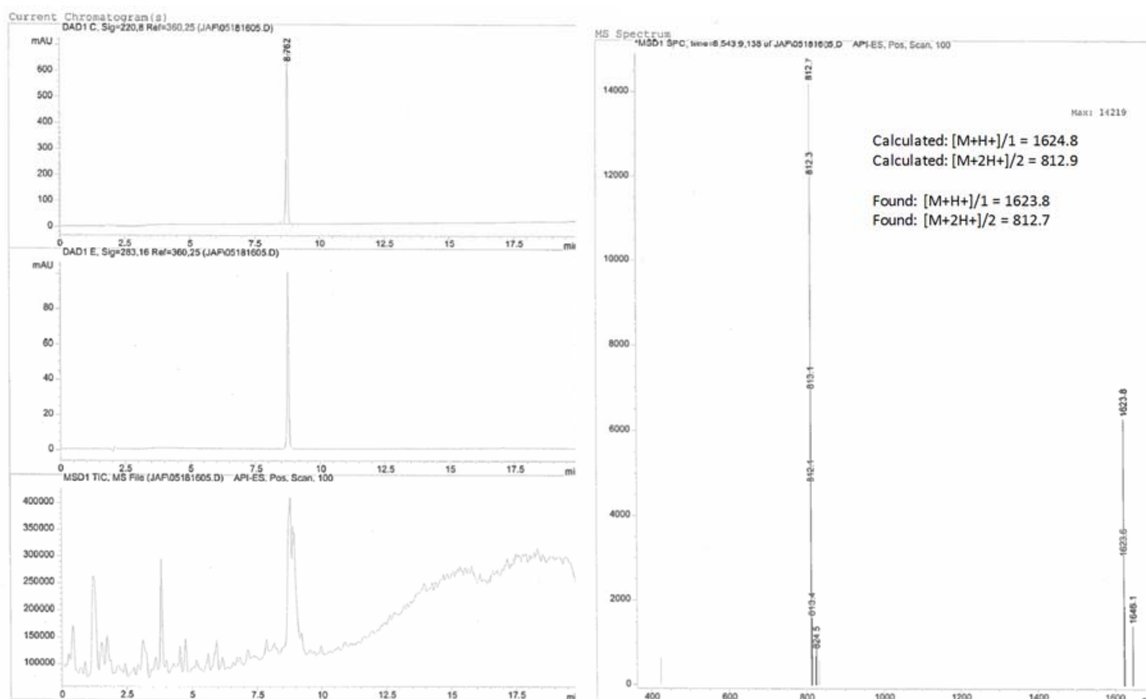
The nucleotide sequences encoding mouse IgG kappa signal peptide followed by mature IL-23 p40 subunit (NP_002178.2) were inserted into a mammalian expression

vector containing CMV promoter. The nucleotide sequences encoding mouse IgG kappa signal peptide followed by mature IL-23 p19 subunit (NP_057668.1) and HIS tag were also inserted into a mammalian expression vector containing CMV promoter. Protein expression was done through transient co-transfection of both p19 and p40 subunits of IL-23 in HEK293F cells cultured in serum-free media, and culture media were harvested 5 days post transfection and stored at 4ºC for subsequent protein purification. IL-23 protein purification was conducted following the same protocol of IL-23R as described above.

Synthesis of compound C

Peptide Compound C in Protagonist Patent WO 2016/011208 A1 was synthesized by solid-phase peptide synthesis using established Fmoc/tBu protocols. Specifically, an automated peptide synthesizer was used (Symphony from Protein Technologies Inc.), Rink Amide AM was the starting resin to generate the corresponding C-terminal amide peptide, and couplings were mediated by diisopropylcarbodiimide/Oxyma in DMF with 5-fold excess of reagents. Fmoc-HomoSer(OTBDMS)-OH was used as the building block for the N-terminal amino acid residue. After completing the solid-phase synthesis of the peptide backbone, solid-phase chlorination was carried out by treating the resin with PPh3 (15 equiv) and Cl3CCN (15 equiv) in acetonitrile for 2 h at room temperature. After final cleavage of the peptide from the resin using established protocols, cyclization in solution to form the thioether linkage was carried in 0.1 M TRIS buffer, pH 8.5 for 12 h at room temperature. Crude peptide after cyclization was purified using reversed-phase chromatography. Purified fractions were pooled and lyophilized to generate final powder as trifluoroacetate salts. Peptide was characterized by LC-MS. Analytical HPLC was carried out on an Agilent 1260 liquid chromatography system equipped with a solvent degasser, quaternary pump, auto sampler, column compartment and a diode array detector (Agilent Technologies, Waldbronn, Germany). The UV wavelength was set at 220 nm. Electrospray mass spectrometry measurements were performed on a MSD quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) interface to the Agilent 1260 HPLC system. MS measurements were acquired in positive ionization mode. Data

acquisition and integration for LC-UV and MS detection were collected using Chemstation software (Agilent Technologies).



