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Wrocław, 2023-03-17

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Review of the doctoral dissertation of MSc. Magdalena Bojko „Searching for peptides interacting with the PD-1 and PD-L1 immune checkpoint proteins for cancer immunotherapies"

The doctoral dissertation of MSc. Magdalena Bojko describes the work carried out by the author under the supervision of prof. dr hab. Sylwia Rodziewicz-Motowidło at the Department of Biomedical Chemistry at the University of Gdańsk. The co-supervisor was Dr. Marta Spodzieja. The results perfectly fit into the research carried out with numerous successes in the group led by prof. Rodziewicz-Motowidło.

The overall aim of the thesis was to design appropriate peptide inhibitors of the PD-1/PD-L1 complex, able to restore the proper functions of the immune system. Before the readers get to the point where they fully understand what this challenging task actually means, they are introduced to a variety of necessary topics – the severity, the societal and economical challenges of cancer diseases (chapter 1), and to the complexities of the human immune system (chapter 2), in which, among others, the readers learn to understand the role of PD-1, a 288 amino acid transmembrane protein, which extracellular soluble domain can be secreted into the serum.

Chapter 3.2 introduces us to the other two key players of the thesis, PD-L1 and PD-L2, 290 and 273 amino acid transmembrane glycoproteins. Both have a variety of functions in immunology and oncology and can serve as a potential treasure troves for novel drug design. Details of PD-1/PD-L1 and PD-L2 interactions are explained, and their recently

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solved crystal structures are also shown. Clinical challenges and already existing solutions are discussed and therapy based on immune checkpoint inhibition is introduced.
Chapter 5.2 is of particular interest, introducing the peptides and peptidomimetics used as inhibitors of PD-1/PD-L1 complex formation. It is important to note that the topic is a scientifically really 'hot' one – most of the discussed literature comes from the last 4 years(!) (Tables 4 and 5).
The young scientist clearly sees a gap in the current state of knowledge that she plans to fill. By the time the reviewer reaches chapter 5.2.1, she is absolutely intrigued by the project and wishes that she had undertaken similar tasks herself.

The aim of the thesis is clearly stated – its Author plans to find peptide inhibitors of the PD-1/PD-L1 complex that will be able to restore the proper functioning of the immune system.
The thesis has a traditional layout –after an introduction to the state-of-the-art of the topic, and a firm statement the aim of the work, the author guides us to one the most interesting parts of the thesis – the experimental results, their interpretation and biological meaning, which, taken together, lead to fruitful conclusions and brief summary. The last part of the work is the "materials and methods" section.

In the first step of the work, the PD-1/PD-L1 complex formation was studied via MD calculations (carried out in collaboration). The role of MSc. Bojko was the final interpretation of the data that resulted in pointing out three PD-L1 fragments and two PD-1 fragments involved in complex formation. This, together with the crystal structure, was the starting point for the design and synthesis of appropriate PD-1 fragments that were potentially able to target PD-L1 and inhibit the PD-1/PD-L1 interactions.
The PhD student obtained 13 linear and cyclic analogues of PD-1 and, using SPR, confirmed the affinity of eight of them to the PD-1 ligand (two of the designed peptides (7 and 10) showed a remarkably high affinity and peptide 6 – a reasonably high affinity). At this point, I am curious **why was norleucine introduced into peptides 1-4 (sequences listed in Table 8)?** And an additional question - **Nle, when found among one letter amino acid code, is easy to be mistaken for asparagine and D-isomers of leucine and glutamic acid. I am aware that a**

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one letter abbreviation does not exist, but shouldn't "Nle" include braces, when put in a one letter code, or is it not strictly required?

The effect of the eight peptides was later tested on cell viability, basing on CHO-K1 (Chinese hamster ovary cell line, deficient in proline synthesis and which do not express the epidermal growth factor receptor), Jurkat E6.1 (human T lymphoblast cell line from a patient with T-cell leukemia) and TCS Ctrl (BW5147?) cell lines. I am curious **what is the difference between TCS Ctrl and BW5147 cell lines – their names seem to be used interchangeably?**

The tested peptides were cytotoxic (especially peptide 10), but only in high concentrations. Peptides 7 and 10 also turned out to partially inhibit the formation of PD1/PD-L1 complex, with peptide 7 being competitive with PD-L1.

An NMR solution structure of peptide 10 was obtained (in collaboration with co-workers from the group; it shows a beta-hairpin like structure), which is quite an impressive achievement, since peptides solution structures are far from being trivial to obtain.

Peptide 10 has further been chosen as a template for peptidomimetic design – loop modifications were introduced in order to improve the compounds' stability and increase their affinity towards PD-L1 (discussed in chapter 4.1). Six of such were designed, based on a very interesting way of controlling the beta-turn via introducing D-proline, D-alanine and glycine in carefully chosen sites. Four of such complexes were further studied. The modifications did not have a major impact on the affinity towards PD-L1, however, they did substantially increase the stability of the peptides in the medium and turned out to be less toxic to regular cell lines. The analogue A3 was able to displace PD-1 from PD-1/L-1 in a dose dependent manner, however, it was not able to restore the eGFP expression. I will, again, ask a difficult question – **why was A3 most successful and how could it be further modified to increase its potency?**

In the second step of the project, the PhD candidate aimed to block the PD-1/PD-L1 interactions, designing seventeen peptides derived from PD-L1 and examined way in a similar manner to that described above. I have to underline, that the design of the peptides and peptidomimetics is true art, and some of the introduced modifications are not really

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clear to me – **why was the change of Nle to Met introduced in L17 (with respect to peptide L13)?**

It turned out that L11 exhibits the strongest affinity for the PD-1 protein and that it prevented PD-L1 from binding to PD-1; the L11 binding is characterised by a fast association constant. Again, at this point, the question which comes to my mind is 'why'? – **Why does L11 inhibit PD-L1/PD-1 binding, while the other analogues do not perform as well? Can we hypothesize which particular modifications may be responsible for this phenomenon?**

Apparently, L11 turned out to be the most "successful" peptides among the studied ones in the second part of the work; because of its inhibitory effect on the PD-1/PD-L1 complex formation, L11 stimulated the expression of eGFP in a concentration-dependent manner.

