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RESEARCH ARTICLE

Proteomic tools for the quantitative analysis of artificial peptide libraries: detection and characterization of target-amplified PD-1 inhibitors

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Abstract: We report a quantitative proteomics data analysis pipeline, which coupled to protein-directed dynamic combinatorial chemistry (DDC) experiments, enables the rapid discovery and direct characterization of protein-protein interaction (PPI) modulators. A low-affinity PD-1 binder was incubated with a library of >100 D-peptides under thiol-exchange favouring conditions, in the presence of the target protein PD-1, and we determined the S-linked dimeric species that resulted amplified in the protein samples versus the controls. We chemically synthesised the target dimer candidates and validated them by thermophoresis binding and protein-protein interaction assays. The results provide a proof-of-concept for using this strategy in the high-throughput search of improved drug-like peptide binders that block therapeutically relevant protein-protein interactions.

Introduction

Programmed cell death 1 (PD-1) is a membrane receptor expressed in T- and B-cell lymphocytes that plays a central role in the adaptive immune system.[1] The interaction of PD-1 with any of its two ligands, PD-L1 or PD-L2, generates a downstream co-inhibitory signal that downregulates T-cell mediated cellular response and inhibits the release of cytokines, thus acting as an immune check-point that prevents autoimmune attacks.[2] This pathway has been consequently hijacked by cancer cells to evade the immune system, leading to a suppression of tumour-killing activity.[3] In recent years, therapeutic targeting of PD-1 and/or PD-L1 with monoclonal antibodies (mAbs) has dramatically improved the prognosis of certain types of malignant tumours, such as melanoma and lung cancer, and many more cancer types are under clinical evaluation (as of September 2019, there were 2,975 active clinical trials involving PD-1/PD-L1 mAbs).[4] Despite their undeniable success, mAbs show high manufacturing costs and often suboptimal pharmaceutical properties, such as poor tissue penetration and immunogenicity.[5] On the other hand, chemically modified peptides have emerged as a new class of therapeutics, since they combine the targeting properties of antibodies with the pharmacological features of small molecules.[6],[7]

As a result of structure-based *in silico* design campaign, we recently discovered a new heterochiral macrocyclic peptide (PD-i6) that binds to the bioactive interface of PD-1 and works as a ligand decoy, blocking the interaction with PD-L1 and thus restoring normal PD-1 activity.[8] Despite having suitable drug-like properties, the *in vivo* application of this candidate is hampered by its relatively weak binding affinity (K_D = 30 μM). To improve the potency of PD-i6, we devised a hit-to-lead strategy that combines solid-phase synthetic peptide libraries and dynamic combinatorial chemistry (DCC).

Protein-directed DCC methods exploit reversible reactions of simple building blocks under thermodynamic control in the presence of a target protein.[9] A library of interchanging species is formed, and if any of the components of the dynamic combinatorial library (DCL) interacts with the target protein, an equilibrium shift will favour the amplification of the interacting components. This method has been applied to the discovery of potent and selective enzyme inhibitors,[10],[11] cellular receptors,[12] and even nucleic acids,[13],[14] but there are very few reports of drugs targeting protein-protein interactions (PPIs).[15] Also, most protein-directed DCC experiments reported to date involve DCLs of a few (<10) building blocks, which typically consist of peptides and small molecules.[16] However, since many PPIs typically feature large and flat contact patches, we aimed to sample larger