Surface plasmon resonance

The SPR experiment for affinity and kinetics of Protagonist peptide (Compound C) against IL-23R was performed on a Biacore T200 (GE Healthcare) with assay temperature set at 25°C. The SPR assay buffer consisted of 10 mM HEPES pH 7.5, 150 mM NaCl, 0.005% P20. A protein A sensor was prepared by immobilizing PierceTM recombinant protein A on to a CM5 chip (GE Healthcare, BR100530) using reagents and protocol of a amine coupling kit (GE Healthcare, BR100050). A 600 seconds activation by EDC/NHS and 500 seconds injection of 10 µg/ml protein A at 5 µl/minute flow rate yielded 2000 RU of immobilized protein A. A multicycle mode was used in SPR experiment where each cycle consisted of protein capture, analyte association/dissociation, and regeneration. During protein capture at the beginning of each cycle, a 90 seconds injection of recombinant human IL-23R Fc chimera protein (R&D systems, 1400-IR) at 5 µl/minute flow rate consistently yielded 900 RU of IL-23R. Analyte association/dissociation consisted of a 60 seconds injection of peptide in known concentration at 50 µl/minute followed by a 90 seconds monitoring of

dissociation at the same flow rate. Regeneration at the end of each cycle consisted of a 30 seconds injection of 20 mM glycine pH 1.5 at 50 µl/minute, which completely removed IL-23R from the protein A sensor. Peptide concentration used in the assay were 1.4, 4.1, 12.4, 37.0, and 111.1 nM. Buffer was used as blank and an additional protein A flow cell without further protein capture was used as a reference flow cell. Using the Biacore T200 Evaluation Software (GE Healthcare), all sensorgrams were blank subtracted and reference flow cell subtracted before being fitting to a 1:1 binding model to extract binding kinetics and affinity.

TR-FRET protein-protein interaction assay

Tested compounds were serially diluted in pure DMSO using an acoustic dispenser directly into the assay microplate. Final concentration of DMSO in the assay was 1%.

TR-FRET assays were performed by preincubation of the diluted compounds with IL2-3R-FLAG for one hour at room temperature in assay buffer containing 25 mM HEPES, 150 mM NaCl, 0.02% Tween 20, 0.1% BSA; pH 7.4. Next, biotinylated IL-23 was added to the assay mix and incubated for an additional hour at room temperature. Finally, the detection reagents, Europium-labeled streptavidin (Perkin Elmer #AD0062; MA, USA) and Anti-FLAG IgG conjugated to SureLight –Allophycocyanin (Perkin Elmer # AD0059F; MA, USA) were added to the assay mix and incubated overnight at room temperature. Then, assay microplates were read in an Envision instrument. Values were collected as the ratio between fluorescence emission at 665 nm and 615 nm (665/615) and were expressed in percentage with respect to the DMSO treated controls. IC_{50} values were calculated using a four parameter logistic equation.

AlphaLISA protein-protein interaction assay

Similarly to the TR-FRET assay, in the Alpha-LISA assay format, compounds were preincubated with IL-23R-FLAG for one hour at room temperature in assay buffer (25 mM HEPES, 150 mM NaCl, 0.02% Tween 20, 0.1% BSA; pH 7.4). Next, biotinylated IL-23 was added to the assay mix and incubated overnight at room temperature. Then,

the detection reagents, Alpha Donor beads conjugated to streptavidin (Perkin Elmer # 6760002S; MA, USA) and Anti-FLAG AlphaLISA Acceptor Beads (Perkin Elmer # AL112M; MA, USA) were added to the assay mix. Microplates were incubated for an additional hour at room temperature. Finally, plates were read in an Envision instrument detecting light production at 615 nm. Raw data was normalized according to the DMSO treated controls and IC50 values were also calculated using a four parameter logistic equation.

Tagged forms of IL-23R and IL-23 were cloned, expressed and purified in-house using standard Molecular biology techniques.

HDX-MS

HDX-MS experiments were performed with an in-house automated modular system interfaced with an Orbitrap Q-Exactive mass spectrometer. Samples were prepared in 50mM Tris buffer containing 50 mM NaCl (adjusted to pH 7.5) at 10uM and diluted into D_2O buffer of an equivalent composition. The exchange reaction was performed with a Tecan liquid handler modified to allow for freezing of samples into Millitubes arrayed in a 96 well plate format. Thus, samples were prepared with three replicates of the following exchange times; Dmin, 10s, 30s, 90s, 270s, 810s, 2430s, 7290s and Dmax. Following this on-exchange, samples were quenched with 320 mM TCEP containing 100 mM NH_2PO_4 solution (pH 2.4) and kept at -80^{0}C until use. The injection and sample thawing was performed in a robotic module equipped with an universal robotics UR5 arm designed to present samples in series to a LEAP CTC-PAL system in a refrigerated cabinet. Samples were thawed and injected into the injection port following digestion an in-house generated immobilized pepsin column. The resulting proteolytic peptide mixture were desalted on a 2.1mm x 2cm C8 trap column and eluted across a 10cm x 2.1mm C18 analytical column into the mass spectrometer.

