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Review report of the PhD thesis entitled
"Design, synthesis, and structural characterization of miniproteins, and incorporation of protein-protein interaction inhibition activity"
written by Juan Lizandra Pérez

Juan Lizandra Pérez's PhD thesis was performed at the Department of Bioorganic Chemistry at the Faculty of Chemistry of the Wrocław University of Science and Technology under the supervision of Prof. Łukasz Berlicki. The topic of the dissertation concerns protein folding and the rational design of miniproteins with a complex tertiary structure - excellent models for studying full-length protein folding and scaffolds for designing molecules with specific biological activities. The dissertation covers 193 pages and consists of six numbered chapters: *Introduction*, *Goals*, *Results and discussion*, *Summary, Experimental Part* and *References* preceded by *Abstract, List of Abbreviations* and *Acknowledgments*. The first part of the 39-page introduction provides a brief overview of the protein folding process, an important role of protein-protein interactions (PPI) in biological pathways and cellular processes, and representative methods for predicting protein structure. The second part of the introduction focuses on naturally occurring miniproteins, which may be a good starting point for structure optimization, as well as *de novo* designed miniproteins characterized by precise folding, including those modified with β-amino acids.

The *Goals* section defines the three main aims of the PhD project: to develop a methodology for *de novo* design of a β-amino acid-containing miniproteins with a well-defined fold, to generate a new binding mode of the inhibitor to PD-L1 based on the WW-domains, and finally to design new inhibitors of the PD-1/PD-L1 immune checkpoint based on the most promising miniproteins from the first part of the study.

The *Results and Discussion* section is divided into two parts. Each part ends with a brief summary. The first one focuses on the study of miniproteins resulting from the combination of two independent subunits: a helix containing β-amino acids, which is a fragment of the gp41 subunit mimetic, and the WW-domain, as the initial β-sheet template (miniproteins with the HEEE structural motif). It is hypothesized that the presence of the helix could potentially stabilize the β-sheet structure. The Rosetta software is employed to predict protein folding. In the subsequent stages of miniprotein design, only residues that could generate interactions between the helix and the β-sheet are mutated. Moreover, the study also includes truncation of the amino acid sequence and rearrangement of the helical fragment of gp1 subunit mimetic with the first strand of the β-sheet of the WW-domain to obtain miniproteins with the EHEE structural motif. In this part of the work, as many as 50 miniproteins are synthesised and examined. Circular dichroism spectroscopy is used to control the secondary structure of all designed miniproteins, as well as the contribution of aromatics to the fold. Temperature-depended CD spectra allow to determine the melting temperatures, while thermochemical denaturation experiments, also monitored by CD, are performed to obtain thermodynamic parameters of folding. The most thermally stable miniproteins are subjected to denaturation and renaturation studies using nano differential

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scanning fluorimetry (NanoDSF). The results reveal that miniproteins refold spontaneously with estimated T_m values consistent with the CD data. The first miniproteins obtained are also analysed with small angle X-ray scattering to indicate their oligomerization state. At the subsequent stages of the design, the doctoral student attempts to crystalize selected compounds to determine their three-dimensional structure. Unfortunately, none of the crystals show diffraction. Attempts to determine the three-dimensional structure using 2D NMR spectroscopy are also unsuccessful, probably due to high flexibility of the loop between the first strand and the helix. The exception is protein 47, for which a preliminary 3D structure is determined by NMR and molecular dynamics simulations using XPLOR-NIH software. The flexibility of the above-mentioned loop is explored using molecular dynamics simulations in GROMACS. The second part of the *Results and discussion* chapter concerns the search for PD1/PD-L1 immune checkpoint inhibitors. In the first phase of search, two WW-domains (WW-Prototype and FBP28WW) are selected as a starting point for the development of inhibitors. The selected positions of the scaffolds are mutated to attain a higher affinity towards selected residues of the target protein. The crystal structure of the PD-1/PD-L1 and opt-PD-1/PD-L1 complexes (opt-PD-1 refers to an optimised mutant of PD-1) are used as references. A total of 27 miniproteins are synthesized and analysed to generate new interactive and binding modes of the inhibitors to PD-L1 target protein. Subsequent miniproteins, similarly to the first part of the study, are analyzed using CD spectroscopy to control secondary structure and the melting point. BioLayer Interferometry (BLI) is used to study the binding kinetics of the inhibitors to the target PD-L1, whereas Homogeneous Time Resolved Fluorescence (HTRF) is performed to assess ability of inhibitors to disrupt the PD-1/PD-L1 complex (estimation of IC_{50} - half maximal inhibitory concentration). Finally, inhibitors based on WW-Prototype are grafted into the HEEE and EHEE miniproteins tested in the initial stage of the study, yielding another 31 miniproteins. For the most promising inhibitors with improved solubility, an immune checkpoint blockade cell-based assay is performed to estimate EC_{50} (half maximal effective concentration) values.

