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De novo design of miniproteins binding to PD-L1

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Objectives

De novo design of miniproteins, ideal drug candidates that cover the space between small molecule drugs and antibodies, has emerged in the past 40 years as an excellent tool for the development, with atomic accuracy, of bioactive molecules. However, a significant challenge remains due to the absence of tools capable of efficiently design sequences incorporating non-canonical amino acids. In the present work, we intend to *de novo* design a miniprotein with a EHEE topology (E – extended; H – Helix) targeting programmed cell death ligand 1 protein (PD-L1). The design approach allows the incorporation of three β-amino acids in an αβααβααβ pattern, ensuring that the structural integrity and binding capabilities of the design remain unaffected.

Materials and methods

Miniprotein design was performed combining Rosetta blue-print design with fast design protocol. Designs were filtered using the Rosetta scores, AlphaFold2, Rosetta *ab initio* calculations and molecular dynamics (MD). Designs were synthesized by Solid Phase Peptide Synthesis, purified by High Performance Liquid Chromatography (HPLC), and characterized with mass spectrometry, analytical HPLC and Circular Dichroism (CD). The affinity studies towards PD-L1 are done with BioLayer Interferometry (BLI).

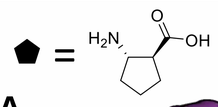
Conclusions

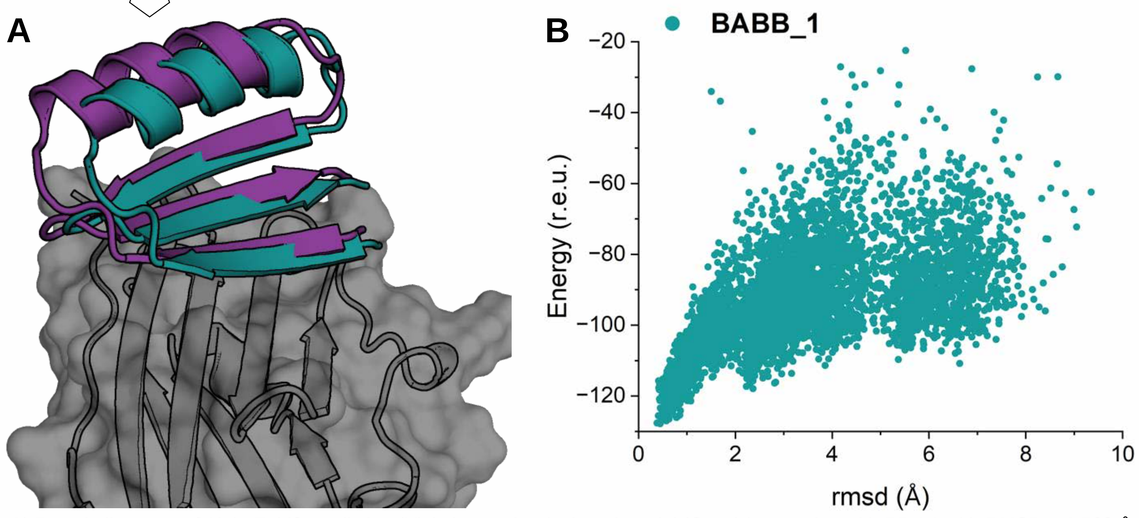
Here, we successfully designed a novel PD-L1 miniprotein binder with a EHEE topology. Moreover, the design approach allowed to incorporate three β-amino acids in the helical region while preserving the structural integrity and improving the binding capacity for the target by 5-fold.

Design

Sequence of inhibitors **BABB_1** and **BABB_T1**.

