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Comments on Ph.D. dissertation of Juan Lizandra Pérez
entitled
"***Design, synthesis, and structural characterization of miniproteins, and incorporation of protein-protein interaction inhibition activity***".

In general, the Ph.D. thesis of Juan L. Pérez, which I have been asked to review, addresses the highly relevant topic of *de novo* design of miniproteins with well-defined biochemical functions. Advancements in computational methods, structural biology and synthetic biology have significantly enhanced the capabilities of *de novo* protein design, including miniproteins. However, it still remains a complex and challenging task due to the intricacies of protein folding and the relationship between sequence and structure. *De novo* design of miniproteins is a promising area of research with applications in drug development, enzyme engineering, and materials science. Ph.D. dissertation of Juan Lizandra Pérez deals with this interesting and current topic and has been prepared under supervision of Prof. Łukasz Berlicki at the Department of Bioorganic Chemistry, Wrocław University of Science and Technology. The subject of the thesis is closely related to the research conducted in Professor Berlicki's group and its title accurately reflects the actual research done.

The dissertation by Juan L. Pérez is written in English, has a total of 193 pages and is enriched with 101 figures and 32 tables. The thesis has a book format, making it comfortable to read. The main part of the thesis consists of six chapters and is preceded by auxiliary sections including abstract, list of abbreviations and acknowledgments. It begins with an introduction to the subject-matter (Chapter 1) followed by the presentation of the goals of the thesis (Chapter 2). Chapter 3 constitutes main part of the dissertation, i.e. results and

discussion, and is divided into two subchapters: 3.1 dedicated to β-amino acid-containing miniproteins and 3.2 focused on PD-1/PD-L1 inhibitors. Chapter 4 offers a brief one-page summary of the results. The experimental section (Chapter 5) provides a detailed description of the methods employed in the work. The bibliography forms Chapter 6, encompassing a total of 258 references.

The literature-based introduction starts with a general information related to protein folding problem and structure prediction. The brief chapter entitled *"Protein-protein interaction"* essentially focuses on interactions between immune checkpoint molecules, PD-1 and PD-L1, based on X-ray crystal structure of the PD-1/PD-L1 complex. The remaining part of the introduction is dedicated to miniproteins. The author classifies miniproteins into those occurring naturally, engineered, *de novo* designed, as well as those with backbone alterations. However, I could not find any information on the criteria that were used to classify these miniproteins into specific categories. Principally, *de novo* designed miniproteins and those with backbone alterations can also be considered as engineered. I expect Mr. Pérez to address this issue during the Ph.D. defense. The last paragraph is dedicated to modulation of biological activity of miniproteins involving molecular grafting or *de novo* activity design.

The "*Results and Discussion*" section is divided into two parts. The first part is dedicated to the *de novo* design of β-amino acids containing miniproteins with different topologies. This study started with the selection of the initial scaffold, a combination of two subunits: triple-stranded antiparallel β-sheet, a well-studied WW-prototype, and β-amino acids containing helix. Based on miniprotein **2** obtained by fragment assembling, over 20 new miniproteins were obtained and carefully analyzed. CD spectroscopy was used to assess the secondary structure content of the obtained mutants and to determine their thermal stability. For the most promising candidates, Mr. Pérez decided to confirm structural characteristics deduced form CD spectra with high resolution methods. For this purpose, investigations were conducted using X-ray and NMR techniques. Unfortunately, neither of these methods yielded the desired information. Crystals were either not obtained, or they were unsuitable for X-ray crystallography. On the other hand, NMR studies were also unsuccessful because the studied miniproteins appeared to be too flexible for structure determination. Nevertheless, careful analysis of miniproteins **1-21** allowed to identify the key residues influencing the overall fold and stability of the HEEE fold. For two miniproteins, **3** and **8**, SAX data were presented, however, it was not clear for me how they contributed to the final conclusions.

Next, miniprotein **21** was circularly permuted to generate miniprotein **25**, expected to adopt the EHEE fold. The successful permutation of the topology was confirmed by the analysis of CD spectra. To further optimize the properties of the permuted miniprotein, Mr. Pérez introduced numerous modifications in different regions of the sequences. A thorough analysis of introduced mutations led to the development of the sequence **46** containing all beneficial mutations. Further optimization and refinement of permuted mutants resulted in sequence **47**, for which a more comprehensive NMR analysis was performed. Analysis of the model, generated based on NMR data, confirmed the presence of the expected compact structure, stabilized by the presence of a hydrophobic core generated between the helix and the β-hairpin. It should be noted that the iterative process of optimizing the miniproteins' properties was supported by continuous analysis of CD spectra, NMR, thermodynamic data and molecular dynamics simulations.