Docking study

The protein model was prepared in the usual way using Schrodinger software (2017.4 suite) using the default protocols included in the Protein Preparation Wizard, except

that missing sidechains were added using PRIME and the protein termini were capped. The SITEMAP program was used to identify likely binding sites using default settings except that shallow pocket detection was enabled, the finest grid was used and the less restrictive definition of hydrophobicity was applied. Using this method, a site was located that included the pocket into which the critical W156 [2018 paper] of IL-23 is seen to bind in the 5MZV structure, as the third-best according to the SITEMAP site score (0.909 vs 0.928 for the “best” site). This site extended across the end of the N-terminal domain of IL-23R, contacting nearly all the regions where major HDX effects were observed upon ligand binding. The dummy atoms defining this site were used to generate a GLIDE docking grid compatible with peptide docking.

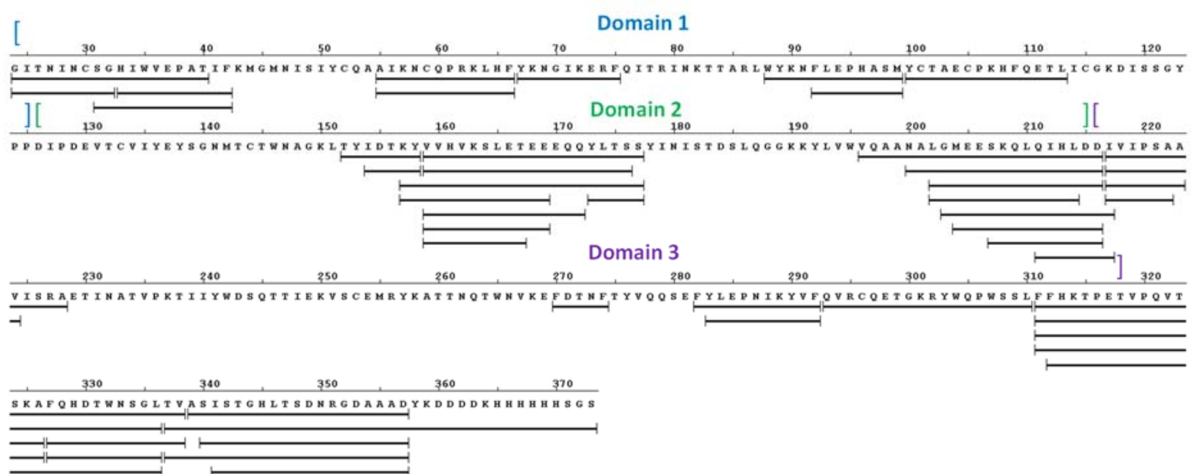
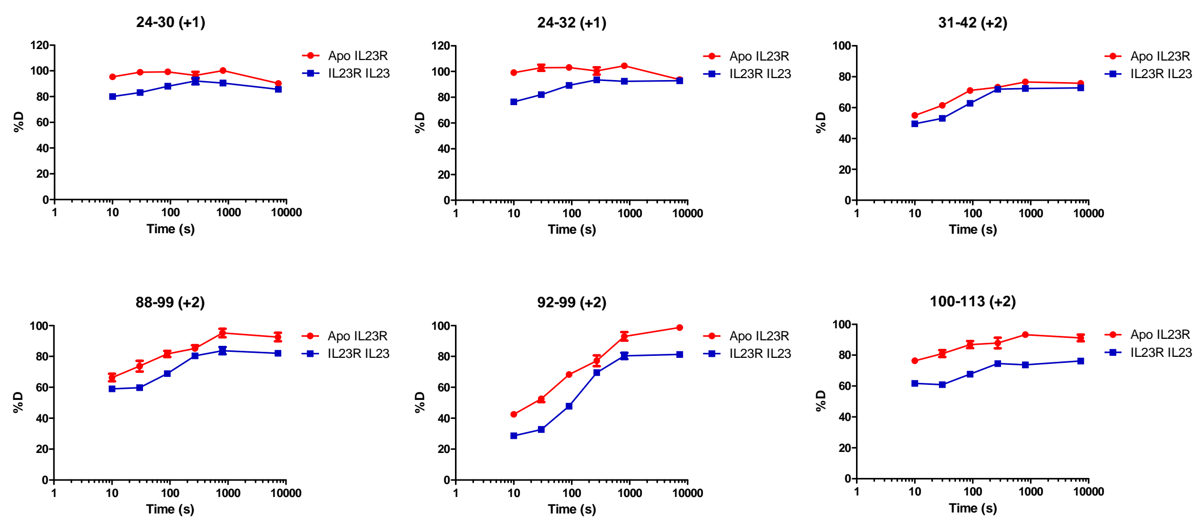


Figure S1. HDX peptide map of IL-23R. Numbering starts at the first IL-23R residue. Bracketed regions indicate peptide coverage by MS.