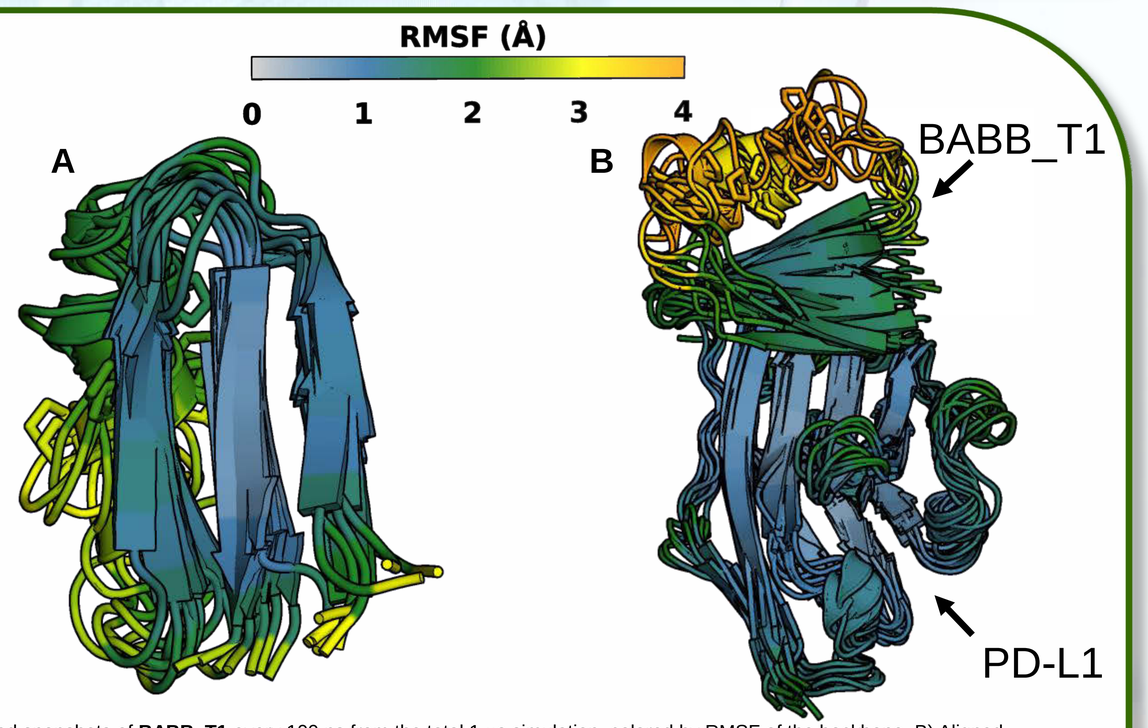
Entry	Sequence
BABB_1	VKITIKQIPTEE**L**AKK**L**AK**L**LLRDPGVHVWIHNGVLIVIKL
BABB_T1	VKITIKQIPTEE⬟AKK⬟AK⬟LLRDPGVHVWIHNGVLIVIKL

⬟ =  (1*S*,2*S*) Aminocyclopentanecarboxylic acid (*trans*-ACPC)



A) Pymol alignment of AlphaFold2 prediction (Violet) over the designed model (Cyan) in complex with PD-L1 with RMSD = 1.563 Å.
B) Folding energy landscape of **BABB_1** from Rosetta *ab initio* structure prediction

Designs were filtered based on Alphafold2 prediction confidence, of the inhibitor as a monomer and in complex with PD-L1. Subsequentially, we performed Rosetta *ab initio* structure prediction simulations, leading to a total of 7 designs from which **BABB_1** was selected for its positively charged character, known to enhance binding capacity for PD-L1.



