DIFFERENT APPROACHES IN OPTIMISATION OF MINIPROTEIN STRUCTURE FOR IMPROVEMENT OF AFFINITY TOWARDS TARGET AND INHIBITORY ACTIVITY

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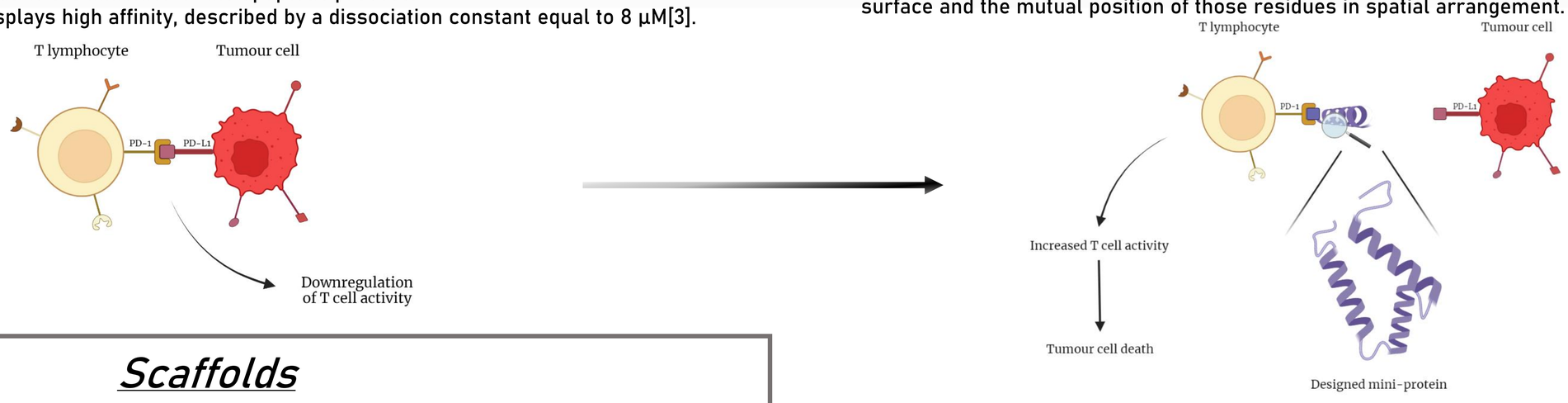
Abstract

The interaction between PD-1 protein (programmed death receptor 1), present on the surface of the immune response system cells, especially T lymphocytes, and PD-L1 (programmed death ligand 1), exposed on the surface of various cancer cells, is thoroughly described in the literature[1,2].

The interplay between these two proteins mediates the modulation of the tumour microenvironment and, as a consequence, leads to the inhibition of the apoptotic process.
PD-L1 binds to PD-1 and displays high affinity, described by a dissociation constant equal to 8 μM[3].

In the pursuit of inhibitors targeting the PD-1/PD-L1 interaction, Rosetta Protein Design software[4] and rational drug design[5] methodologies were employed to generate novel miniproteins. Designed sequences underwent synthesis and evaluation to assess their efficacy in disrupting the aforementioned interaction. The optimisation process focused on leveraging the physicochemical properties of the exposed amino acid residues towards the target protein surface and the mutual position of those residues in spatial arrangement.



Scaffolds

In the beginning, two different scaffolds of well described topologies, ENH and MvaT, were applied to the Rosetta Protein Design software[4] to better fit to the PD-1 protein cavity which is known to bind PD-L1. Rational drug design was in use to suggest further modifications. More than 86 miniproteins were obtained and investigated in terms of affinity towards the target molecule, folding pattern and thermal stability.

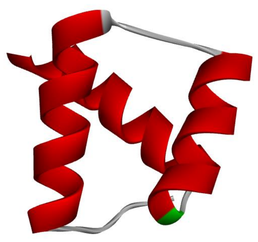


Figure 1 - Structure of the *Drosophila melanogaster* engrailed homeodomain [4] (pdb: 1enh)

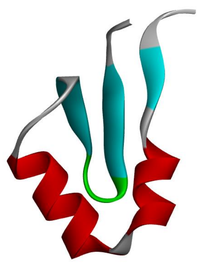


Figure 2 - C-terminal domain of MvaT protein [3] (pdb: 2mxe)

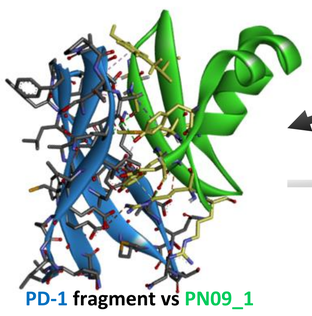


Figure 3 - Result of the Rosetta Protein Design Software optimization [Discovery Studio Visualizer]. Green ribbon represents PN09_1 miniprotein derived from MvaT scaffold. It is shown in complex with PD-1 (light blue ribbon). Yellow sticks represent residues which are introduced in PN09_1 instead of those which are originally placedin the MvaT sequence.