Chapter four of the doctoral dissertation is a concise summary of the research. The following chapter, *Experimental part*, contains information about the methods and scientific equipment used as well as details about the syntheses along with MS analyzes of all miniproteins and the NMR analysis (chemical shifts and NOE contacts) of miniprotein 47. The dissertation ends with a bibliography (258 references, reference 220 seems to be incomplete).

I consider the strength of the work to be logical in planning subsequent stages of research and interdisciplinary nature of the work. In my opinion, the most important scientific achievements of the reviewed thesis are:

- Selecting appropriate starting models for structure optimization, folding studies and design of inhibitors, which proves good knowledge of the subject;
- Rational design of miniproteins with protein-like tertiary structures, which required the synthesis of as many as 108 miniproteins (including both scaffolds and inhibitors) with an average length of 40 amino acid residues and extensive studies of their secondary structure and stability;
- Optimization of the fold through a hydrophobic core between the triple-strand antiparallel β-sheet and the helix (HEEE and EHEE structural motives);
- Confirmation that the incorporation of β-amino acids allows to control the overall shape of the molecule, not only due to the induction of a helical structure, but also as a contributing factor to the stabilization of the tertiary structure;
- Displaying that miniproteins with residues responsible for proper folding facing the hydrophobic core endow them with the potential to be redesigned into active PD-1/PD-L1 immune checkpoint inhibitors without compromising their 3D conformation;
- Designing of a new interactive surface for PD-L1 target proteins and identification of promising inhibitors (miniproteins I38 and O_I13 created on the EHEE scaffold);

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- Investigation of the effect of net charge on solubility and inhibition properties. It has been shown that a higher positive charge may induce higher inhibition;
- Finally, proving that combining precisely folding miniproteins with an active motif is a promising strategy for obtaining biologically active molecules.

While reading the doctoral dissertation, I had a few questions and minor comments, which I listed below. I would like to ask the candidate for the PhD degree to respond to the following issues during the public defence of the dissertation as they require explanations or comments.

- p. 57, Figure caption 29. Panel C shows ellipticity as a function of temperature for miniproteins 21, 22 and 24, excluding 23. Therefore the entry 21-24 is incorrect. The table in D panel shows T_m values for miniproteins 20-24, not 14-19;
- p. 63, Figure 34. All entries in the table (panel B) correspond to enthalpy, but according to the main text they should represent: enthalpy, entropy, Gibbs free energy, heat capacity and sensitivity to denaturation;
- p. 66 According to the data in Table 10 (p. 67), the total contribution of mutations to the stability of compound 36 compared to compound 37 was $\Delta\Delta G°$ -0.6 kcal/mol, not -1 kcal/mol;
- P. 88 Table 20 shows the amino acid sequences of two WW-domains. What does the colour blue mean?
- p. 115 Figure 76D. What does the asterisk mean? Does this mean that T_m was an approximation obtained from data collected before miniprotein precipitation?
- p. 120 Figure 83. The caption to the figure suggests that the scaffold is miniprotein 46. However, the sequence is not consistent with that in Tables 13 and 27.
- The experimental section lacks details regarding protein 47 structure calculations using XPLOR-NIH and NMR data.
- p.164 The HSQC experiment has no mixing time. Did the PhD student mean the HSQC-TOCSY spectrum?
- In the text of the dissertation, I also found a few typographical errors and minor stylistic and editorial errors, which are so few that it is not worth replacing them.