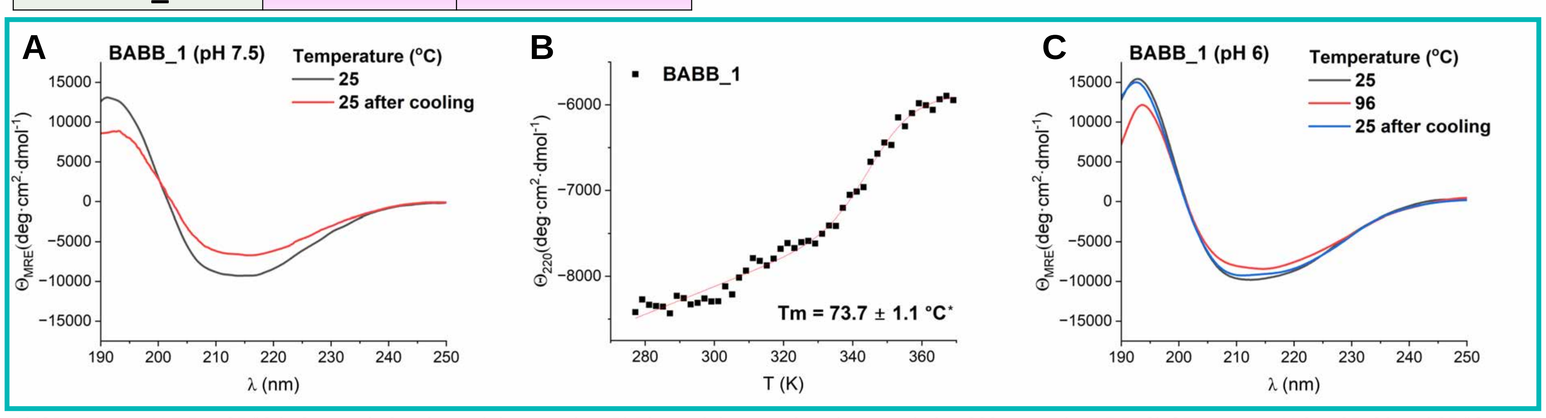
A) Aligned snapshots of **BABB_T1** every 100 ns from the total 1 μs simulation, colored by RMSF of the backbone. B) Aligned snapshots of **BABB_T1** in complex with PD-L1 every 100 ns from the total 1 μs simulation, colored by RMSF of the backbone

Amber/20 was used for MD simulations of **BABB_T1**, both as a monomer and in complex with PD-L1, due to the lack of structure prediction tools for β-amino acid-containing miniproteins. With a total simulation time of 1μs, it is visible that the structure remains stable after the introduction of the *trans*-ACPC as well as the complex predicted from the design. However, the helical region showed increased flexibility, likely due to *trans*-ACPC constrained nature limiting its ability to accommodate electronic density compared to leucine.