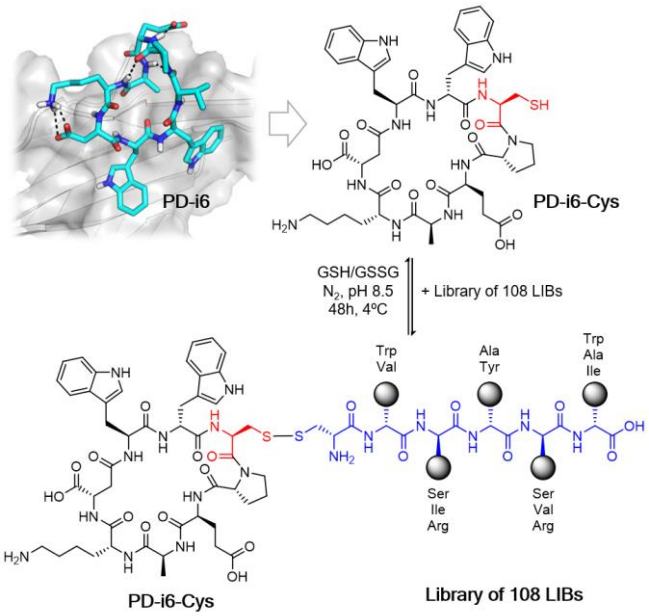

Figure 1. A Val to Cys mutation in a solvent exposed residue of the anchor ligand (PD-i6-Cys) was introduced to allow for the protein-directed thiol-exchange DCC assay to occur with a library of 108 Cys-containing D-peptides.

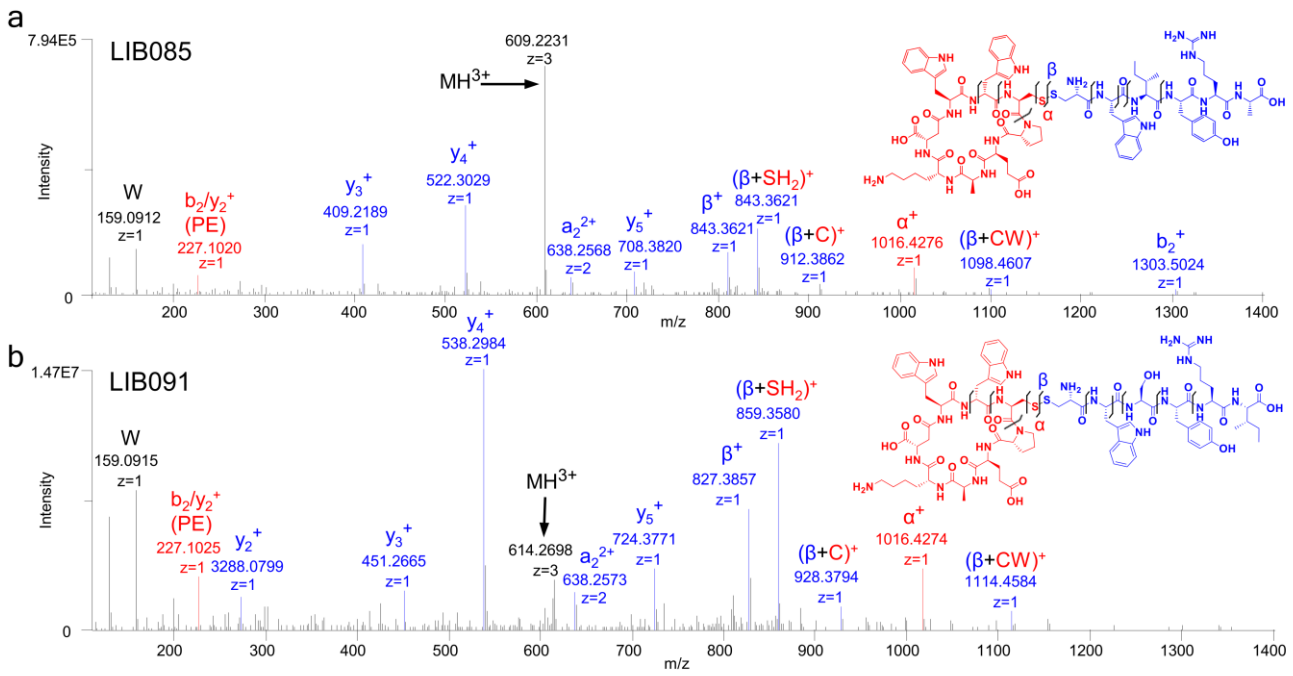

Figure 2. Validation of MS/MS spectra identified by Andromeda. MS/MS manually annotated spectra, selecting +3 charge states a) *m/z* 609.22 and b) *m/z* 614.27 as precursor ions for HCD fragmentation on a Fusion Lumos Orbitrap. A representative spectrum is shown.

libraries of interchanging species (>100) composed of peptidyl building blocks.

