REVIEW

by

Assoc. Prof. Maya Margaritova Zaharieva, PhD –The "Stephan Angeloff" Institute of Microbiology, Bulgarian Academy of Sciences

Subject: *Dissertation for awarding the educational and scientific degree Doctor Professional field 4.3. "Biological Sciences", scientific specialty "Microbiology"*

Author: *Simeon Emilov Dimitrov*

Form of PhD study: *full-time*

Department: *"Infectious Microbiology" of the "Stephan Angeloff" Institute of Microbiology, Bulgarian Academy of Sciences*

Topic: *"Evaluation of the potential of new derivatives of ethambutol and isoniazid in relation to their antitubercular activity"*

Supervisors: *Assoc. Prof. Violeta Valcheva Ruseva, PhD and Prof. Milka Milcheva Mileva, PhD, "Stephan Angeloff" Institute of Microbiology, Bulgarian Academy of Sciences*

I hereby declare that I have no conflict of interest under Article 4, paragraph 5 of the Development of Academic Staff in the Republic of Bulgaria Act (DASRBA). I have no joint publications on the topic of the dissertation with Dr. Simeon Dimitrov, Master of Veterinary Medicine. The review has been prepared in accordance with the requirements of DASRBA and the Regulations of the "Stephan Angeloff " Institute of Microbiology, BAS, for applying the Development of Academic Staff in the Republic of Bulgaria Act. I have not used artificial intelligence software in preparing this review of the dissertation.

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1. General presentation of the procedure and the PhD student

The submitted set of materials on paper/electronic media complies with the Development of Academic Staff in the Republic of Bulgaria Act and with the regulations of the "Stephan

Angeloff" Institute of Microbiology, BAS, and includes all required documents. The documents are arranged clearly, in the order described in the candidate's application.

The PhD student has attached ***3 publications*** *with SJR and/or impact factor (ICI-IF), which have been cited 5 times in the scientific literature.*

I have no comments or remarks regarding the documents.

2. Short biographical data on the PhD student

PhD student Simeon Emilov Dimitrov was born on March 7, 1991, in Sofia. In 2016, he graduated from the Faculty of Veterinary Medicine at the University of Forestry – Sofia, with a "very good" grade on his state examinations. He was enrolled as a full-time doctoral student in the Department of Infectious Microbiology at SAIM-BAS in 2022. During his doctoral studies, he completed courses in computer science, microbiome analysis, and bioinformatics. He accumulated a credit of total 545 points, more than two and a half times the required minimum.

3. Relevance of the topic and feasibility of the objectives and tasks

The dissertation topic is highly relevant. Tuberculosis remains a socially significant problem with numerous challenges, especially due to the rise of drug resistance and the spread of multidrug-resistant tuberculosis strains. The COVID-19 pandemic has further complicated the situation, as according to WHO it has led to an increase in undiagnosed and untreated cases, which are a major source of infection and carry a higher risk of complications and mortality. The development of new drugs is a difficult and lengthy process, with many candidates failing due to high toxicity, poor bioavailability, or other pharmacological issues. Therefore, pharmacological screening of newly synthesized substances to evaluate their efficacy and mechanisms of action against different Mycobacterium tuberculosis strains is particularly relevant and essential in the fight against tuberculosis.

4. Knowledge of the problem

In the literature review of the dissertation, the problem of tuberculosis, the availability of drugs, vaccines, and preventive measures are examined in great detail. Special attention is given to the development of multidrug resistance to the two most effective antitubercular

drugs, rifampicin and isoniazid, as well as resistance to several antibiotics used in regions of Africa, Asia, and some Eastern European countries. The bibliography includes 346 sources, which demonstrates the tremendous effort made by the PhD student in reviewing the available scientific literature. The candidate has analyzed the published information and provided a critical summary of the current achievements in the field, outlined pressing problems, and scientifically substantiated the aim of the dissertation. The text shows that the candidate has learned how to work with scientific literature and, based on processed information, to formulate scientific hypotheses.