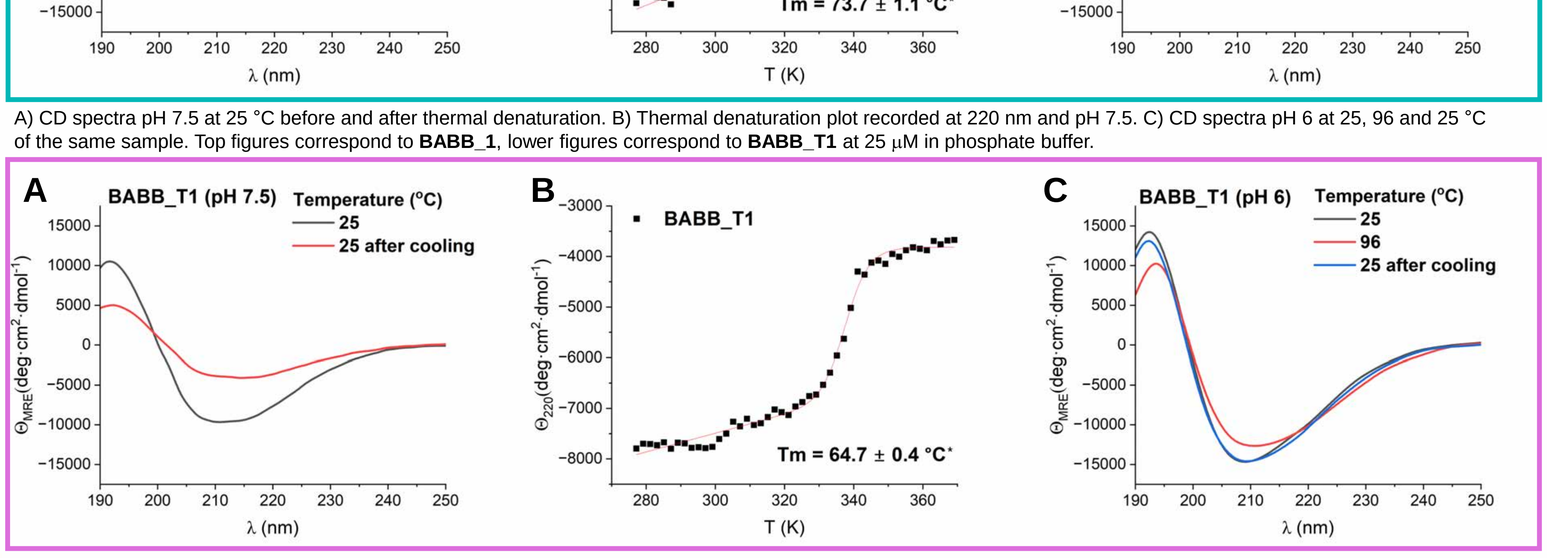
Characterization

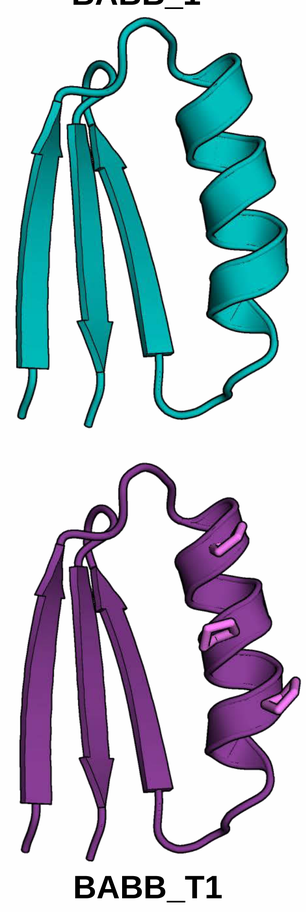
T_m and K_D, of inhibitors **BABB_1** and **BABB_T1**, *precipitation during measurement.

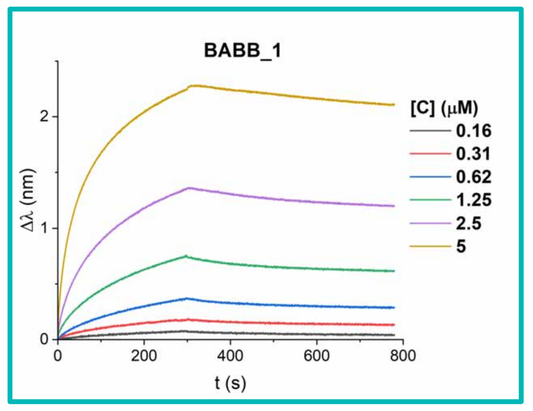
Entry	T_m (°C)	K_D (nM)
BABB_1	73.7 ± 1.1*	961 ± 11.6
BABB_T1	64.7 ± 0.4*	192 ± 2.2



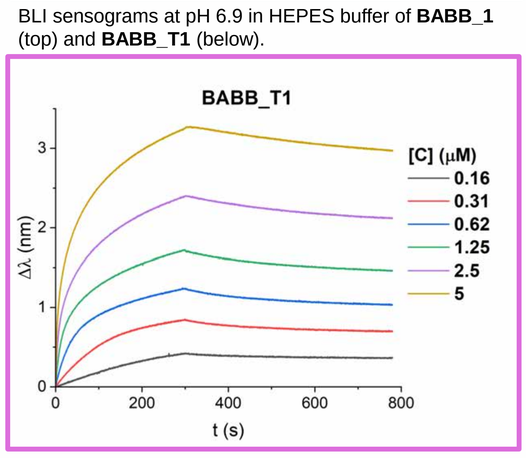
A) CD spectra pH 7.5 at 25 °C before and after thermal denaturation. B) Thermal denaturation plot recorded at 220 nm and pH 7.5. C) CD spectra pH 6 at 25, 96 and 25 °C of the same sample. Top figures correspond to **BABB_1**, lower figures correspond to **BABB_T1** at 25 μM in phosphate buffer.







BLI sensograms at pH 6.9 in HEPES buffer of **BABB_1** (top) and **BABB_T1** (below).



CD structural determination indicated the presence of folded structures in solution. However, at pH 7.5, precipitation was observed during thermal denaturation, precluding accurate T_m determination. Modification of pH to 6 showed high thermal stability of both inhibitors (>96 °C) and no apparent precipitation. Affinity studies by BLI at pH 6.9 reflected nanomolar affinities for PD-L1 with a 5-fold higher binding affinity of the β-amino acid-containing miniprotein than the designed partner.

Acknowledgments and references

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