To the CHAIRMAN OF THE SCIENTIFIC JURY

Determined by order RD 38-469/21.07.2023

Of the rector of SU "St. Kliment Ohridski"

R E V I E W

A dissertation on the topic:

Design and Application of Functional Nucleic Acids for Synthetic Control of Gene Expression

by Georgi Yordanov Miloshev, candidate for the acquisition of the educational and scientific degree "Doctor" in direction 4.3. Biological Sciences in the scientific specialty Genetics, doctoral program "Bioinformatics" with scientific supervisor Prof. Robert Penchovski

The Review was prepared by: Assoc. Ph.D. Iliana Ivanova, lab. Bionanotechnology at the Department of General and Industrial Microbiology, Faculty of Biology

I declare that I have no joint publications or conflict of interest of any other nature with the applicant in the sense of $ 1, item 2a of the DP of the law for the development of the academic staff in the Republic of Bulgaria as well as lack of circumstances under Art. 33.

The dissertation work submitted to me for review contains 132 pages of text, and the standards for the ratio between individual parts are not observed (49 pages of literature review, 1 page of aim and tasks, 8 pages of materials and methods; 37 pages of results and discussion; 3 p. discussion, 1 p. conclusions, 1 p. contributions) including 4 tables, 61 figures, appendices and a bibliography with 184 titles - all in Latin. In my opinion, the prepared list of abbreviations should have been placed at the beginning after the table of contents, in order to facilitate the reader's familiarity with the various and unfamiliar terms in the work. The PDF format of the PhD thesis also hinder finding the meaning of the numerous abbreviations and to understand the text.

1. **Relevance of the topic:** Antibiotic resistance of pathogens causing significant diseases is continuously increasing worldwide. The European Antimicrobial Resistance Surveillance Network (EARS-Net) collects data from all countries in the European Union (EU) every year on the most dangerous pathogens. The most commonly reported bacterial species were *E. coli* (39.4%), followed by *S. aureus* (22.1%) and *K. pneumoniae* (11.9%). In 2021, more than half of *E. coli* isolates reported to EARS-Net and more than one-third of *K. pneumoniae* isolates were resistant to at least one antimicrobial group under surveillance, and combined resistance to several antimicrobial groups was common. *E. coli* remains an important pathogen in the EU and the world, with cases still increasing by nearly 3% between 2021 and 2022 and often combined resistance. Evidence clearly shows that the social burden of infections caused by some antibiotic-resistant bacteria is now comparable to the total damage caused by socially significant diseases such as tuberculosis, influenza and AIDS (Cassini 2018). On the other hand, mortality associated with antimicrobial resistance (AMR) in the EU alone amounts to over 33,000 cases per year, and losses are measured in billions. The development of new methods and approaches to limit and combat these infections is the subject of the work of scientific teams worldwide and is enshrined in the WHO Global Plan of Action against AMR.

The design of new compounds with antibacterial activity is one of the most important issues of modern biology, biotechnology and medicine. Despite the wide spectrum of antimicrobial agents, the problems of drug resistance and side effects remain unresolved so far. A promising strategy to overcome these problems may be the synthesis of low-molecular-weight antimicrobial compounds attacking basic bacterial biomolecules and bacterial nucleic acids. Creating oligonucleotides that specifically interact with bacterial RNAs, block their functions and thereby inhibit bacterial growth is a modern method of fighting infections. One of the main advantages of this approach is the highly selective suppression of bacterial cells, safely due to the differences between eukaryotic and bacterial enzymes. Oligonucleotide inhibitors targeting prokaryotic biomolecules are not expected to cause off-target effects on eukaryotic cells. The ability to suppress bacterial growth by disrupting mRNA and regulating gene expression is the most recent trend in the search for antimicrobial agents. Given the above, I believe that the work is extremely necessary and provides new and significant data regarding the antimicrobial effect of antisense oligonucleotides and their effects on prokaryotic cells.

2. **Literature review.** The overview is presented on 49 pages and is logically structured into separate parts. A thorough review of the scientific achievements in the field has been carried out, combined with the use of good scientific style. The attached 22 figures are informative and contribute to the clarification of all aspects of the text. The use of modern genetic methods and approaches for the management of gene expression in microorganisms has been traced, and the possibilities for accelerating and stopping transcription processes have been examined in detail. At the end of the review, directions and modern approaches of interest are indicated, which logically points to the purpose of the work. The failure to observe the division of words, as well as the grammatical rules for full and incomplete articles, makes an unpleasant impression, inadmissible for a person striving for the highest scientific and educational degree. It is proper for the scientific supervisor to pay more attention to the correctness of the expressions in the dissertations of his PhD students, which will only contribute to the better perception of these extremely valuable achievements described in the scientific theses of his PhD students.

3. **Purpose and tasks.** The objective and the six tasks are well formulated on one page, reflecting the main aspects and essence of the dissertation work. The tasks are skillfully selected to achieve the set goal.