5. Research methodology

The dissertation stands out with a rich methodology, from obtaining and characterizing the derivatives of EMB and INH to testing their pharmacological activity. A consistent and well-argued link between the conducted chemical and pharmacological tests is notable. The chosen methods are modern and logically integrated into the sequential experimental cascade of the research, supporting the substantiation of the results and revealing the potential of the compounds for advancing to the next pharmacological stages of development.

The PhD student carried out a significant volume of experiments to establish the antitubercular activity of the studied compounds. He determined their minimum inhibitory concentration (MIC) using REMA according to a standard protocol described in the scientific literature. In parallel, molecular docking was performed with the software Molecular Operating Environment (MOE, 2022). The interactions of the compounds with four crystallographic protein structures from Mycobacterium tuberculosis were studied: InhA with ligands TCU (PDB: 2X22) and 641 (PDB: 4TZK), GlfT2 (PDB: 4FIY), and oxidoreductase (PDB: 4NXI)

Pharmacological tests also included determining transmembrane permeability by a modern method, evaluating in vitro cytotoxicity on a panel of non-tumorigenic and tumorigenic cell lines. In vivo acute and subacute toxicity was also assessed according to OECD (2001) guidelines and ISO 10993-2/12 standards. Pathomorphological evaluation of organ preparations from test animals provided evidence of a lack of toxicity in the selected compounds.

The PhD student mastered a number of methods for determining antioxidant activity and redox-modulating capacity. These included determining total protein, total glutathione, glutathione peroxidase (GPx) and glutathione reductase (GRd) activity, as well as malondialdehyde as a marker of lipid peroxidation. The activity of superoxide dismutase was also monitored. In vitro antioxidant capacity was assessed using the DPPH and ABTS tests.

Of particular interest is the in vitro analysis of mutagenesis in resistant mutants. Whole-genome sequencing was performed after isolating DNA from them.

Statistical analysis was carried out using several modern software programs, demonstrating that the candidate acquired in-depth knowledge of computational methods applied in the quantitative evaluation of pharmacological data.

6. Characteristics and evaluation of the dissertation

The presented dissertation is written in a high scientific style and spans 154 pages. The texts are richly and successfully illustrated with 40 figures and 20 tables. The bibliography contains 346 references, most from the past 5 years. The work includes a detailed analysis of the scientific literature on the topic. Six tasks were formulated in relation to achieving the stated goal. The materials and methods used are described with detailed protocols. The results and their discussion form the main part of the work. The results are presented accurately and thoroughly. Ten conclusions have been drawn. These conclusions are logical and encompass the entire diversity of the obtained experimental data. Four contributions are identified.

The dissertation of Simeon Dimitrov follows the general structure established in the country, maintaining the balance between its parts: all essential sections are included – introduction, objectives and tasks, materials, methods, results, discussion of results, conclusions, and references.

7. Contributions and significance of the work for science and practice

The contributions in the dissertation of Simeon Dimitrov are scientific in nature. They summarize the original experimental data obtained by the PhD student regarding a highly relevant scientific problem – new derivatives of ethambutol and isoniazid with antitubercular activity, which are potential drug candidates.

For the first time, the candidate investigated the antitubercular activity of these derivatives and proved their activity against MDR-TB. For this purpose, mutant clones of the reference strains M. smegmatis and M. tuberculosis H37Rv resistant to increasing concentrations above the established MIC of the selected compound were obtained for the first time. The two most active compounds were characterized in terms of: 1) their toxicity on non-tumorigenic cell lines, and 2) their acute and subacute toxicity in test animals (rats).

It was also established that aroylhydrazone 3d interacts with NAD+ and Tyr158 and binds to InhA without prior activation by KatG, making it effective against MDR-TB.

