

PhD DISSERTATION REVIEW

from Associate Professor Yordanka Ivanova Uzunova, PhD (Bioorganic Chemistry)

Medical University, Plovdiv

on a dissertation submitted under the procedure for awarding the educational and scientific degree of DOCTOR in the field of higher education 4. Natural Sciences; Professional Field 4.2. Chemical Sciences; Doctoral Program in Organic Chemistry

Author of the dissertation: Miglena Zlatkova Milusheva

Title of the dissertation: Synthesis of novel antispasmodic agents with impact on memory functions in experimental animals

Research supervisors: Assoc. Prof. Stoyanka Nikolova Atanasova, Plovdiv University Paisii Hilendarski and Assoc. Prof. Iliyana Dimitrova Stefanova-Kancheva, Medical University, Plovdiv

1. General overview of the procedure and PhD student

This review was prepared following Order No. RD-22-1134/15.05.2025 of the Rector of Plovdiv University Paisii Hilendarski (PU), whereby I was appointed as a member of the Scientific Jury to ensure the procedure for the defense of a dissertation entitled “Synthesis of novel antispasmodic agents with impact on memory functions in experimental animals” for the acquisition of the educational and scientific degree of Doctor of Philosophy in the field of higher education 4. Natural Sciences; professional field 4.2. Chemical Sciences; doctoral program in Organic Chemistry. The author of the dissertation is Miglena Zlatkova Milusheva, a doctoral student in full-time study at the Department of Organic Chemistry with scientific supervisors Assoc. Prof. Dr. Stoyanka Nikolova Atanasova, Department of Organic Chemistry, Plovdiv University Paisii Hilendarski and Assoc. Prof. Dr. Iliyana Dimitrova Stefanova-Kancheva, Department of Medical Physics and Biophysics, Medical University, Plovdiv.

The set of materials submitted by the doctoral student meets the requirements of the Law on Academic Staff Development in the Republic of Bulgaria, the regulations for its implementation and is in accordance with Art. 36 (1) of the Regulations for the development of the academic staff of PU (dated 16.12.2024).

2. Actuality of the subject matter

In recent years, there has been a growing trend in pharmacy towards repurposing or “reviving” well-known drugs for new therapeutic applications and modifying their active substances to enhance their pharmacological effects. In that respect, synthesizing new structures that combine different pharmacophores with a well-known pharmacological effect as potential drug

candidates with antispasmodic activity is a topical theme and the presented research work is aimed at crafting such types of compounds. Apart from their primary antispasmodic (spasmolytic) function, additional effects influencing cognitive functions are also explored and evaluated.

3. Knowledge of the research problem

PhD student Miglena Milusheva demonstrates in-depth knowledge of the topic related to her dissertation work. The dissertation cites 394 sources, 285 of which are in section Literature Review, with a significant part of them published in the last 10 years. The review encompasses summarized data on the etiology and epidemiology of inflammatory bowel diseases and the drugs used for their treatment. The properties of anthranilic acid and its derivatives are described, as well as methods for their synthesis pertinent to the topic of the dissertation. The need for hybrid molecules as potential drugs acting on several targets to treat complex diseases is substantiated in a succinct and clear way. Experimental models for preclinical evaluation of biologically active substances are considered. The Literature Review subsections conclude with an analysis and summary of the literature, which evidences that the doctoral student is capable of searching, systematizing and critically analysing scientific literature.

4. Research methodology

To achieve the aims and objectives of the dissertation, mono- and diamides of anthranilic acid were synthesized after a preliminary *in silico* assessment of the pharmacokinetic and toxic potential of the drug candidates. Three monoamides and 15 diamides of anthranilic acid were selected and synthesized. Each structure was identified and characterized by several analytical methods (thin-layer and preparative chromatography, ^1H - and ^{13}C -NMR-, mass-, IR - spectrometry), as well as by their melting point. To evaluate the biological effects, Wistar rats and smooth muscle preparations derived from these animals were used with the relevant permits from the appropriate authorities. The evaluation of antimicrobial activity was conducted on ten bacterial strains (five of them gram-positive and five gram-negative), two yeast strains and five fungal strains.

PhD student Miglena Milusheva has a thorough grasp on the methods and laboratory techniques used in the field of organic synthesis and has acquired knowledge and skills in the methods applied to assess the biological activity of small molecules.

5. Characterization and evaluation of the dissertation and its contributions

The dissertation is interdisciplinary. It is 170-page long and contains 12 tables, 32 figures and 13 diagrams. The References List has 394 literary sources. The dissertation includes the following sections: Introduction, Literature Review, Aim and Objectives, Own Research, Experimental (materials and methods), Conclusions and Contributions. The emphasis is on the part dedicated to the author's own research. The conclusions and contributions are summarized as follows:

Design of small molecules such as antispasmodics and approaches for their synthesis has been conducted. For the first time, 18 anthranilic acid hybrids were obtained using the specified method with very good yields, of which 16 were novel structures, unpublished in the scientific literature so far. All synthesized compounds were purified, isolated and characterized by their melting points and spectral methods, including FT-IR, ¹H-NMR, ¹³C-NMR, HRMS.