A major hurdle to the success of such protein-directed dynamic combinatorial methods is the quantitative analysis of the complex mixtures that are formed between DCL components, and the subsequent identification of those DCC-amplified species.[17] In our case, the analysis became even more challenging due to multiple factors: 1) excess of certain elements on the reaction media—such as disulfide-linked PD-i6-Cys homodimers and reduced/oxidised glutathione pairs; 2) presence of non-natural amino acids; 3) presence of side-chain modifications on the peptides; 4) presence of amino acid combinations yielding identical molecular weights in the library of D-peptides; and 5) formation of disulfide-linked heterodimers involving the different DCL components.

In this scenario, the combined use of proteomic tools comprising nanoLC-MS/MS methods, MaxQuant software with Andromeda search engine,[18] for identifying and quantifying peptide species, and MSstats for statistical analysis,[18,19] enabled us to efficiently separate, characterise and quantify complex protein/DCL mixtures without the need of re-synthesizing sub-libraries of compounds showing identical molecular weights.

Results and Discussion

PD-1/peptide DCC assay

For the protein-directed DCC experiment, we selected thiol-disulfide exchange as reversible reaction due to its dynamic nature under mildly basic aqueous conditions (pH 8.5) and its biocompatibility with proteins devoid of free Cys residues, such as PD-1.[20] Our previously identified PD-1 binder, PD-i6, was used as anchor ligand.[8] A solvent-exposed Val residue in PD-i6, which was not involved in either inter- or intramolecular interactions, was mutated to Cys to allow the reversible reaction to occur (Figure S1). The DCL was composed of a mixture of 108 hexapeptides, all of them bearing an N-terminal D-Cys residue. The library was synthesised on solid phase by “split-and-mix” methodology, which yields a multicomponent and closely equimolar mixture of peptides. The composition of the DCL was designed to include polar (Ser), apolar (Ile, Val), aromatic (Tyr, Trp), and charged (Arg) residues, which are typically enriched in protein-protein interaction hotspots.[21] While maximising sequence diversity, we tried to minimise the number of redundant molecular weights by using the Tags-Free method.[21, 22] Finally, we synthesised the library with D-amino acids in order to endow our final peptides with high proteolytic and metabolic resistance.

The protein-DCC experiment was carried out at relatively high concentrations of protein (PD-1) and anchor ligand (PD-i6-Cys), 200 and 500 μM respectively, in order to drive complex formation given the weak affinity of the PD-1/PD-i6 interaction (K_D = 30 μM). To enhance the thiol-disulfide reaction rate, the protein-DCC experiment was performed in a glutathione redox buffer (4:1 reduced to oxidized glutathione ratio, as previously optimised for this type of experiment).[10] This mildly reducing redox state ensures the existence of a large pool of monomer building blocks and creates stringent selection conditions that allow only the best disulfide bridges to be amplified. The DCC mixtures (with and without the target protein, as control) were reacted for 48 h to ensure that the systems reached equilibrium.[10] TFA 20% was subsequently added to quench the thiol-disulfide exchange and precipitate the protein before analysing the mixtures (Figure 1). Due to the excess of PD-i6-Cys homodimers compared to the newly formed DCL-PD-i6-Cys heterodimers, we added an off-line HPLC purification step to remove homodimers and to have a more suitable dynamic range for detecting the protein-amplified species.

