SCIENTIFIC OPINION

from **Prof. Maria Bogomilova Angelova, DSc**, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences
on a dissertation presented to a Scientific Jury, formed by order No. RD-38-469/21.07.2023 of the Rector of Sofia University "St. Kliment Ohridski" for obtaining the educational and scientific degree "Doctor" in Professional field: 4.3. Biological Sciences (Microbiology)
Doctoral candidate: **Georgi Yordanov Miloshev**
Dissertation topic: Design and application of functional nucleic acids for synthetic control of gene expression
Scientific supervisors: **Prof. Robert Penchovsky, PhD**

Relevance and significance of the PhD thesis subject

Nowadays, drug resistance has emerged as a global threat to the treatment of infections caused by bacteria, viruses, and mycetes. This phenomenon is not only a serious complication in medical practice but also one of the greatest public health challenges worldwide. The scenario regarding the adverse effects of resistance is much larger than our imagination. Resistance is increasing at an alarming rate and is a problem, especially for immunocompromised patients. Alexander Fleming warned about it as early as 1942. One key solution to the problem is antisense technologies to create antisense oligonucleotides (ASOs) with applications in the treatment and prevention of various diseases by inhibiting the expression of selected genes. This undoubtedly topical area of synthetic biology is the main focus of Georgi Miloshev's thesis.

Knowledge of the subject

The dissertation is constructed in a traditional form with the relevant sections. It includes 131 standard computer pages and is illustrated with 4 tables and 61 figures. The literature review is based on 184 literature sources, 42% of which are from the last 5 years. The review discusses the main aspects of the doctoral thesis. The known data on the place of ribozymes in synthetic biology as synthetic riboswitches in the cell for exogenous control of gene expression are presented in detail. Knowledge of the identification of indicator RNAs for cancer detection, the design of high-speed oligonucleotide-sensitive ribozymes, and their use are also included. The subsections on the nature and importance of ribozymes and their mechanism of action, as well as information on antisense oligonucleotides and externally targeted RNA sequences as agents for synthetic control of gene expression, are very useful to the reader. The review concludes by outlining the prospects for RNA synthetic biology and its relationship to pharmaceutical biotechnology, metabolic engineering, cellular reprogramming, genome editing, and more.

This PhD thesis aims to develop a new universal method to control gene expression in *Escherichia coli* using synthetic antisense oligonucleotides that inhibit LacZ expression. In my opinion, it corresponds to the relevance and importance of the problem and highlights the

innovative character of the study. The aim unites all directions of the experimental work. For its realization, 6 specific, interrelated, and logically following tasks have been formulated, which include all mandatory stages of such a study.

Methodology of the study

The section Materials and Methods demonstrates a very wide range of methods tailored to the specific requirements of the experiment. These are bioinformatic, biochemical, and molecular genetics. As bioinformatic tools, our and foreign databases and software products are used. Worth mentioning are the acquisition of competent cells, PCR, and enzymatic assays, etc. The methods are described in great detail (in some places even with superfluous details) and can be reproduced.

Characterization and evaluation of the dissertation and contributions

The Results section is presented on 36 pages. The first part of the experimental work focuses on the design of antisense oligonucleotides to inhibit gene expression in *E. coli*. In my opinion, the first two passages have no place in this section. Some of the writing should be included in the literature review and some in the discussion. Here, the creation of the 1st DNA fragment, the introduction of the KpnI restriction site, the cloning of the PL promoter sequence, and the creation of another fragment containing an antisense oligonucleotide that inhibits bacterial growth and its secondary structure are presented in great detail, and with the necessary evidence. The next stage continues with the cloning of the synthetic gene expression control construct into a plasmid for expression in *E. coli* with a reporter gene for LacZ. The results demonstrate that the generated reporter gene construct using plasmid pRS414 works efficiently and gives the expected results in terms of beta-galactosidase enzyme activity. By combining first and second-generation antisense oligonucleotides (ASOs), a strategy to control gene expression with an OFF switch in *E. coli* was created, which was experimentally verified at 9 increasing concentrations. It was found that an increase in the concentration of ASO resulted in a decrease in the expression rate of LacZ. The use of allosteric hammerhead ribozyme showed different behavior of the system in terms of gene expression depending on its interaction with ASO. The newly designed system by ASO to control the OFF switch of gene expression using synthetic hammerhead ribozyme showed high efficiency in beta-galactosidase assays.

Section Discussion departs from the conventional sense, lacking discussion of the data against the background of what has been published in the literature to date. This is rather a summary of the results obtained by the author, which is also very useful but cannot replace the discussion. This summary enables the reader to see the overall picture of all the experimental work and to perceive more clearly what has been achieved.

I accept the formulated conclusions and contributions. I would like to emphasize that the dissertation work of Georgi Miloshev has a pronounced theoretical character with a strong applied aspect.

Critical remarks, recommendations, and questions

In addition to the comments noted above in the text, I have the following recommendations:

1. The 3 scientific publications presented in the dissertation should not be included in the literature review. By presumption, such articles present a part of the obtained results. These are proprietary data and should find a place in the section “Results”.

2. Articles included in the thesis should not be part of the reference list.

3. The review needs to present data on the control of gene expression in *E. coli* through the use of synthetic antisense oligonucleotides that inhibit LacZ expression. It is necessary to note what has been done so far and what is new that is targeted by this work.

4. I recommend that the passages describing the experiments and calculation formulas be included in the section Materials and Methods rather than in the Results section.

5. I recommend improving writing style, observing verb tense, and avoiding 1st person singular sentences. In some places, the translation from English to Bulgarian does not reflect the real meaning of the phrase (I am referring to the author's published articles submitted to the thesis).

As can be seen from the above, the remarks and recommendations are not substantive. They are of a technical character and do not diminish the scientific value of the dissertation presented. I note them to be useful for the future scientific work of the PhD student.

I have the following questions for Georgi Miloshev:

1. Why are bacterial riboswitches considered new and very promising targets for antibacterial drug discovery?

2. Inhibition of LacZ expression in E. coli has also been the subject of previous bioinformatics studies. What is new in the presented thesis work?

3. Can you specify where, for example, the lacZ reporter system newly developed in the thesis will find application for the control of gene expression in synthetic designer ribozymes and antisense oligonucleotides?

Conclusion

In conclusion, I would like to emphasize that the PhD student has fulfilled the requirements ZRASRB and the additional criteria of the SU “St. Kliment Ohridski” for obtaining the educational and scientific degree "Doctor". The material presented by Georgi Miloshev is dissertable, the topic is relevant and offers a contemporary level of an important issue for theory and practice. The conducted experiments are methodologically correct, the obtained results are reliable, and are a solid basis for further scientific and applied studies. The problem posed is multifaceted and studied in detail at a modern level, significant theoretical and applied contributions are made. In my opinion, Georgi Miloshev comes out of the PhD as a well-trained specialist in the field of genetics and bioinformatics, has mastered modern methods, and has gained experience in interpreting scientific data.

Based on the analysis made and the proven growth of the PhD student, I propose to the distinguished members of the scientific jury, formed by order № RD-38-469/21.07.2023 of the Rector of Sofia University "St. Kliment Ohridski, to award **Georgi Yordanov Miloshev** the educational and science degree “**DOCTOR”** in the scientific field 4.3 Biological Sciences (Genetics-Bioinformatics).

25. 09. 2023

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/Prof. M. Angelova, DSc/