The effect of the introduction of various substituents on the antispasmodic activity is theoretically investigated. The antispasmodic activity of all synthesized compounds was measured, and for five of them (6b and 6d of Group 1, four in Group 2, 8d and 8e in Group 3) it was better than that of the antispasmodic mebeverine. The anti-inflammatory activity of all synthesized compounds was evaluated by two methods *in vitro* and *ex vivo*. All compounds studied showed promising protection against thermal denaturation of albumin, with compounds 3 and 6d demonstrating the strongest activity. *Ex vivo* studies revealed hybrids 6e, 6d and 5 as substances with a significant inhibitory effect on the synthesis of the pro-inflammatory cytokine IL-1 β , while hybrids 6e, 4 and 7a showed the highest activity against nNOS expression.

Three of the compounds with very good antispasmodic activity in influencing memory functions of experimental animals were studied, with one of them (6d) having a significant effect on memory and learning processes.

For compound 4, a very good antimicrobial activity was established against the pathogenic fungi *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Rhizopus* sp., and moderate activity against *Candida albicans* and *Saccharomyces cerevisiae*. The tested gram-positive bacterial strains *Listeria monocytogenes*, *Enterococcus faecalis*, *Micrococcus luteus* and gram-negative bacteria *Salmonella enteritidis*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* exhibited moderate sensitivity towards hybrid 4 and its diamide derivatives 7a–e.

For each group of synthesized compounds, the structure-biological activity relationship is established, which can serve as the basis for future rational design of biologically active compounds.

One of the hybrids was proven to have a certain degree of biological activity, characterizing it as a substance with the functional characteristics of an antispasmodic with anti-inflammatory action enhancing memory functions in experimental animals.

6. Assessment of the publications and personal contribution of the doctoral student

Three scientific reports on the dissertation research are presented, published in specialized and multidisciplinary international journals of the first quartile (Q1) – the International Journal of Molecular Sciences (IF 4.9), Pharmaceuticals (IF 4.3) and Molecules (IF 4.2). The doctoral student is the first author of all three publications. At the time of review, two of them have a total of 22 citations. The total scientometric result of the published articles is 75 points (Q1 – 3x25 points),

exceeding the national minimum requirements for acquiring the educational and scientific degree of Doctor and the requirements of the Regulations for the development of the academic staff of PU (required points 30). In addition, the dissertation results were presented at 18 national and international scientific forums, where Miglena Milusheva was the first author of 11 posters and reports.

7. Author's summary

Written clearly and succinctly, the author's summary reflects the sequence of her research work described in the dissertation and logically comes to the formulation of the main achievements of the doctoral student. Proper scientific language is used, and the conversions discussed and the results obtained are duly illustrated with relevant schemes, figures and tables.

As a critical note to the the author's summary would be the lack of a list of cited literature, which is a mandatory requirement for any independent scientific work.

8. Recommendations for future use of the dissertation contributions and results:

I have the following critical remarks to the doctoral student. I believe that the format of the dissertation will benefit if the reagents used are presented in the chapter Materials and Methods, as well as the concentrations of all solutions. In my view, figure legends will be more conveniently located after the figure captions. In Figure 22, there is a discrepancy between the values for compounds 7e and 8e and those presented in Table 9, which I attribute to a technical error. Although the methodology indicates the number of experimental animals used in the learning and memory tests, it is necessary to give that number in the figures with the results (e.g. Figs. 26,27,28,29). The above remarks do not diminish the significance of the dissertation work.

I have the following questions to Miglena Milusheva:

1. Why is it important that newly synthesized molecules should not be substrates for P-glycoprotein?
2. To what would you attribute the lack of relaxation effect of 8e at a concentration of 10^{-6} mol/L? (Fig. 17)
3. What statistical test was applied to the distribution of the biological test results? What is the statistical method used for analyzing the results of the memory and learning tests?

The results obtained provide a number of opportunities for future development, both from a synthetic and biological point of view. I would recommend further work on the synthesis with new hybrid molecules, not only anthranilic acid derivatives. The role of the derivatives with the greatest antispasmodic effect in the inflammatory process, using other methods for assessing NOS activity or NO concentration and interleukin expression should be further clarified.

CONCLUSION

The dissertation contains scientific, applied sciences and applied results that represent an original contribution to science and meet all the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for the Implementation of that Law and the relevant Regulations of PU. Miglena Milusheva demonstrates solid theoretical knowledge and practical skills in both organic chemistry and in the field of methods for assessing biological activity, which is evidence of her ability to independently plan, conduct and report scientific research. Considering the above, I am confident in my positive assessment of the research, results and contributions presented in the dissertation and author's summary, and I recommend that the esteemed scientific jury award the educational and scientific degree of Doctor to Miglena Milusheva in the field of higher education: 4. Natural Sciences, professional field 4.2. Chemical Sciences, Doctoral Program in Organic Chemistry.

12.06.2025

Review prepared by:

Assoc. Prof. Yordanka Ivanova Uzunova, PhD