MS proteomic identification of amplified species

We applied proteomics data analysis workflows to protein-directed DCC in order to identify the best disulfide-linked dimeric peptide inhibitors of the PD-1/PD-L1 interaction. Since there is, to our knowledge, no available database search engine to identify dimeric cyclic peptides, we applied a widely accepted and freely available software for protein identification and quantification in the proteomics field: MaxQuant with Andromeda as a search engine. As Andromeda could not assign internal fragments of cyclic peptides, we used a database with all the linear DCL

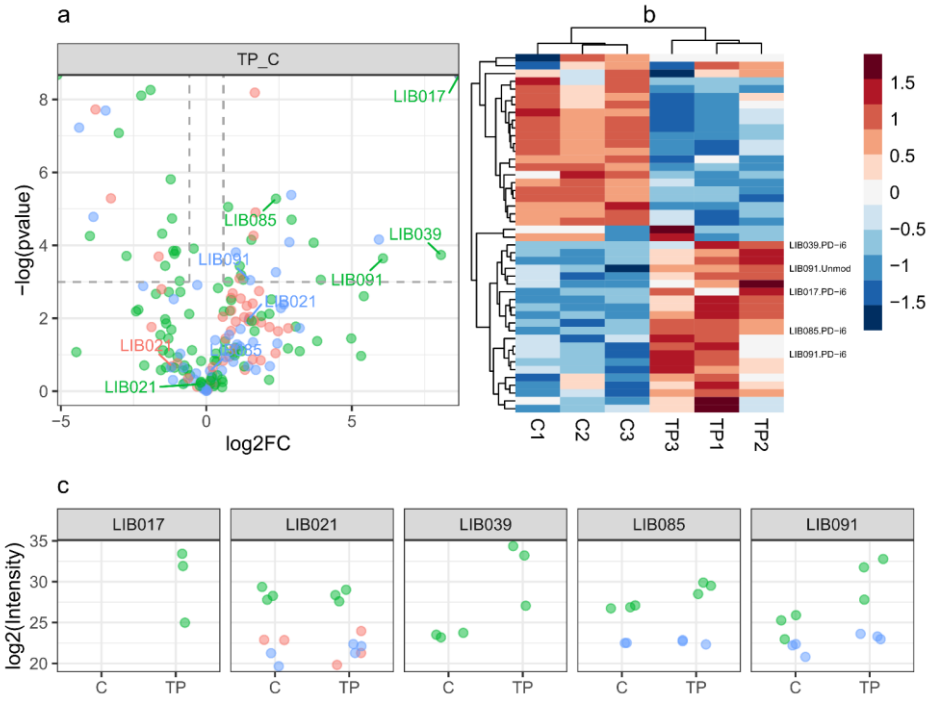

Figure 3. a) Volcano plot reporting the peptide expression logarithmic fold-change (log2FC; x-axis) and the p-value (-log(pvalue); y-axis) when analyzing the target protein (TP) vs control (C) samples (n = 3). Dashed lines represent significant cutoff: |FC| > 1.5 and p-value <0.05. Dimeric cyclic peptides used for validation are highlighted together with their corresponding unmodified or GSH peptide. b) Heatmap of significant peptides between TP and C. Each peptide is represented by a row of colored tiles (red, up-regulated; blue, down-regulated). c) Strip charts of selected peptides for further validation. Log2(Intensity) for each replicate (y-axis) is represented by TP and C groups. Colors in a) and b) plots represent peptide modifications (PD-i6 = green, GSH = red, unmodified = blue).

peptides (108 entries) and introduced PD-i6-Cys (1013.408 Da) as a dynamic Cys modification in the database search queries. With MaxQuant searches, we found 100 out of 108 possible different sequences, regardless of the PD-i6-Cys or glutathione (GSH) modification. We identified 213 peptides, 99 of which were PD-i6-Cys-bound heterodimers, 62 unmodified peptides and 52 GSH-modified species. For peptide quantification, we took advantage of the match between runs algorithm of MaxQuant to transfer feature information from one run to another and quantify the maximum number of peptides.[18]