The second part of the thesis focuses on the design of PD-1/PD-L1 immune checkpoint inhibitors. The search for inhibitors was based on the sequences of two different WW domains, the WW-Prototype, and FBP28WW. New inhibitor sequences were designed with the help of Rosetta FastDesign protocol. After characterization of these inhibitors with CD spectroscopy, their binding to the target PD-L1 was studied using BLI and HTRF techniques. As a result, two best mutants, **I6** and **I9,** were selected for further optimization. Histidine residue was tested as an affinity enhancer and was introduced into **I6** sequence. In contrast to the results obtained for mutants of the **I6** sequence, analogs derived from the **I9** sequence displayed a limited capacity to accommodate new mutations without impacting the miniprotein fold. This constraint hampered its potential for further optimization. In the next step, Mr. Pérez decided to graft best optimized inhibitors into miniprotein **21**. The resultant inhibitors with the HEEE fold, derived through this process, exhibited improved binding characteristics, with K_d and IC_{50} values at a low micromolar level. Nonetheless, despite these encouraging properties, they demonstrated a tendency to aggregate or exhibited poor solubility, thus precluding further studies. After the circular permutation of miniprotein **21** to **25** adopting the EHEE fold, a series of new inhibitors (**I36-I39**) were designed. The permutation of the topology improved physicochemical properties of those inhibitors. For **I38** an EC_{50} of 27.4 μM was determined, making it a very promising inhibitor candidate.

Taking into account the encouraging results obtained for inhibitors adopting the EHEE topology, a hybrid approach was subsequently employed to design another set of PD-1/PD-L1 immune checkpoint inhibitors. The method involved combining mimicry of the interactive

surface of optimized PD-1 with mutations intended to increase the affinity for the target, while preserving the EHEE fold. Among all the inhibitors investigated in this series, **O_I13**, was recognized as the most active inhibitor in cell assays. It is also regarded as the most potent β-amino acid-containing miniprotein-based inhibitor of the PD-1/PD-L1 immune checkpoint, stabilized by a hydrophobic core.

As I have demonstrated, in his Ph.D. dissertation Mr. Perez conducted an extensive experimental work related to the design and characterization of several dozen of miniproteins with specific topology and properties. To achieve the goals, Mr. Pérez had to employ a variety of experimental and computational methods. The miniproteins required for the research were obtained using solid-phase peptide synthesis (SPPS). The information regarding the secondary structure of miniproteins relied mainly on the analysis of CD spectra. This technique was also used in thermal denaturation measurements and thermodynamic studies. The attempts to determine three-dimentional structure of the selected miniproteins by X-ray crystallography or NMR spectroscopy were unsuccessful due to the bad quality of obtained crystals or due to the flexibility of the studied structures, respectively. Optimization of the fold was supported by computational methods. The binding affinity of the designed inhibitors to PD-L1 protein was evaluated using Bio-Layer Interferometry (BLI) experiments. The affinity studies were followed by inhibition studies applying Homogeneous Time Resolved Fluorescence (HTRF) that provided information on the ability of inhibitors to disrupt the PD-1/PD-L1 complex. Cell-based assays were done in cooperation with Dr. Łukasz Skalniak from the Faculty of Chemistry, Jagiellonian University.

In summary, the dissertation of Mr. Pérez focuses on a highly significant topic of a rational design of β-amino acid containing miniproteins and the design of new inhibitors that disrupt the PD-1/PD-L1 interaction. While a substantial amount of new material presented in this thesis is its strong point, presentation of the results and discussion is, in contrary, its weak point. It was difficult to follow all the arguments regarding the subsequent steps of optimization process of miniproteins. When reading "*Results and discussion*" I often found myself lost in descriptions of many experimental details (e.g., crystallization conditions, NMR parameters, description of SAXS experiment and BLI technique etc.) that should be included in the experimental section.

Other comments:

- Generally, the abstract of a Ph.D. dissertation should be a concise summary of the research work, highlighting its key elements. I would also expect it to include a summary of the main conclusions drawn from the research. Unfortunately, this information is missing, and the abstract of this Ph.D. thesis can serve mainly as a general introduction to the issues discussed in the dissertation.
- Since the design of PD-1/PD-L1 immune checkpoint inhibitors is one of the main topics of the dissertation, why the information regarding the current state of knowledge on known PD-1/PD-L1 inhibitors is practically missing?
- In my opinion, the rationale for using two different structures of the PD-1/PD-L1 complex, 4ZQKI and 5IUS, in the design of inhibitors requires explanation.
- The dissertation gives the impression of being written in a hurry, under time pressure. It contains a large number of typos and editorial mistakes.

Despite some critical notes mentioned above I should stress that the dissertation of Mr. Pérez addresses very interesting and important aspects of *de novo* design of miniproteins. The results of his study contribute significantly to our knowledge about this intriguing group of molecules with implication in basic biological research and drug development. In order to accomplish this work Mr. Pérez had to acquire the necessary theoretical knowledge and all practical skills required to propose such diverse and advanced experiments. Based on the opinion given above I conclude that the Ph.D. thesis presented by Mr. Juan Lizandra Pérez meets all the necessary requirements for the Author being awarded a Ph.D.

Zofia Daniel