Sequences

Table 1 - List of selected MvaT-derived sequences and their physico-chemical properties. Residues of MvaT highlighted in bold blue indicate the substituted spots. Residues in bold red did not improve the measured parameters, whereas those in bold black are proven to have positive influence - either giving lower dissociant contant value (K_d) or higher thermal stability point of the product.

name	sequence	CD		BLI
		proper folding pattern	thermal stability point [°C]	K_d [μM]
mvaT	KVKQYKNPHT GEVIETKGGN HKTLKEWKAK WGPEAVESWA TLL			
PN01	KRKMYSNPHT GAAIYTRGGN HKTLKEWKAK WGPEAVESWA YLE	Y	36	231
PN06	KRKMYYNPHT GAAIYTRGGN HKTLKEWKAK WGPEAVESWA YLE	Y	49	6.61
PN09	KRKMYSNPHT GAAIYTRGGN HKLLKEWKAK WGPEAVESWA YLE	Y	36	2.63
PN09_1	KRKMYYNPHT GAAIYTRGGN HKLLKEWKAK WGPEAVESWA YLE	Y	50.6	11.22
PN09_2	KRKMYYNPHH GAAIYTRGGN HKLLKEWKAK WGPEAVESWA YLE	N	<30	n/a
PN09_4	KRKMYYNPHT GAAIYTQGGN HKLLKEWKAK WGPEAVESWA YLE	Y	46.4	12
PN91_H17	KRKMYYNPHT GAAIYTHGGN HKLLKEWKAK WGPEAVESWA YLE	Y	55	250
PN91_N21	KRKMYYNPHT GAAIYTRGGN NKLLKEWKAK WGPEAVESWA YLE	Y	51.5	26.5
PN91_S21	KRKMYYNPHT GAAIYTRGGN SKLLKEWKAK WGPEAVESWA YLE	Y	49.5	24.6
PN91_F13	KRKMYYNPHT GAFIYTRGGN HKLLKEWKAK WGPEAVESWA YLE	Y	55.5	404.7

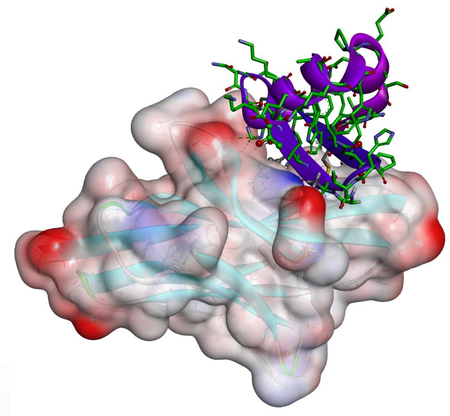


Figure 4 - Docking of MvaT mini-protein structure (violet ribbon) to PD-1 protein molecule.

Rational drug design strategies were then applied to propose further structural modifications. The changes in sequences included single and multiple substitutions of residues at certain positions and the alteration of the total resultant charge of the miniprotein being designed.
Considering the observation that positively charged miniproteins were shown to have a greater attraction to the PD-L1 surface, a hypothesis regarding altering the resultant charge to negative was tested, in terms of potentially obtaining a highly effective PD-1 protein inhibitor.
Over 80 miniproteins were synthesized and subjected to comprehensive analysis to determine their affinity towards the PD-1 receptor, folding characteristics, and thermal stability.