Peptide identifications after database search were based on linear peptide fragmentation of the corresponding DCL heterodimer peptide with PD-i6-Cys, which was considered as modification. We did not have any *a priori* information about the fragmentation pattern of the cyclic peptide PD-i6-Cys thus we manually validated the fragmentation pattern for two dimeric peptides, LIB085 and LIB091, and tried to assign all ion fragments from the linear and the cyclic peptides (Figure 2 and Figure S2). We named the generated fragment ions containing the cross-linked peptides using the Schilling nomenclature,[23] which designates the longer chain as α (cyclic peptide) and the shorter as β (linear peptide). For both peptides we easily assigned the y ion series and observed that the disulfide bond, which linked the cyclic peptide α and the linear peptide β, was fragmented in the gas phase. The cyclic α ion with *m/z* 1016.4 generated by disulfide fragmentation was common for both peptides as they share the cyclic peptide PD-i6-Cys. The ions at *m/z* 843.4 and 827.4 corresponded to the linear β peptide ions after the disulfide bridge cleavage for LIB085 and LIB091, respectively. We also observed a common fragmentation in the Cys β carbon of PD-i6-Cys, leading to α + SH2 ions (*m/z* 843.4 and 859.4 from LIB085 and LIB095, respectively). In addition, a common ion from peptide α at *m/z* 227.1 corresponded to the internal ion PE, fragments b2 and y2. Interestingly, ions at *m/z* 1016.4 and 227.1 were consistently observed in all MS/MS spectra from DCL peptides containing PD-i6-Cys. These ions can be used as diagnostic ions for disulfide-linked cyclic heterodimer molecules and thus support the MS/MS interpretation

To perform the statistical analysis, we used peptide intensities from MaxQuant and ran MSstats.[19] We first conducted a preliminary analysis to explore the results after data processing with MSstats, which summarized features into peptides by median polish. A principal component analysis (PCA) showed the separation for Control (C) and Target Protein (TP) conditions in the first component, which accounted for the 31% of variance (Figure S4a). The scatterplot of log2(intensities) TP vs C showed that most peptides did not change between conditions, which had a TP/C ratio close to 1. However, most changes were observed toward TP condition and for PD-i6 heterodimers (Figure S4b).

We found 46 peptides differentially amplified between TP vs C (Figure 3a, |FC| > 1.5 and pval < 0.05, n = 3), 16 up-regulated and 20 down-regulated. From the 20 down-regulated ones 70% were PD-i6 heterodimers and 15% GSH-modified peptides. The heatmap for these 46 differentially amplified peptides shows a good hierarchical discrimination between TP and C conditions, (Figure 3b). We were interested in those PD-i6 heterodimers that were amplified in the TP condition, meaning that PD-1 would promote their formation. 13 out of 16 up-regulated peptides (81%) were PD-i6 heterodimers (Table S1), and showed a fold change ranging from 1.68 for heterodimers LIB019 and LIB025, to 267.2 for LIB039. The heterodimer LIB017 was only present in TP condition and had an infinite fold change. The p-values ranged from 0.0051 for LIB085 to 0.0497 to LIB025.

Within all amplified heterodimers, we chose the following peptides for further validation: the two heterodimers with the highest fold change (LIB039 and LIB091), the heterodimer exclusively present in the TP condition (LIB017), the heterodimer with the lowest p-value (LIB085) and one heterodimer that did not change—thus unaffected by the presence of the target protein in the reaction—as negative control (LIB021). We tracked the behaviour of these peptides by highlighting them in the scatterplot, the volcano plot, and the hierarchical clustering