The above-mentioned comments and minor shortcomings that arose during the writing of the dissertation in no way diminish the scientific value of the reviewed doctoral thesis, which I rate highly. The candidate for the PhD degree has proven that he is an experienced specialist in the field protein folding, is able to properly design interdisciplinary research using a variety of experimental and computational techniques - as well as skillfully interpret the obtained results and approach them critically.

There is no information about the PhD student's publications. However, according to the information contained in the DONA database (WUST scientific output), the candidate for PhD degree is a co-author of two papers from JCR list, one paper in conference proceedings and three international conference presentations:

Papers:

- Violeta Marković, Jeelan B. Shaik, Katarzyna Ożga, Agnieszka Ciesiołkiewicz, Juan Lizandra Peréz, Ewa Rudzińska-Szostak, Łukasz Berlicki. Peptide foldamer-based inhibitors of the SARS-CoV-2 S protein–human ACE2 interaction. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 2023, 38, 2244693 (IF_{2023}=5.756).
- Agnieszka Ciesiołkiewicz, Juan Lizandra Peréz, Łukasz Berlicki. Miniproteins in medicinal chemistry. *Bioorganic & Medicinal Chemistry Letters*, 2022, 71, 128806 (IF_{2023}=2.94).

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Proceedings:

- Monika E. Szefczyk, Juan Lizandra Peréz, Anna M. Szczepańska, Paulina I. Fortuna Alternative carriers in drug delivery systems - peptide foldamers. *Proceedings of the 8th World Congress on Recent Advances in Nanotechnology* (RAN'23), March 23-25, 2023, Lisbon, Portugal.

Conference presentations:

- Juan Lizandra Peréz, Łukasz Berlicki. WW-domain based inhibitors of PD-1/PDL-1 interaction. EFMC-ISMC International Symposium on Medicinal Chemistry, Virtual event, Aug. 29-Sep. 2, 2021.
- Juan Lizandra Peréz, Łukasz Berlicki. From WW-domains to foldameric miniproteins, suitable scaffolds for PD-1/PD-L1 interaction inhibitors. ChemBiotIC, Chemistry & Biotechnology International Conference, June 24-25 2021, Wrocław, Poland.
- Natalia J. Świerczek, Katarzyna Ożga, Violeta Marković, Jeelan B. Shaik, Agnieszka Ciesiołkiewicz, Juan Lizandra Peréz, Ewa Rudzińska-Szostak, Łukasz Berlicki. Design, synthesis, inhibitory activity and NMR studies of helical peptide foldamers against SARS-COV-2 protein S - human ACE2 interaction. 13th Young Medicinal Chemist Symposium = Nuove Prospettive in Chimica Farmaceutica, April 26-29, 2021.

Is there anything more? The results of the research presented in the doctoral dissertation have been presented at two international conferences, but have not yet been published. Does the PhD student plan to publish these results in the nearest future?

In conclusion, the PhD thesis of Mr. Juan Lizandra Pérez contains essential elements of scientific novelty and makes a significant contribution to the development of miniproteins as scaffolds for inhibitor design. The results of the research conducted under his doctoral dissertation are of great cognitive value and may be of practical importance in terms of designing new compounds with pharmaceutical importance based on miniproteins with precise tertiary folding. In my opinion, the doctoral dissertation submitted by Juan Lizandra Pérez fully meets the formal (in the light of the Act of July 20, 2018 Law on Higher Education and science; consolidated text: Journal of Laws of 2023, item 742, as amended) and customary requirements for doctoral dissertations.

Considering the above, I am applying to the Scientific Discipline Council of Chemical Science at the Wrocław University of Science and Technology to admit Mr. Juan Lizandra Pérez to the next stages of the doctoral procedure.

Emilia Sikorska