Table 2 - List of PN09_1-derived mini-proteins with lowered resultant charge. The substitutions in sequences were made stepwise by altering the amino acid residue with positively charged side chain to amino acid residue with negatively charged side-chain.

name	sequence	CD		BLI	resultant charge
		proper folding pattern	thermal stability point [°C]	K_d [μM]	z
PN09_1	KRKMYYNPHT GAAIYTRGGN HKLLKEWKAK WGPEAVESWA YLE	Y	50.6±1.3	11.2±5.1	+3.217
PN91_K28Q_K30E	KRKMYYNPHT GAAIYTRGGN HKLLKEWQAE WGPEAVESWA YLE	Y	39.0±1.2	n/a	+0.226
PN91_K28E_K30E	KRKMYYNPHT GAAIYTRGGN HKLLKEWEAE WGPEAVESWA YLE	Y	41.7±1.2	n/a	-0.773
PN91_QEE	KRKMYYNPHT GAAIYTRGGN HKLLQEWEAE WGPEAVESWA YLE	Y	39.9±0.8	n/a	-1.769
PN91_EEE	KRKMYYNPHT GAAIYTRGGN HKLLEEWEAE WGPEAVESWA YLE	Y	38.0±2.5	n/a	-2.768
PN91_QEEE	KRKMYYNPHT GAAIYTRGGN HQLLEEWEAE WGPEAVESWA YLE	Y	35.6±1.9	n/a	-3.764
PN91_4E	KRKMYYNPHT GAAIYTRGGN HELLEEWEAE WGPEAVESWA YLE	Y	34.0±4.5	n/a	-4.762

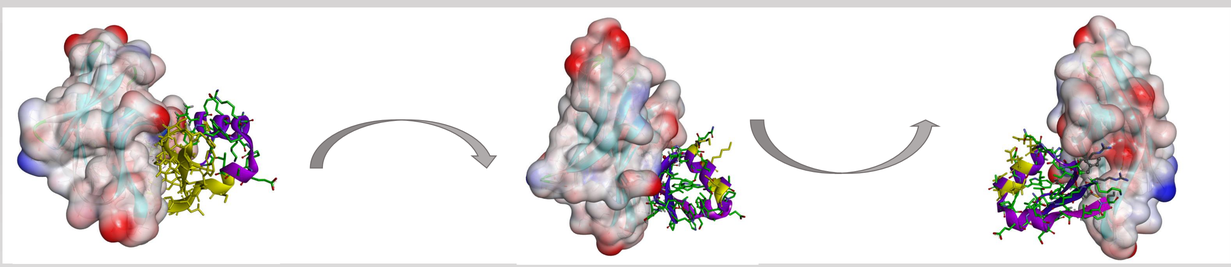


Figure 5 – PN09_1 docked to PD-1 protein, displayed from the perspective showing the region that had to remain unchanged (yellow colour)

Figure 6 – Another visual representation of PD-1 vs PN09_1 after rotation and with highlighting of four residues submitted to substitution with negatively charged side chain.

Figure 7 – Visualization displaying PN91_4E docked to the PD-1 protein, with four Glu residues instead of positively charged amino acid side chains.

Results

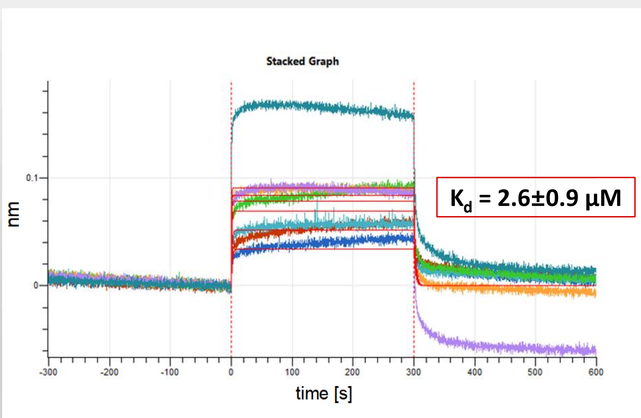


Figure 8 - Biolayer interferometry measurements for MvaT-derived PN09 miniprotein in concentrations from 1.25to 30 μM [Octet K2, Sartorius]. The calculated K_d value is close to 2.6 μM.

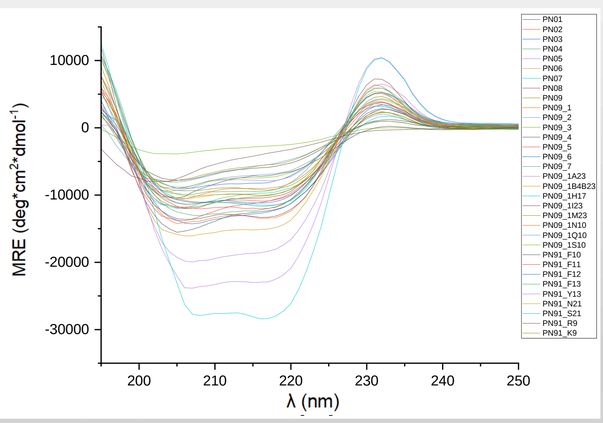


Figure 9 - CD spectra of 33 peptides derived from MvaT mini-protein scaffold. Minima seen around 205-210 nm and 215-220 nm indicate on the proper folding pattern due to presence of α-helices and β-sheet in the structures.

