

OPINION

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Elected as a member of the scientific jury by Order No. 168/29.11.2024 of the Director of the Institute of Microbiology of the PhD dissertation for the award of the educational and scientific degree "**Doctor**"

In the field of higher education **4. Natural Sciences, Mathematics and Informatics**

Professional direction **4.3. Biological sciences**

Scientific Specialty "**Immunology**"

Author: Emiliya Zahariya Stoyanova

Topic: A novel approach for treatment by epitope - specific tumor inhibition of mouse model of melanoma

Research supervisor: Prof. Andrey Tchorbanov, PhD

1. General Description of the Presented Materials

The author of the dissertation is Emiliya Zaharia Stoyanova, a PhD student in regular training at the Department of Immunology, Stefan Angelov Institute of Microbiology, Bulgarian Academy of Sciences (BAS), under the supervision of Prof. Dr. Andrey Tchorbanov. The set of materials presented for review, both in paper and electronic format, complies with the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the regulations for the application of LDASRB, and the BAS regulations for the application of DRASRB. It meets the criteria of the Regulation for the conditions and procedures for obtaining scientific degrees and holding academic positions at the Stefan Angelov Institute of Microbiology, BAS, for obtaining the educational and scientific degree "Doctor."

The PhD student has attached 2 publications on the dissertation topic and the corresponding evidence, certificates from participation in scientific conferences. Her total scientific production includes 4 publications in international journals, cited 10 times; her h-index is 2, according to Scopus.

2. Relevance of the Topic and Appropriateness of the Set Goals and Tasks

In recent decades, skin melanoma has shown a sharp increase in incidence and tends to transform from a relatively rare oncological disease into one of growing medical significance. Recent statistical data indicate a significant increase in melanoma cases worldwide. From 2012 to 2020, the number of cases increased by 41% – from 230,000 to 325,000. There is a worrying trend of annual increases in incidence by 3-5% globally, including in Bulgaria. This rise is most pronounced in regions predominantly populated by fair-skinned people, such as Australia and New Zealand. Annually, around 600 people in Bulgaria are diagnosed with malignant melanoma. According to the European Commission, the highest incidence of melanoma in Europe is in the age group 45-69, while statistical data from the USA show that melanoma is one of the most common diagnoses among young adults (especially young women) and adolescents (15-39 years old). Malignant melanoma can develop even in early childhood. Major risk factors for the disease include ultraviolet rays and sunburn from natural and artificial sources like tanning beds, as well as genetic predisposition.

The dissertation topic addresses an important and current area, focusing on the search for new and specific therapeutic approaches to melanoma, presenting experimental data on the effect of protein epitope-specific vaccines containing hemocyanins. Hemocyanins are large respiratory proteins found in the hemolymph of mollusks and arthropods, intensively studied in recent years for their potential against various socially significant diseases like tumors, viral infections, etc., or their possible use as vaccine adjuvants.

One of the best-studied and widely used hemocyanins in biomedical applications is Keyhole Limpet Hemocyanin (KLH). Due to its large size and numerous epitopes, it generates a strong immune response, and the abundance of lysine residues makes it very suitable as a carrier protein for haptens to generate hapten-specific antibodies. It is used in various cancer vaccines, including non-Hodgkin's lymphoma, breast cancer, and bladder cancer. These vaccines contain specific tumor-associated antigens conjugated with KLH to stimulate anti-tumor immune responses that can destroy tumor cells.

Testing new therapeutic approaches for all malignant diseases expands possibilities and gives hope for addressing this global health problem. For this reason, I believe the presented dissertation topic is extremely relevant and important.

3. Characteristics and Evaluation of the Dissertation Work

The dissertation work is structured according to the requirements of the Regulation for the Application of the Law for the Development of the Academic Staff at the IMicB - BAS,

containing 129 pages, 52 figures, and a bibliographic reference including 134 literature sources. It is structured as follows:

- **Title**
- **Table of Contents**
- **Used Abbreviations**
- **Introduction** – 1 page. The PhD student purposefully introduces the main aspects and obtained results in the dissertation work.
- **Literature Review** – presented on 40 pages, including 17 illustrative figures.

My impression of the presented literature review is that Emiliya is very well acquainted with the current state of the researched problems. 114 sources were used, 45 of which are from the last 5 years. The literature reference is divided into ten subsections, with 17 figures that illustrate and complement the presented information.

For a smooth introduction to the topic, the PhD student goes through the general aspects of cancer diseases, particularly melanoma, summarizing important aspects of the anti-tumor immune response, presenting key moments and immune cells with a crucial role in innate and adaptive immune responses against cancer.

In a separate subsection, Emiliya presents the dual role of the immune system in the development of these diseases, specifically the provision of an immunosuppressive environment that supports tumorigenesis and promotes tumor growth. She summarizes modern approaches to melanoma therapy, also noting the shortcomings of these therapies. Space is given to the possibilities of natural, biologically active molecules as anticancer products, thereby introducing the dissertation topic. The significance of in vivo models for testing new potential therapeutic agents is also noted.

