SUPPLEMENTARY INFORMATION

Fragment-based screening of programmed death ligand 1 (PD-L1)

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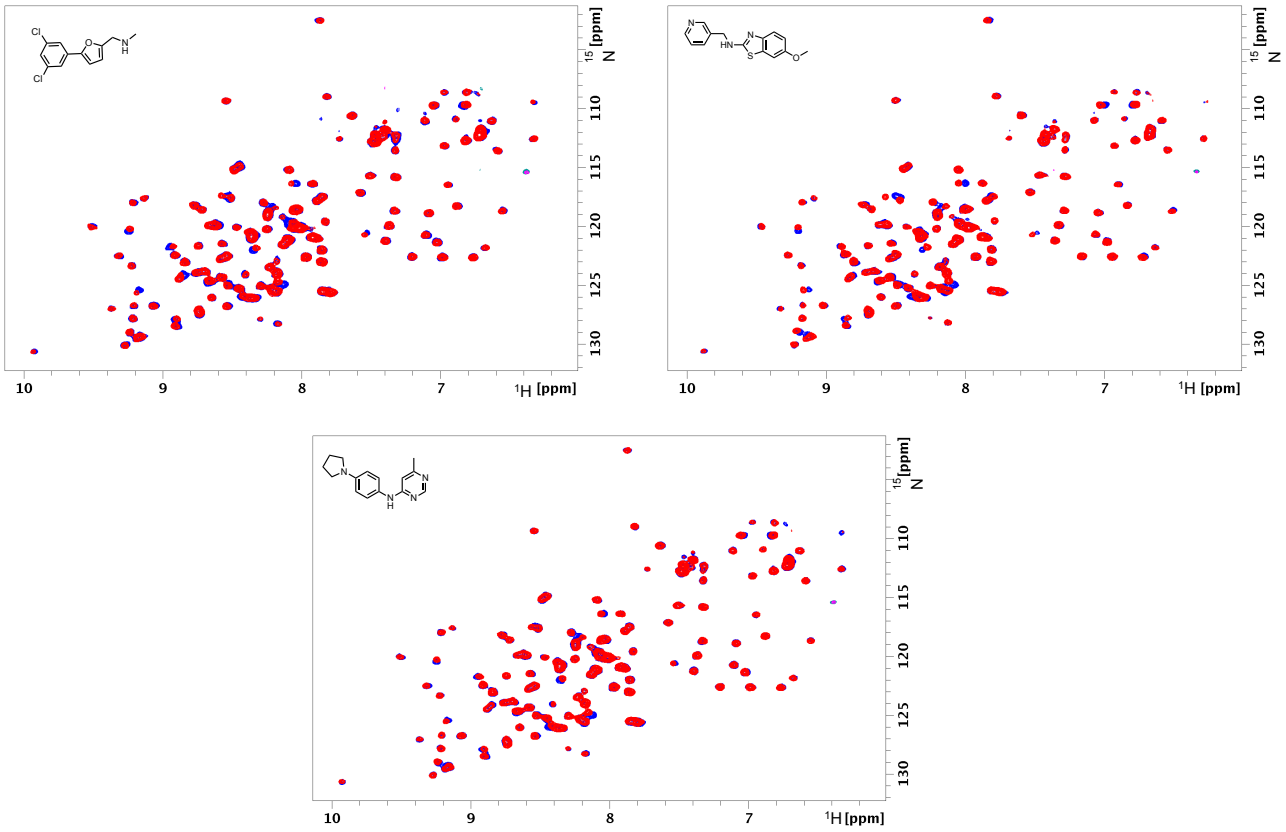
Supplementary Information



Supplementary Figure 1: SOFAST HMQC spectra PD-L1-6His-H140E in the absence (blue) and presence of 800 µM fragment (red) for fragment 3 (top left), fragment 6 (top right) and fragment 7 (bottom).

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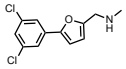
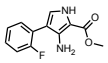
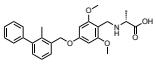
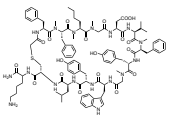
Supplementary Table 1: X-ray statistics of PD-L1 crystal structures.