I find chapter IV to be the most impressive one in the thesis – it discusses the obtained results together with those from the newest literature, thus putting them in a "bigger frame". This is done with an impressive scientific maturity, for which I have to truly congratulate the PhD candidate.

Scientific maturity also means being able to put complex ideas in simple words and summarize them on one page – this is exactly what happens in chapter V, "Summary".

The doctoral thesis is written in beautiful, linguistically correct English and it is a real pleasure to read. Its editorial side is very careful - the print, formulas and drawings are legible, and the doctoral student is obviously a skilled writer. Despite my sincere intentions, I did not find any grammatical or stylistic errors. I found (literally) one typo: "Van der Waals", on page 66, should be "van der Waals", to the best of my knowledge.

It is worth to note that the skills that the PhD student acquired are truly interdisciplinary, ranging from basic organic chemistry (peptide synthesis) through physical and structural chemistry (affinity calculations, MD studies, NMR-based structure solving) to biological experiments on cell lines. I am really happy to see this kind of approach to science.

The fulfilment of the project definitely took a lot of diligence and hard work, and I clearly see why the 'late night sessions and moral support' of the co-supervisor were necessary.

The scientific information they provided in this dissertation is extremely valuable and clearly contributes to basic science. The publishable result of the work are (so far) two

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excellent papers in the most renowned journals in the field – Bioorganic Chemistry and Bioelectrochemistry. The leading role of the PhD candidate is obvious in both of them.
MSc. Bojko was clearly involved in a series of side projects, which resulted in a book chapter and in numerous oral and poster presentations on international conferences (8 and 16, respectively, most of them are thematically connected with the scope of the doctoral thesis). She was also the PI of four small research grants and the contractor of a large Polpharma grant.
The PhD studies of MSc. Bojko were rich in fruitful collaboration; one of the most important one for her may be her stay in Prof. Steinberger's laboratory at the Medical University of Vienna, where she performed studies on the inhibitory properties of the newly designed systems on a cellular assay constructed on a Jurkat E6.1 line. At this point, I have to point out that her stay must have taken more than one day, as stated in the thesis (20.09.2021-20.09.2021) – this certainly is a typo that has no impact on the work itself.

The purpose of the dissertation, i.e. design appropriate peptide inhibitors of the PD-1/PD-L1 complex, able to restore the proper functions of the immune system, was fully achieved. The topic is absolutely up to date, the experiments have been correctly designed, and their effects are clearly visible and reproducible. Their conclusions are clear and sound. I see the work as a significant input into the field of PD-1/PD-L1 complex antagonists – a solid "brick in the wall" that may serve as a foundation for the design of clinically significant immune modulators, paving way for further studies on this rapidly developing topic.

Taking into account the novelty and exceptionally high quality of the of scientific work, it has to be summarized that it meets (and significantly exceeds) the requirements for doctoral dissertations in accordance with Article 13 of the Act of March 14, 2003 on academic degrees and academic titles and on degrees and title in the field of art (Journal of Laws of 2003 No. 65, item 595; of 2005 No. 164, item 1365; of 2010 No. 96, item 620; No. 182, item 1228, of 2011 No. 84, item 455). In this regard, I am applying to the Scientific Council of Chemical Sciences of the University of Gdańsk for the admission of the PhD candidate Magdalena Bojko to the next stages of the doctoral procedure.
I expect that a significant part of the results described in the work will be published in at least a few reputable journals in the field. Taking into account the above very positive assessment of the work, its outstanding scientific quality, novelty, the huge amount of valuable data, the potential possibility of using them in therapy, and above all the fact that

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the conclusions of the work definitely enrich the chemistry of therapeutic peptides, I am applying for a distinction of the work.

The Polish version of the last paragraph follows:

„Biorąc pod uwagę nowatorstwo i wybitnie wysoką jakość pracy naukowej, należy podsumować, że spełnia ona (a nawet znacznie przewyższa) wymogi stawiane rozprawom doktorskim zgodnie z artykułem 13 Ustawy z dnia 14 marca 20 03 r. o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki (Dz. U. z 2003 r. Nr 65, poz. 595; z 2005 r. Nr 164, poz. 1365, z 2010 r. Nr 96, poz. 620, Nr 182, poz. 1228, z 2011r. Nr 84, poz. 455). W tym odniesieniu wnoszę do Rady Dyscypliny Nauk Chemicznych Uniwersytetu Gdańskiego o dopuszczenie Magdaleny Bojko do kolejnych etapów przewodu doktorskiego. Spodziewam się, że znaczna część opisanych w pracy wyników zostanie jeszcze opublikowana w co najmniej kilku renomowanych czasopismach z dziedziny. Biorąc pod uwagę powyższą bardzo pozytywną ocenę pracy, jej wybitną jakość naukową, nowatorskość, ogrom wartościowych danych, potencjalną możliwość wykorzystania ich w terapii, a przede wszystkim fakt, że wnioski z pracy zdecydowanie wzbogacają chemię terapeutycznych peptydów, zwracam się z wnioskiem o wyróżnienie pracy."

With my best regards,

M. Rowińska-Żyrek

Magdalena Rowińska-Żyrek