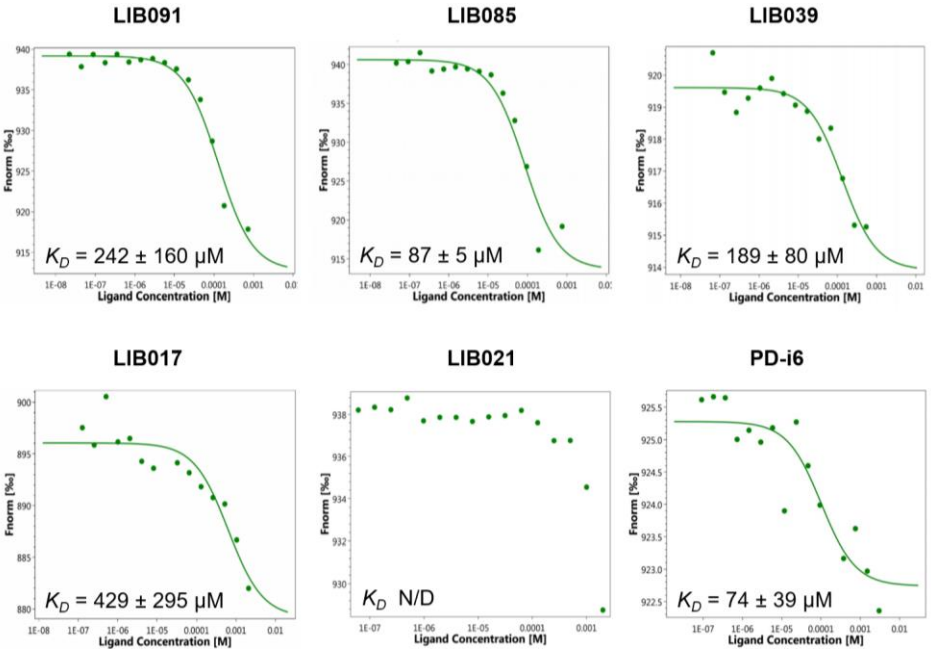

Figure 4. MST binding assays of amplified peptides to PD-1 and their affinity constants (K_D). Experiment performed in duplicate (Figure S5). A representative trace is shown.

heatmap. We also extracted individual stripcharts of their intensities (Figure 3c). All selected peptides had signals in the three replicates. For LIB039 and LIB017, we did not identify neither the unmodified peptide nor the GSH-peptide. In both cases, the intensities of two of the three replicates were very high (log2(intensity) > 32) showing the highest fold change for LIB039. LIB091 and LIB85 were identified as PD-i6 heterodimers and their unmodified counterparts. The unmodified peptides did not show a significant change between TP vs C for LIB085, but they did for LIB095. The heterodimer LIB085 showed the lowest p-value and good reproducibility despite its moderate fold change. Peptide LIB021 did not change between TP and C in any of the modified forms (PD-i6, GHS, or unmodified).

Chemical synthesis of the target candidates

Five protein-amplified heterodimers, together with a negative control, were individually synthesised via a convergent strategy that combined solid-phase and in-solution peptide chemistry. The thiol-modified macrocyclic peptide used as scaffold, PD-i6-Cys, was assembled and cyclized on resin as previously reported.[8] Special attention was paid during the cleavage and purification process to avoid the early oxidation of the thiol group (Cys side chain). On the other hand, each of the selected D-peptide linear moieties was synthesised on solid phase with an acetamidomethyl (Acm) modified Cys at the N-terminus. After peptide cleavage, the Acm group was converted to its electrophilic S-carbomethoxy sulfenyl (Scm) derivative, which enabled the Cys(Scm) residue to undergo a selective inter-molecular crossed-disulfide coupling with PD-i6-Cys. For this last step, several reaction concentrations were tested for dimer formation (0.5 mM, 0.2 mM and 0.1 mM), resulting in near quantitative yields for all of them in <60 min reaction time (Figure S3).

PD-1 binding assays

The binding affinity of each heterodimeric peptide to PD-1 was first assessed by microscale thermophoresis (MST), which allows the in-solution quantification of interactions based on the movement of molecules in response to a microscopic temperature gradient. To this end, the extracellular domain of PD-1 was fluorescently labelled with Alexa-647 and titrated with increasing concentration of peptides. In this assay, clear thermophoresis changes could be observed for all samples except for the negative control, LIB021. However, low water solubility and potential aggregation of some of the dimers hampered the accurate determination of the fluorescence intensity at the highest concentrations. Fitting of the binding data to a 1:1 Langmuir model showed that LIB091, LIB085 and LIB039 had similar binding affinities to PD-i6. Nevertheless, none of the amplified dimers significantly improved the affinity of PD-i6 (Figure 4).