- **Goals and Tasks** – The goal is precisely defined, with four clearly formulated and logically connected tasks derived for its achievement:
 1. Development of an experimental mouse model of melanoma.
 2. Investigation of the anti-tumor properties of RtH and HaH in a B16F10 mouse melanoma model.
 3. Synthesis and chemical conjugation of a peptide (GD3P4), a mimotope of the tumor-associated carbohydrate epitope (GD3) characteristic of melanoma cells, to hemocyanin molecules and analysis of the obtained chimeric molecules (RtH-GD3P4 and HaH-GD3P4).

4. Investigation of the therapeutic effect of the constructed anticancer vaccines RtH-GD3P4 and HaH-GD3P4 in the B16F10 mouse melanoma model and its significance for the survival of experimental animals.

- **Materials and Methods** – The methodology used in the development of the dissertation work is presented on 16 pages and 8 figures and reflects a diverse set of molecular biological, immunological, and histological methods, well described and enabling reproducibility. The illustration of experimental setups with schemes makes a good impression, greatly facilitating the reader.

Undoubtedly, the dissertation work represents serious and thorough research conducted at a high methodological level.

- **Results** – 34 pages, 27 complex figures. The presented results are divided into three subsections and reflect the set tasks.

The choice of an appropriate scheme for working with the model cell line is crucial for subsequent experiments, specifically the number of cells to inoculate to achieve maximum disease manifestation while allowing a window to measure the maximum therapeutic effect. The presented results in the first subsection show precisely this – injection of the B16F10 melanoma cell line at a concentration of 1.5×10^5 cells/animal leads to 100% mortality in untreated animals and provides a 3-month therapeutic window to assess treatment effectiveness.

The next part of the results presents data on the anti-tumor potential of the tested hemocyanins in the already determined mouse melanoma model, but with three different treatment approaches – therapeutic, intensive, and a pre-treatment scheme.

The third part of the results describes the design and construction of anti-tumor vaccines containing the GD3-mimicking peptide and the hemocyanins RtH or HaH. The constructed protein molecules are tested again using the three treatment approaches. A considerable amount of work has been carried out. The results are presented in 27 figures, many of which are complex.

- **Discussion** – consisting of 10 pages, synthesizing and summarizing the information from the obtained results in the dissertation work.

The PhD student makes a skillful attempt to analyze the mechanisms of action of the hemocyanins and the constructed vaccines and how different immunization regimes affect tumor behavior and immune system communication with the tumor. The use of such large and complex molecules containing various carbohydrate components and the complexity of the immune response suggest diverse and sometimes contradictory results, which the PhD student and her supervisor interpret scientifically. This diversity of data provides new opportunities for

future studies aimed at deepening the understanding of specific control mechanisms over tumor development.

- **Conclusions** – 1 page
- **References** – 12 pages; 134 used sources

4. **Evaluation of Publications and Personal Contribution of the PhD Student**

Two articles related to the dissertation topic have been published in the peer-reviewed international journal *Marine Drugs* (Q1), with a total Impact Factor of 10.03. In Scopus, 10 citations are found excluding self-citations. During her dissertation, Emilia Stoyanova has made 7 presentations at national and international scientific forums.

Personal participation of the PhD student – In both presented articles on the dissertation topic, Emiliya Stoyanova is the first author. I also have personal impressions and observations of Emiliya 's work over the years, which gives me a basis to unequivocally accept the personal contribution of the PhD student in the conducted research.

5. **Critical Remarks, Recommendations, and Questions**

I have no critical remarks regarding the conducted research. Some minor technical errors and inaccuracies (e.g., "infection" of mice with the cell line) can be noted. A more appropriate term would be "inoculation". "Infection" is generally used in the context of the spread of infectious diseases among organisms. These inaccuracies do not in any way diminish the quality of the presented dissertation work.

From the presented results and the systematic conclusions, it is evident that, in addition to the constructed vaccines, pure hemocyanins also have a strong anti-tumor effect.

Could the PhD student, based on the data and analyses, determine under what conditions (e.g., tumor development stage, immune status of the organism, etc.) it would be more appropriate to apply therapy with pure hemocyanin and when therapy with epitope-specific vaccine?

6. **Author's Abstract** The author's abstract for Emilia Stoyanova's dissertation reflects the main achieved results and meets all generally accepted requirements of the Regulation for the Application of the Law for the Development of the Academic Staff in the Republic of Bulgaria.

CONCLUSION

The dissertation **contains scientific and scientifically applied results**, which constitute an original contribution to science and meet all the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the regulations for the application of LDASRB, and the regulations for the application of LDASRB at BAS. The presented dissertation results **fully comply** with the specific requirements of the Regulations of IMicB - BAS for the application of LDASRB.

The dissertation demonstrates that Emiliya Stoyanova **possesses** in-depth theoretical knowledge and practical skills, showing qualities and abilities for conducting independent scientific research.

Based on the above, I confidently give my *positive evaluation* of the conducted research and *recommend that the esteemed jury award Emiliya Zaharia Stoyanova the educational and scientific degree "Doctor" in the field of Biological Sciences (4.3)*.

January 8, 2025

Reviewer:.....

[Assoc. Prof. Dr. Nikolinka Mihailova]