8. Assessment of publications related to the dissertation

The scientific publications related to the dissertation amount to 3. An exceptional achievement is the fact that the papers were published in prestigious international peer-reviewed journals with Q1 and Q2 impact ranking and have a total impact factor of 23. The candidate is first author of one of the publications and co-author of the other two. The publications cover a substantial part of the dissertation results. They have been cited a total of 5 times (excluding self-citations) in renowned international journals, which is proof of their high quality and relevance.

The candidate has participated in 6 scientific forums: 2 international, 1 with international participation, and 3 national. He is the principal investigator of an intramural project at MU-Plovdiv entitled "Biopolymer nanosystems for targeted drug delivery in anticancer therapy" and participates in two other projects – one national, funded by the Research Fund, and one international under the Strategic Research and Innovation Program for the Development of MU-Plovdiv (ÏÑÍÈÈÐ-ÌÓÏ).

9. Abstract (Author's dissertation summary)

The abstract fully meets the requirements of the SAIM-BAS regulations. It concisely and clearly presents the achieved results without omissions. It is 49 pages long and contains 30 figures and 18 tables. The summary provides a clear idea of the objectives, tasks, and implementation of the dissertation. It includes discussion of the results, conclusions and contributions, as well as the publications and participation in scientific forums related to the dissertation.

10. Critical remarks and recommendations

I have no critical remarks or recommendations concerning the conducted research and the submitted set of materials. In some places, the expression and terminology in the texts referring to pharmacological studies on cytotoxicity and acute and subacute in vivo toxicity could be improved.

11. Personal impressions

I have known PhD student Simeon Dimitrov since his enrollment. I have attended seminars where he presented his research reports related to the dissertation. During his presentations, I was impressed by the way he presented and summarized the results, as well as the variety of methods used. He presented his results and their significance briefly, clearly, and comprehensively, in logical sequence. The candidate demonstrated commitment to the research, answered questions, and left a positive impression as a motivated young scientist ready to dedicate himself to science.

12. Recommendations for future use of the dissertation contributions and results

It is more than obvious that the obtained results provide a solid foundation for future studies at higher pharmacological levels, aiming to develop a pharmaceutical product with medical application. Most importantly, they significantly enrich the available global scientific literature with data on the activity of new derivatives of ethambutol and isoniazid with higher antitubercular activity and lower toxicity. The results are highly encouraging and promising for further research in this direction. Given their high quality, I recommend continuing the scientific work with testing of other newly synthesized compounds. It would also be interesting to deepen pharmacological testing on a broader range of in vitro cell systems to assess cytotoxicity and effects on cellular metabolism regarding CYP450 enzymes. This would help in progressing to suitable in vivo models for studying pharmacokinetic properties. I would be pleased if in the future Dr. Simeon Dimitrov continues his scientific career, as I consider him already an established young researcher with excellent abilities and talent.

CONCLUSION

The dissertation of Assistant Simeon Dimitrov ***contains numerous scientific results, representing original contributions in the field of antitubercular activity****. The presented scientific research* ***meets all*** *requirements of the Development of Academic Staff in the Republic of Bulgaria Act (DASRBA), the Regulations for its implementation, and the regulations of SAIM-BAS for dissertations. I believe that the dissertation, together with three publications in prestigious international journals with high impact factor and/or SJR, cited 14 times, meets the qualitative and quantitative criteria for awarding the educational and scientific degree Doctor.*

The dissertation demonstrates that PhD student Simeon Dimitrov ***possesses*** *in-depth theoretical knowledge and has acquired valuable professional skills in the scientific specialty "Microbiology," and he* ***demonstrates*** *qualities and abilities for independent scientific research.*

For these reasons, I confidently give my ***positive assessment*** *of the research presented in the dissertation, abstract, results, and contributions, and* ***I propose to the esteemed academic jury to award the educational and scientific degree "Doctor"*** *to Assistant Simeon Dimitrov in the specialty "Microbiology," professional field 4.3. Biological Sciences.*

29.09.2025

Reviewer: .....

(Assoc. Prof. Maya Zaharieva, PhD)