Given the therapeutic relevance of blocking the PD-1/PD-L1 interaction in cancer, as shown by several clinically approved monoclonal antibodies, we aimed to evaluate the capacity of our DCL-amplified dimers to inhibit this protein-protein interaction, using a bead-based AlphaScreen assay. In this setup, which mimics the protein display found *in vivo*, a concentration-dependent blockade of the PD-1/PD-L1 interaction was observed for all the selected hits. Some of them, such as LIB091, seemed to exert a more potent effect than the parent PD-i6 peptide, although the overall activity of the dimers remained in the mid micromolar range (Figure S6).

Conclusion

We subjected a combinatorial library of >100 D-peptides to a protein-directed DCC experiment under thiol-exchange favouring conditions in the presence of a Cys-modified peptide ligand (PD-i6) of immune checkpoint protein PD-1. In this assay, the target protein selects in situ its affinity ligands through a thermodynamic templated effect in which the library composition shifts to the formation of specific PD-i6-Cys-disulfide-linked dimers, at the expense of other non-binding species. The resulting mixture was efficiently processed and quantitatively analysed by nanoLC-MS/MS and proteomic-based data analysis pipelines, which allowed the identification of the protein-amplified peptide dimers. The new hit dimers were successfully synthesised by SPPS and tested for binding to human PD-1 by MST and AlphaScreen. All these amplified hits (but not the negative control dimer) showed binding to PD-1 and disrupted the interaction with PD-L1 at low-µM concentrations, but their potency was not substantially improved compared to the parent PD-i6 peptide. The lack of affinity improvement in the amplified dimers, which we acknowledge to be a limitation of our study, might be a consequence of i) the relatively small size of the DCL; ii) the redox conditions defined at the start of the DCC experiment–which might be still not sufficiently stringent to drive the amplification of

target-specific high-affinity binders; and iii) the intrinsically challenging properties of PD-1 as drug target, given the lack of cavities and hotspot residues on its binding interface.[5] Despite this limitation, our analytical pipeline has proven useful to unambiguously identify amplified dimers in complex mixtures of peptides featuring non-natural amino acids, macrocyclic structures and redundant molecular weights. The application of proteomics workflows to protein-directed DCC experiments allows direct and rapid identification of large-scale libraries in a direct and rapid way. Therefore, we are confident that our approach will simplify the analytical challenges involved in the deconvolution of complex libraries and will facilitate the development of high-throughput combinatorial screening approaches.

Experimental Section

Peptide synthesis and DCC

The synthesis of the PD-i6-Cys cyclic peptide was performed as previously described.[8] Each DDC experiment was performed with a DCL of 108 D-peptides and human recombinant PD-1 (the target protein) in DCC buffer (Tris 100 mM, NaCl 50 mM, oxidised glutathione 0.375 mM, reduced glutathione 1.5 mM, pH 8.5) in the case of control samples and PD-i6-Cys anchor ligand (bearing a Cys) was added in treated samples. (Detailed protocol is described in *Supplementary Methods*).

nanoLC-MS/MS, data processing and analysis

Resuspended fractions were injected onto a nanoAcquity UPLC BEH 130™ C8 column (Waters, 75 µm × 25 cm; 1.7 µm) using a nanoAcquity UPLC system (Waters Corporation). Peptides were separated on the analytical column and eluted into the mass spectrometer Orbitrap Fusion Lumos™ Tribrid (Thermo Scientific). For peptide identification and characterization, we performed a database search using MaxQuant 1.6.7.0 software with Andromeda search engine against a database constituted with DCL-peptide sequences. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [24] partner repository with the dataset identifier PXD028412. Statistical analysis of modified peptides amplification was performed using MSstats[19] (v3.20.2) with equalised medians for normalisation, Tukey's Median Polish and imputation of missing values. (See Supplementary Methods for more details).

