SCIENTIFIC OPINION

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Member of the Scientific Jury, formed by Order No. I-116/04.08.2025 of the Director of The Stefan Angelov Institute of Microbiology, Bulgarian Academy of Sciences

Regarding: Dissertation for awarding the educational and scientific degree "**Doctor**"
Field of higher education: **4. Natural Sciences, Mathematics, and Informatics**
Professional field: **4.3. Biological Sciences**
Scientific specialty: **Microbiology**
Author: Simeon Emilov Dimitrov
Topic: Testing the potential of new derivatives of ethambutol and isoniazid to study their antituberculosis activity
Scientific supervisors: Assoc. Prof. Violeta Valcheva Ruseva, PhD – Institute of Microbiology, BAS, and Prof. Milka Milcheva Mileva, PhD - – Institute of Microbiology, BAS

Relevance of the scientific problem being developed

The topic of the presented dissertation work by Simeon Emilov Dimitrov is related to the conduct of pharmacological screening of newly synthesized derivatives of ethambutol and isoniazid with a view to their toxicity and antimycobacterial activity, as well as to clarify some aspects of the mechanism of their action against *Mycobacterium tuberculosis*.

Despite the developed vaccines and chemotherapeutics, tuberculosis continues to pose a serious threat to public health due to the increasing number of resistant strains. Global efforts to control resistant tuberculosis are linked to the WHO Global Strategy to reduce morbidity and mortality by 2035. However, to control this globally widespread epidemic, there is also an urgent need to develop new anti-tuberculosis agents that can reduce treatment time, specifically affect resistant strains at low therapeutic doses. Most of the studied structures fail for a number of reasons, for example, they show specific or cumulative cytotoxicity towards individual human tissues and cells, form toxic metabolites, have poor bioavailability due to problematic pharmacodynamics and pharmacokinetics. The main requirements for new chemotherapeutics are that they have low toxicity and high antimycobacterial activity, thereby achieving good efficiency and minimal unwanted side effects.

Structure of the dissertation and assessment of results and contributions

The presented dissertation is written on 156 standard A4 pages of text according to the generally accepted scheme, as follows: Introduction (2 pages), Literature review (41 pages), Aim and objectives (1 page), Materials and methods (13 pages), Results and discussion (53 pages), Conclusions (1 page), Contributions (1 page), Publications related to the dissertation, Open citations, Reports to scientific forums of scientific results (2 pages), References (32 pages) with 329 cited literary sources. The recommended ratios between the individual parts of

the work have been observed. The work is richly illustrated with 21 tables and 40 figures summarizing the obtained results.

The literature review shows the in-depth knowledge of doctoral student Simeon Dimitrov on modern research on the topic of the dissertation. Special attention is paid to oxidative stress in the pathogenesis of *M. tuberculosis* infection. The various mechanisms of antibiotic resistance are described. The classification and mechanisms of action of the currently existing anti-tuberculosis drugs are presented.

Aim and objectives – The aim is precisely and clearly defined, and six well-formulated and logically related tasks have been derived to achieve it:

1. To establish *in vitro* antimycobacterial activity of newly synthesized chemical compounds against reference strains *Mycobacterium tuberculosis* H37Rv and *Mycobacterium smegmatis* MC2155;

2. To analyze the energy interactions between protein targets in *M. tuberculosis* and the synthesized compounds and to identify molecules (ligands) with high affinity to these targets by molecular docking;

3. To study the transmembrane permeability of selected compounds;

4. To determine *in vitro* cytotoxicity, *in vivo* acute and subacute toxicity in an experimental mouse model, to monitor pathomorphological changes and markers of oxidative damage in target organs;

5. To study the redox-modulating capacity of the compounds in chemical model systems;

6. To induce *in vitro* mutagenesis and isolate DNA from the resistant mutants, with subsequent whole genome sequencing and bioinformatics analysis.

Materials and methods – The methodology used in the development of the dissertation is well described and allows for reproducibility of the results. Methods at different levels of assessment of the expected result were used. Both *in vitro*, *in vivo*, *in silico* models and pathomorphological assessment of tissue samples were used. Without a doubt, this section confirms that the dissertation represents a serious and in-depth study at a high methodological level.

Results and discussion – The presented results are divided into eight main subsections and are summarized by a short conclusion. The synthesis of selected analogues of ethambutol and isoniazid is presented and their toxicity *in vitro* and *in vivo* is determined. Their antimycobacterial activity *in vitro* is determined. Their transmembrane permeability was determined by molecular docking. A pathomorphological assessment of the changes in target organs after administration of the selected analogues was made. The antioxidant activity and redox-modulating capacity after treatment with the selected compounds were monitored. *In vitro* mutagenesis and isolation of resistant mutants of *M. tuberculosis* H37Rv were performed with subsequent complete genome sequencing.