4. **The materials and methods** (8 pages) are described in detail and correctly, supplemented by 1 table, which shows the high level of competence of the candidate. A very good impression is made by the skillful handling of a significant range of traditional microbiological, biochemical, modern bioinformatics and statistical methods that the PhD student has mastered. All standard microbiological and genetic techniques are precisely described. Five foreign databases and four bioinformatics programs created by Prof. Penchovski were used to analyze and select the most suitable sequences for the anti-sense oligonucleotides and hammerhead ribozymes chosen to attack bacterial cells. Methodical approaches correspond to the set tasks and ensure their realization.

5. **Results and discussion.** Splitting the results and the discussion into two sections I consider a correct decision, given the better follow-up of the logic of the results and the innovative nature of the work. The rich theoretical justification corresponds to the experimental solutions and the achievement of the set goal – creation of a new universal method for controlling gene expression in *Escherichia coli* by using synthetic antisense oligonucleotides that inhibit LacZ expression. Our results present the construction of antisense oligonucleotides for Lac-Z-operon regulation in *Escherichia coli*. The PhD student chose this gene because its function is easily demonstrated with a biochemical color reaction that measures beta-galactosidase activity. This gene was already used nearly 35 years ago as a reporter system for monitoring transcription in operon fusions (Lewandoski, Smith, 1988). In his work, the dissertation uses a 10 kb plasmid with an inserted bacteriophage lambda promoter element (Tomich et al., 1988). The introduction of a restriction site between two enzymes KpnI and BAMH1 was verified

by restriction mapping of the clones generated by the respective endonucleases. Another fragment was then created containing an antisense oligonucleotide that inhibited bacterial growth. The putative secondary structure of the sequence was established with the freely available RNA Fold software. The purpose of cloning is to obtain enzymes to be tested as allosteric ribozymes for the synthetic control of gene expression. The synthetic gene expression control construct was cloned into an *Escherichia coli* expression plasmid with a reporter gene for Lac Z. Beta-galactosidase activity was tested at various time points. A new plasmid pRS414ge was created as a unique tool for genetic control experiments in the field of RNA synthetic biology, with one-step cloning of different allosteric ribozymes. The plasmid was sequenced and its nucleotide sequence elucidated.

Another contribution of the PhD student was the design of an antisense oligonucleotide to shut down gene expression in *Escherichia coli* linked to the cell-penetrating peptide pVEC. This ASO was tested at 9 different concentrations and a concentration-dependent suppression of gene expression was found in the bacterium. The PhD student also used first- and second-generation ASOs inserted into an allosteric hammerhead ribozyme to shut down gene expression in *Escherichia coli*. He tested the strategy to turn off gene expression in a ribozyme in bacteria by 5 different mutations of ACO (mismatch). Testing was done using the freely available software RNAcofold Webserver - http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi. The mutations were shown to prevent gene expression from being turned off, and the exact working sequence of ASO was confirmed. This was also checked with the thermodynamic value of the different sequences as well as with the beta-galactosidase activity. It would also be interesting to follow the cytotoxicity of this antisense oligonucleotide to different eukaryotic cell lines. The remarks made do not reduce the scientific value of the work performed.

I have the following questions to the PhD student:

1. Are there other approaches besides the cell-penetrating protein pVEC to deliver antisense oligonucleotides?

2. What routes of administration of the created antisense nucleotides as drugs you could offer?

3. You are working with a special strain of bacteria, and how the standard ones in the intestines of humans and animals would be affected by such an intervention?

Acquired competence and compliance with the requirements of the educational and scientific degree "Doctor"

The PhD student has acquired new knowledge in a specific scientific field: studying the structure, properties and effect of antisense oligonucleotides in relation to their antimicrobial activity; mechanisms of their effect and their targets in prokaryotic cells. Contributed to two publications and one book chapter, which is enough to achieve the educational and scientific degree of Doctor.

The PhD student has acquired the skills to apply a variety of methodological approaches to solving specific research tasks. He has mastered microbiological, biochemical, bioinformatics and molecular genetic methods. In addition to specialized techniques, he has acquired skills in teaching students, students and prospective students. He also participates in numerous specializations and trainings. His awards as PhD student and teacher of the year speak for his successful realization.

The PhD student demonstrates independence in the development of innovative ideas, independence and taking responsibility for solving scientific and practical problems, successful adaptation to a work environment and assimilation of new knowledge.

CONCLUSION

Based on the arguments mentioned above, I support the relevance of the problems that are the subject of the dissertation work. Regarding the structure, specific tasks for solving added scientific problems, methodological approaches, experimental solutions, realized results and their interpretation, I express the opinion that the dissertation of PhD student Georgi Yordanov Miloshev is a fully completed author's work that meets the criteria of volume, content and creativity.

In connection with this, I recommend that the honorable Scientific Jury, appointed by Order No. RD 38-469/21.07.2023, award Georgi Yordanov Miloshev the educational and scientific degree "Doctor".