Structure	IgV-6xHis-H140E	Fragment 1	Fragment 3	Fragment 9	BMS Small molecule	BMS Macrocyclic Peptide
Ligand	n/a					
Parameter						
PDB ID Code	6NP9	6NM7	6NOJ	6NOS	6NM8	6NNV
no. of chains	1	2	2	2	2	4
Data collection						
space group	C2221	P21212	P21212	P21212	P21212	P21
a,b,c (Å)	53.33, 54.11, 85.72	82.91, 94.92, 32.15	84.01, 97.40, 33.18	83.80, 96.94, 32.65	33.45, 54.60, 141.2	56.84, 61.68, 80.89
α,β,γ (deg)	90, 90, 90	90, 90, 90	90, 90 90	90, 90, 90	90, 90, 90	90, 90.17, 90
resolution (Å)	40-1.27(1.29-1.27)	41.95 - 2.43 (2.47 - 2.43)	40-2.7(2.75-2.7)	40-2.33(2.39-2.33)	20.26 - 2.79 (2.995 – 2.79)	40-1.92(1.96-1.92)
no. of unique reflections	32928(1604)	10155 (511)	7548(354)	16647(803)	6839 (332)	49872(2469)
completeness (%)	99.6(99.8)	100 (100)	94.2(89.6)	99.9(99.8)	99.3 (100)	96.3(95.1)
redundancy	9.2(7.1)	7.7 (7.7)	4.1(2.6)	8.9(7.9)	11.7 (12.6)	3.6(2.5)
Rsym	0.05(0.28)	0.05 (0.021)	0.09(0.46)	0.08(0.5)	0.235 (0.03)	0.06(0.33)
I/σ(I)	61.3(7.2)	15.2 (2.16)	7.3(2.1)	11.3(2.6)	18.8 (4.0)	10.8(2.4)
Structure Refinement						
no. of reflections test set	2016	492	601	1190	345	1677
Rwork/Rfree	20.16(21.83)	20.22 (26.86)	18.53(27.54)	19.00(24.54)	19.23 (31.31)	21.47(25.31)
Ramanchandran						
preferred regions (%)	99.15	93.15	92.31	95.14	93.93	94.41
allowed regions (%)	0.85	6.05	7.29	4.86	6.07	4.97
disallowed regions (%)	0.00	0.81	0.40	0.00	0	0.62
rms deviations						
bond length	0.006	0.009	0.01	0.011	0.010	0.004
bond angle	0.895	0.967	1.45	1.259	1.391	1.046
coordinate error	0.21	0.34	0.34	0.28	0.31	0.23

Experimental

Purification of PD-L1: The IgV domain of human PD-L1 (residues 18 – 134) was codon optimized and cloned into the pET28b vector using *NcoI* and *NotI* restriction sites to include a C-terminal 6His tag (Genscript). The resulting vector was used to transform *E. coli* BL21 (DE3) GOLD cells (Strategene). Unlabeled PD-L1 for X-ray crystallography was expressed in LB media. Uniformly ^{15}N labeled PD-L1 for NMR studies was expressed in M9 media containing ^{15}N ammonium chloride (Cambridge Isotope Laboratories) as the sole nitrogen source. Protein expression was induced for 5 hours at 37 °C following addition of 1 mM IPTG at OD = 0.8. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH = 8.5, 150 mM NaCl, 20 mM DTT, 2 mM EDTA and 1 mM PMSF) at 10 mL/g of cell pellet and lysed by homogenization at 4 °C (APV-2000, APV). Inclusion bodies were collected by centrifugation (10,000 x g for 20 minutes). Inclusion bodies were washed using a series of washes containing lysis buffer and 2% Triton X-100, 1% Triton X-100, 0.5% Triton X-100 and 1.5 M NaCl wash steps (5,000 x g for 10 minutes per wash). Purified inclusion bodies were solubilized at 2 mg/mL in denaturing buffer (50 mM Tris-HCl pH = 8.5, 250 mM, 6M Guanidine HCl) for 4 hours at room temperature. The solubilized inclusion bodies were filtered through a 0.45 μm filter and refolded dropwise into refolding buffer (100 mM Tris pH = 8, 1 M arginine, 2 mM EDTA, 0.25 mM oxidized glutathione and 0.25 mM glutathione) at 4 °C overnight.[1] The final protein concentration in the refolding buffer was 0.1 mg/mL. Refolded PD-L1 was dialyzed 3 times over 48 hours into dialysis buffer (10 mM Tris pH = 8, 20 mM NaCl). Buffer exchanged PD-L1 was concentrated using a stirred ultrafiltration cell with a 10 MWCO filter (Amicon, Millipore). Concentrated PD-L1 (0.5 - 1 mg/mL) was loaded to a size exclusion column (Superdex75 26/60) pre-equilibrated with NMR buffer (50 mM Sodium Phosphate pH = 7, 25 mM NaCl) using an AKTA purifier. Monomeric PD-L1 was pooled, concentrated to 1 mg/mL (60 μM), aliquoted and flash frozen using liquid nitrogen. All protein was stored at -80° C until needed for experiments.