Conclusions – a page and a half of clearly formulated 10 conclusions reflecting the main results of the study.

Contributions – within half a page, the doctoral student and his supervisors make four contributions:

1. For the first time in Bulgaria, a pharmacological screening of selected derivatives of EMB and INH was conducted. Lead compounds were identified - two aroylhydrazone and two nitrofuranilamide compounds with the highest *in vitro* antimycobacterial activity

2. The interactions of aroylhydrazone 3d with NAD+ and Tyr158 prove that 3d can be an inhibitor that binds directly to InhA, without requiring activation by KatG, making it effective against MDR-TB.

3. The two aroylhydrazone compounds (3a and 3d) and the two nitrofuranilamide compounds (DO190 and DO209) exhibit low cytotoxicity and good intracellular accumulation in normal and tumor cell lines, making them suitable drug candidates for subsequent preclinical studies.

4. For the first time in Bulgaria, mutant clones of the reference strains *M. smegmatis* and *M. tuberculosis* H37Rv were obtained by the method of induced *in vitro* mutagenesis, resistant to increasing concentrations above the established minimum inhibitory concentration of the selected compound. The identified mutation in the Rv3755c gene is associated with mediated drug resistance and drug tolerance.

The set of materials presented for consideration is in accordance with the requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADASRB), the Regulations for the Implementation of the ADASRB and the Regulations of the Bulgarian Academy of Sciences for the Implementation of the ADASRB and meets the criteria of the Regulations on the Conditions and Procedure for Acquiring Scientific Degrees and Holding Academic Positions at the Institute of Microbiology "Stefan Angelov", BAS for Acquiring the Educational and Scientific Degree "Doctor".

The doctoral student has attached 3 publications on the topic of the dissertation and the relevant evidentiary material. His total scientific output is 5 publications in international journals, the h-index of the doctoral student is 4, according to Scopus data. 4 participations with a report and presentation of 3 posters at international conferences have been presented.

Personal participation of the doctoral student

Proof of the personal participation of the doctoral student is one article on the topic of the dissertation, in which Simeon Dimitrov is the first author. I also have a personal impression of Simeon's work over the years. A good impression is made by his ability to work in a team, which is my personal conclusion from my observations and from impressions shared with his supervisors during the years of his doctoral studies. A positive influence on the development of Simeon as a promising young scientist is also the ability to combine the professional experience of the two supervisors, working in different directions, the result of which is the presented comprehensive and methodically rich dissertation work. Throughout the entire period of the dissertation, he showed dedication, initiative and a desire to solve the problems that arose in the course of the work. This gives me reason to accept without doubt the personal contribution of the doctoral student to the research carried out.

Critical remarks, recommendations and questions

The summaries presented so far regarding Simeon Dimitrov's dissertation work are indicative of the fact that we have before us a promising young scientist. Therefore, I will allow myself to make a few critical remarks that he can use in his future works.

1. When including diagrams, tables and figures, one must carefully ensure that each one is numbered and titled, so that it sufficiently presents the information included in it. In the dissertation work, I noticed on page 60 a table that is not numbered and titled.

2. When presenting diagrams, tables and figures, the sequence of their numbering must be carefully followed and a number must not be repeated or skipped. In this regard, I noticed several inaccuracies in the presented dissertation:

a) there are three tables with number 16 (on pages 97, 98 and 110), which changes the total number of tables;
b) on page 104, according to the logic by which the figures are numbered, it should be figure 28, but it is figure 29. Figure 29 is also on page 106 (which is the real figure 29);
c) from figure 21 to the end of the dissertation, there is a repetition of the numbering of the figures. There are two figures 21 (on pages 93 and 111), two figures 22 (pages 93 and 111), two figures 23 (pages 94 and 112) and the same repeated numbering until the last figure. This is obviously a typographical error, which misleadingly also leads to incorrect numbering of the figures in the text.

As a result of these omissions, instead of the stated 30 figures in the dissertation, there are 40 figures presented, and instead of 18 tables, there are actually 21 tables.

Conclusion

The dissertation work presented by Simeon Dimitrov represents an original contribution to science and meets all the requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADASRB) and the Regulations on the Conditions and Procedure for Acquiring Scientific Degrees and Academic Positions of the Stefan Angelov Institute of Microbiology - BAS. The presented materials and dissertation results in terms of relevance, volume of research, achieved scientific contributions and publication activity fully comply with the specific requirements adopted in connection with the Regulations of the BAS and the IMicB-BAS for the application of the ADASRB. Based on the above, I confidently give my positive assessment of the conducted research and propose to the esteemed scientific jury to award Simeon Dimitrov the educational and scientific degree of "doctor" in Professional field 4.3. Biological Sciences, scientific specialty Microbiology.

16.09.2025 Prepared the opinion:

/Assoc. Prof. Dr. Neli Vilhelmova-Ilieva/