A



B

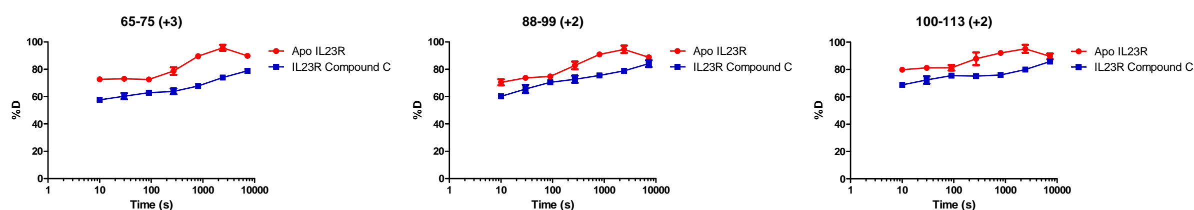


Figure S2. HDX kinetic plots for the peptides fragments exhibiting difference in deuterium exchange of the differential HDX study of IL-23R±IL-23 (A) and IL-23R±Compound C (B).

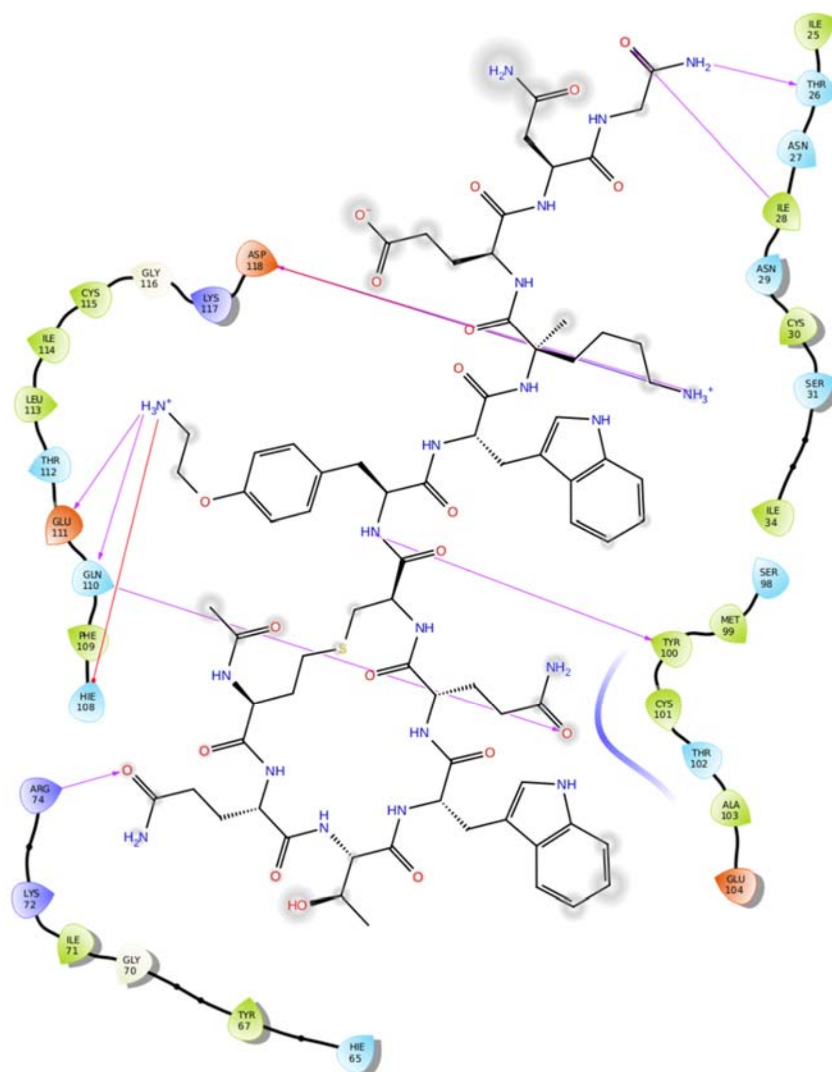


Figure S3. 2D representation of the interactions between compound C and IL-23R in the hypothetical pose consistent with HDX.