Binding assays

Binding affinities were experimentally determined between the selected dimers and the extracellular domain of human PD-1 in solution by microscale thermophoresis (MST). The competitive inhibition of the peptides on the PD-1/PD-L1 interaction was measured by AlphaScreen (See Supplementary Methods for extended details)

Associated Content

Supporting Information

Supplementary Methods, Figures S1-S7, Table S1 and Supporting Data.

Acknowledgements

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Keywords: proteomics • peptides • protein-protein interactions • DCC • combinatorial chemistry

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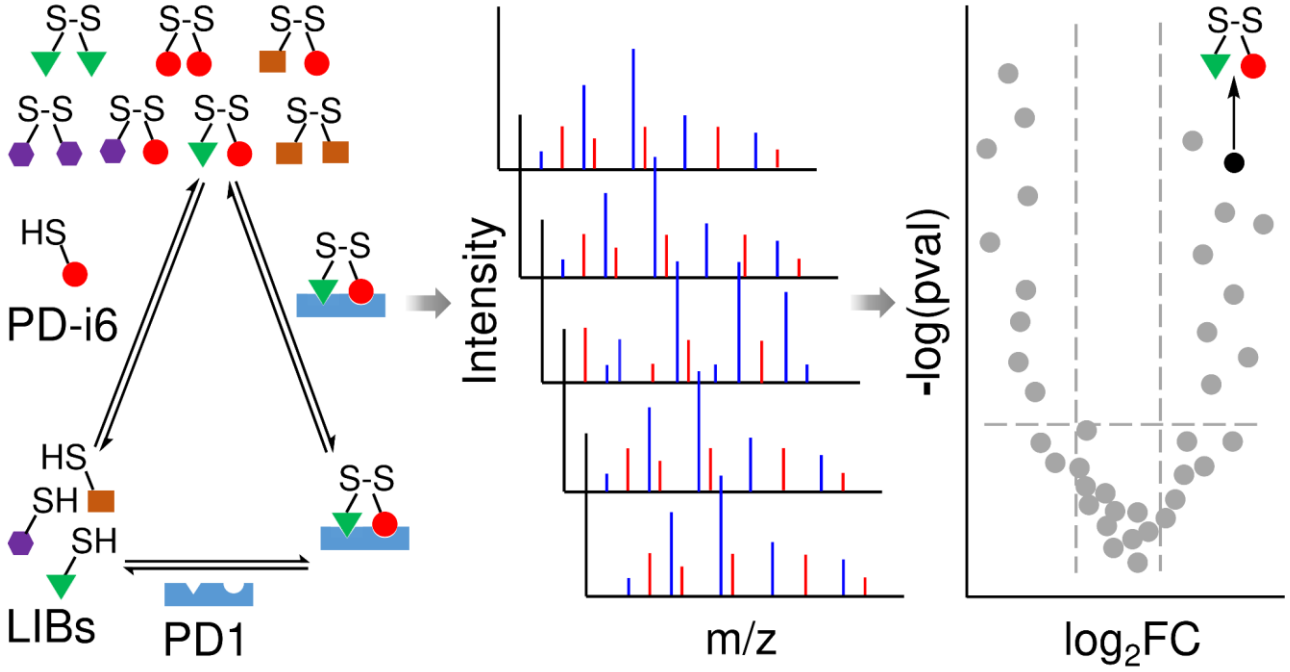

We report a proteomics data analysis method coupled to protein-directed dynamic combinatorial chemistry (DCC) to discover interface blockers of the PD1/PD-L1 interaction. The S-linked dimeric species that were significantly amplified in the protein-templated mixture were identified, synthesized, and subjected to biophysical screening.