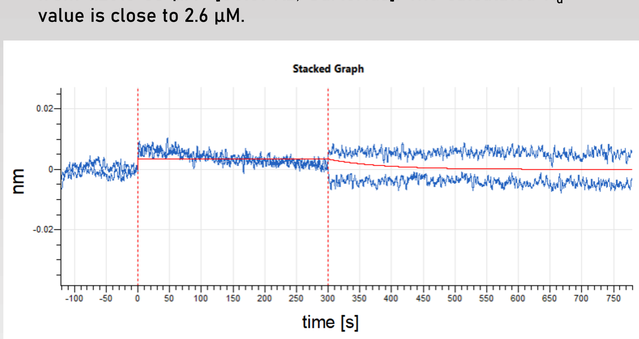


Figure 10 – Example of biolayer interferometry measurement: PN91_QEE shows no specificity towards the PD-1 protein. Unfortunately, all the other peptides with lower resultant charge showed the tendency to lose the specificity even though the changes in the sequence were not made in the binding fragment of mini-protein and the CD spectra have proven that the folding pattern is preserved.

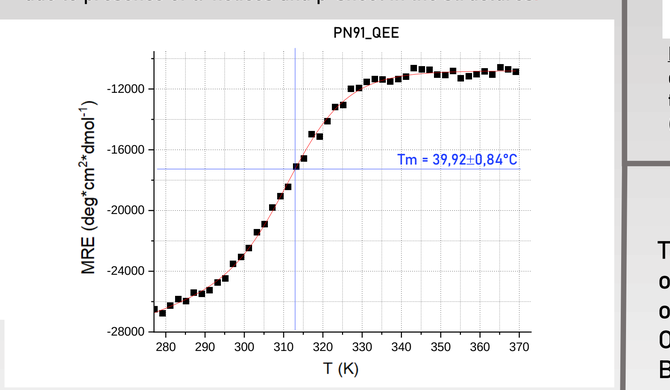


Figure 12 – Thermal stability point is calculated basing on the CD spectra measured in various temperatures – interval measurements from 4°C to 96°C. Here the value for PN91_QEE, of lower resultant charge than PN09_1, is presented.
For the calculation of the parameter a normalised fitting method is applied [OriginLab software], but the the highest temperature in which the mini-protein preserves the folding pattern might be also estimated from the curve: in the half of the slope in between two plateaux.
In general, as seen in the Table 2, the changes in non-binding region applied for lowering the resultant charge, seemed to decrease the thermal stability of designed mini-proteins.

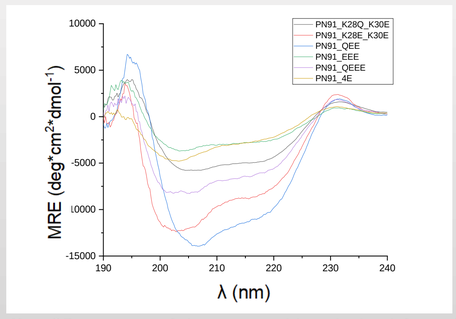


Figure 11 - CD spectra of 6 new peptides derived from PN09_1 mini-protein, but with altered resultant charge. As the minima are present, it is stated that the folding pattern is preserved.

Conclusions

To make the picture complete, it must be underlined that the data presented above constitute a fragment of the whole idea, initially thought to be presented. This work is mainly focused on the issue of the resultant charge modulation and its influence on the physicochemical properties of the products. On the way to these parameters and results, two different scaffolds, ENH and MvaT, were tested. Besides these two statregies, also peptides based on short helices similar to 7ARR (pdb: 7arr) were synthesised and investigated. In eight sets of mini-proteins and peptides, a total number of products will soon reach circa one hundred different sequences.

Based on the data presented here, it can be concluded that the modulations made in order to make the resultant charge lower - knowing that resultant charge of the PD-1 protein is positive – did not serve to obtain an inhibitor of PD-1/PD-L1 interaction. Probably the main problem is the fact that the substituted residues, even though they were not playing crucial role in the mode of binding, had too significant impact on the thermal stability of the next products and that, in turn, made the new mini-proteins unable to display any inhibitory activity. The research will be continued in order to possibly find a solution to this problem.

Acknowledgement

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