

# REVIEW

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Subject: Approbation of dissertation work for defense and acquisition of the educational and scientific degree "Doctor", in Professional field: 4.3. "Biological Sciences", in scientific specialty: "Immunology" - 01.06.23.

Topic of the dissertation: "A novel approach for the treatment of a mouse model of melanoma through epitope-specific tumor inhibition"

Author of the dissertation: Emilia Zaharia Stoyanova, full-time doctoral student at the Institute of Microbiology "Stefan Angelov" BAS

Scientific supervisor: Prof. Dr. Andrey Ivanov Chorbanov, db

Relevance and significance of the dissertation:

Melanoma is one of the most aggressive forms of skin cancer with high metastatic potential. Despite advances in therapies, the prognosis for patients with advanced melanoma remains poor. The development of new treatment approaches, especially those that focus on specific tumor antigens and activate the immune system, is of utmost importance. Emilia Stoyanova's dissertation addresses precisely this need, proposing a new approach for immunotherapy based on hemocyanin vaccines in a mouse model of melanoma. The use of hemocyanins in melanoma immunotherapy is not new, but the relevant mechanisms of immunomodulation have not been fully elucidated. This makes the topic relevant and significant for modern oncoimmunology. Of regional importance is the study of hemocyanins specifically from *Rapana thomasiana* and *Helix aspersa*, species distributed in Bulgaria, for which there is a school in our country with interesting results.

Structure of the dissertation:

The dissertation is structured in a standard format, including introduction, literature review, aim and objectives, materials and methods, results, discussion, conclusions and references. The total volume is 139 pages, with 134 literary sources used, of which 34% were published in the last 5 years. The dissertation is illustrated with 52 figures.

The literature review is extensive and well-structured, covering key aspects of tumor pathophysiology, skin cancer, cancer immunology, as well as modern approaches to the treatment of melanoma. Particular attention is paid to tumor-associated carbohydrate antigens and the potential of natural products as anticancer agents, with a focus on hemocyanins. A special section examines mouse models in oncology. The richness and depth of the review of this extremely complex and rapidly developing interdisciplinary field is impressive. The PhD student skillfully covers the broad topic of tumor immunology and, without unnecessary

verbosity, manages to focus on the specific problem of the functionality of hemocyanins as epitope carriers and their potential role in the creation of tumor vaccines.

The goal of the dissertation - development of a new therapeutic approach for the treatment of melanoma using hemocyanins and epitope-specific vaccines, is very specific and clear, with a strongly applied nature. In fact, the interpretation of the results points to interesting theoretical challenges and in general the work has a more theoretical nature than the goal suggests. To achieve the goals, 4 groups of tasks have been defined: 1) development of an experimental mouse model of melanoma, 2) study of the anti-tumor properties of RtH and HaH in the B16F10 mouse model of melanoma, 3) synthesis and chemical conjugation of a peptide mimotope of GD3 to hemocyanin molecules and analysis of the resulting chimeric molecules (RtH-GD3P4 and HaH-GD3P4), 4) study of the therapeutic effect of the constructed anti-cancer vaccines RtH-GD3P4 and HaH-GD3P4 in the B16F10 mouse model of melanoma. The tasks are logically structured and cover both in vitro and in vivo experiments.

The methodology includes an impressively wide range of comprehensively described modern immunological, biochemical (development of conjugated vaccines with hemocyanins) and molecular biological techniques as well as a mouse model of melanoma. The abundance of experimental approaches distinguishes this dissertation and suggests a high methodological level reached by the dissertation.

The results are presented in detail and are excellently illustrated. The key experiments resulted in:

1. Successful isolation and purification of hemocyanins from *Rapana thomasiana* (RtH) and *Helix aspersa* (HaH).

2. Demonstration of the antitumor effect of hemocyanins in a mouse model of melanoma.

3. Development of conjugate vaccines containing GD3-mimetic peptide and hemocyanins. The design of the peptide mimotope and its synthesis are not part of the dissertation. This includes the successful conjugation of hemocyanins with the peptide.

4. Introduction and determination of the parameters of a mouse model of melanoma and a therapeutic immunization regimen. Several regimens were tested: classical therapeutic immunization in a developed tumor, intensive therapy with administration in parallel with the development of the tumor from its earliest stages, and pretreatment therapy (sensitization).

4. Demonstration of the effectiveness of vaccines in slowing tumor growth and prolonging survival of experimental animals. It was found that the pretreatment regimen gave a weaker effect. These results support the previously established fact that hemocyanins, administered alone, have the effect of antitumor vaccines.

5. Analysis of the immune response induced by the therapy, including the generation of tumor-specific antibodies and cytotoxic T-lymphocytes.

The discussion is in-depth and critical, with the author successfully interpreting the results obtained in the context of modern scientific knowledge.

The conclusions are clearly formulated and reflect the main achievements of the dissertation. Notably, the dissertation lacks an otherwise traditional section – contributions. I view positively this fact as the achievements must be determined by the scientific community and the effect of the research on the development of the field. This requires some time to take into account, for example, the level of citation. In my opinion, the main contributions of the dissertation are 1) the characterization of the effect of hemocyanins from species widely distributed in Bulgaria on the development of a mouse model of melanoma and 2) data on the relationship between stimulation of the IgM response, NK cell activation and antitumor effect of hemocyanins without an added epitope. The contribution of the dissertation is also significant as a source of many new questions and potentially very interesting hypotheses that have yet to be tested.

Overall, the volume and significance of the dissertation work corresponds to and exceeds that required for a dissertation for the award of the scientific degree of Doctor.

Critical notes and questions:

1. It is interesting that the antitumor effect correlates with the induction of IgM antibody response with hemocyanins alone, but not with vaccines. Does the candidate have a hypothesis for the reasons for this difference?
2. How are the results of serum cytokine levels interpreted for hemocyanins and conjugates separately and in comparison?
3. What are the likely target antigens of the cytotoxic cells induced by the conjugates even in animals without tumors?
4. How does the doctoral candidate see the possibility of translating these results into clinical trials in humans?

Conclusion:

Emilia Zaharia Stoyanova's dissertation represents a thorough and innovative study in the field of melanoma immunotherapy. The results obtained have both fundamental and potentially applied significance. The author demonstrates excellent knowledge of the scientific literature, mastery of modern experimental methods and the ability to critically analyze the results.

Based on the above, I strongly recommend awarding Emilia Zaharia Stoyanova the scientific and educational degree of "doctor". I categorically vote FOR the awarding of the degree and wish the doctoral student to continue her studies in the field of tumor immunology.

Assoc. Prof. Dr. Anastas Dimitrov Pashov

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