NMR Spectroscopy: NMR screening was performed at 30° C using a Bruker Avance III 600 MHz spectrometer equipped with a 5 mm single-axis z-gradient cryoprobe and Bruker SampleJet sample changer. Screening samples (500 µL) contained 35 µM (0.5 mg/mL) of ^{15}N PD-L1 IgV-6His-H140E and fragment mixtures containing 400 µM of each fragment and 5% DMSO-d_6. ^{1}H,^{15}N SOFAST-HMQC spectra were obtained using 26 scans (12 minutes) and analyzed using Topspin (Bruker BioSpin).[2] Deconvolution of hit mixtures were performed as a single fragment at 800 µM concentration. NMR titration experiments were performed by monitoring the change in ^{1}H and ^{15}N chemical shifts of 6-point 2-fold serial dilutions (3 mM, 1.5 mM, 0.75 mM, 0.375 mM, 0.188 mM, 0.093 mM). K_d measurements were calculated by fitting using an in-house script.[3] Signal intensity of ligand peaks in 1D-^{1}H NMR spectra (zgesgp) of each sample prior to HMQC acquisition was used to monitor ligand solubility. Data points with poor ligand solubility were excluded from analysis.

Antagonist induced dissociation assay (AIDA): PD-1 reference sample contained 20 µM ^{15}N labeled PD-1 in NMR buffer (50 mM $NaPO_4$, 25 mM NaCl, 4% d6-DMSO). PD-1 / PD-L1 reference sample contained 20 µM ^{15}N labeled PD-1 and 23 µM PD-L1 IgV-IgC in NMR buffer. Test samples contained 20 µM ^{15}N labeled PD-1, 23 µM PD-L1 IgV-IgC and 800 µM fragment in NMR buffer. ^{1}H,^{15}N SOFAST-HMQC spectra were obtained of all samples. Qualitative displacement scores were assigned by overlaying the HMQC spectra of test samples to the broadened PD-1 / PD-L1 samples. Gly90 was used to assess the rescued signal intensity. Higher amounts rescued signal intensity of G90 (> 15%) was assigned a score of 2 while weaker rescued signal intensity (1% to 15%) was assigned a score of 1. No rescued signal intensity was assigned a score of 0.

X-ray Crystallography: Unlabeled IgV PD-L1 in X-ray buffer (10 mM Tris pH 8, 20 mM NaCl) was concentrated to 3 – 5 mg/mL (200 – 345 µM) and centrifuged at 13,000 rpm for 5 minutes to remove precipitation. The Hampton Index HT screen was used to test 96 diverse conditions for crystallization. Ligand free crystals of PD-L1 IgV-6His-H140E were obtained in conditions containing 100 mM HEPES pH 7.5 and 2M ammonium sulfate overnight at 18 °C. Fragment bound crystals were generated by incubating PD-L1 IgV-6his-V76T at 35 µM (0.5 mg/mL) with 2 – 4 mM of fragment on ice for 3 hours in X-ray buffer. Precipitated fragment was removed by centrifugation (13,000 rpm for 5 minutes). The complex was concentrated to 200 µM (3 mg/mL) using 3 kDa MWCO amicon ultra- 0.5 mL centrifugal filters (Millipore). Crystals of the all fragment / PD-L1 complexes were formed in conditions containing 0.02 - 0.1 M NaH_2PO_4, 1.0 – 2.0 M K_2HPO_4 within 48 hours at 18 °C. Crystals of the PD-L1 BMS small molecule inhibitor were obtained by incubated 35 µM PD-L1 IgV-6his-V76T and 1 mM BMS inhibitor on ice for 3 hours. The complex was concentrated to 350 µM (5 mg/mL) before plating. Crystals were obtained in conditions containing 29% PEG_{4000}, 0.28 M NaCl, 0.01 M Tris pH = 8.5 within 48 hours at 18 °C. The macrocyclic peptide was incubated with 35 µM PD-L1 IgV-6his-V76T, H140E and 200µM peptide. The complex was concentrated to 200 µM (3 mg/mL) and screened against the Hampton Index HT screen. Crystals of the PD-L1 macrocyclic peptide was obtained in 3.5 M NaFormate within 48 hours at 18 °C.

All crystals were cryoprotected in mother liquor containing 20% glycerol prior to freezing in liquid nitrogen. Data were collected on the Life Sciences Collaborative Access Team (LS-CAT) beamlines at the Advanced Photon Source (APS), Argonne National Laboratory. Indexing, integration, and scaling were performed with HKL2000. The phases were determined by molecular replacement (Phaser-MR) using the PD-L1 IgV-6His construct (PDB code 5C35). Refinement of the models were done using phenix.refine and manual fitting the model to electron density using COOT.[4,5] All figures were generated using PyMOL.[6]

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Supplementary References:

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