To Prof. Penka Petrova DSc -
Chairman of the Scientific Jury,
determined by Order № I-46/28.03.2025
of the Director of the Institute of Microbiology
„Stefan Angelov" – Bulgarian Academy of Sciences
„Acad. Georgi Bonchev" str, bl. 26, Sofia 1113

Attached I submit: A review
of dissertation work of Assoc. Prof. IVANKA NIKOLOVA NIKOLOVA, PhD, on the topic "*In vitro* and *in vivo* study of the antiviral activity of a series of new diaryl ethers and their analogues - promising chemotherapeutics in anti-enterovirus therapy"
for awarding the scientific degree "Doctor of Science"
in professional direction 4.3 Biological sciences (Virology),

Reviewer: Prof. Neli Stoyanova Korsun, MD, DSc
Specialty: Virology
Institution: National Centre for Infectious and Parasitic Diseases, Sofia

Address and contacts: 44A Stoletov Blvd.
1233 Sofia
Email: neli_korsun@abv.bg
Phone: (02) 9318132

The review was compiled in accordance with the requirements of the Law for the development of the academic staff in the Republic of Bulgaria (LDASRB) and Section III of the Rules of LDASRB - Conditions and procedure for awarding the scientific degree "Doctor of Science"

I have no joint publications or participation in scientific forums and projects with Assoc. Prof. Ivanka Nikolova.

Materials received

I received all materials related to the dissertation by e-mail: CV; copy of the PhD diploma; PDF copies of the dissertation, abstract and publications; list of publications included in the dissertation; list of citations of publications; participation in conferences on the topic of the dissertation; certificate of compliance with the minimum national requirements according to the LDASRB.

Career development

Assoc. Prof. Ivanka Nikolova graduated from the Russian Language School in Burgas in 1990. She completed her higher education with a master's degree in 1995 at Sofia University "St. Kliment Ohridski", specialty biology and chemistry. From 1995 to 1998, she worked as a biologist-specialist at the Institute of Microbiology (IMicB) "Stefan Angelov" at the Bulgarian Academy of Sciences. From 1998 to 2001, she was a full-time doctoral student at the same institute under the supervision of Prof. Dr. A. S. Galabov, MD. She was named the best young scientist in the field of microbiology in Bulgaria for 2003. In 2004, she defended her dissertation titled "Resistant and dependent mutants of Coxsackie B1 virus to the picornavirus inhibitor deoxaryl" and earned a PhD in virology, classified under code 4.3 "Biological Sciences." In the same year, she was appointed as a third-grade research associate in the Department of Virology at the IMicB. In 2008, she was promoted to chief assistant. From 2012 to the present, she has been the head of the laboratory "Experimental chemotherapy of enterovirus infections", and since 2016, she has been the head of the Department of Virology. In 2020, she was appointed to the academic position of Associate Professor at the IMicB. She is a member of the Union of Scientists in Bulgaria. From 2015 to 2022, she was the secretary of the "Acad. Stefan Angelov" Foundation, and from 2024 to the present, she is the Chairman of the Scientific Council of the IMicB.

Relevance of the topic of the dissertation

Enteroviruses are one of the most common viral pathogens in humans, causing significant morbidity with a wide range of clinical manifestations, including several severe, even fatal diseases of the central nervous system, heart, pancreas, liver, etc. Currently, poliomyelitis is the only infection successfully controlled through effective vaccines, allowing for the possibility of its eradication. Regarding non-polio enteroviruses, which are about 280 serotypes, there are no preparations for specific prophylaxis and antiviral treatment. Only 3 vaccines against enterovirus A71 are available, but they are licensed only in China. The high incidence of enterovirus infections is associated with severe clinical manifestations, including meningitis, encephalitis, myocarditis, and type 1 diabetes. The absence of broadly protective vaccines and effective antiviral treatments, along with the rapid emergence of resistance among these viruses to existing antiviral drugs and the unacceptable toxicity of some options, underscores the ongoing need for research focused on discovering new antiviral agents that offer both a strong therapeutic effect and good tolerability.

General characteristics of the dissertation work

The dissertation work is structured according to the requirements of the Regulations for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB). It has a volume of 189 pages. It contains: title page; notes and thanks (1 page); table of contents (1 page); abbreviations used (2 pages); introduction (2 pages); literature review (48 pages); goal and objectives (2 pages), materials and methods (11 pages); results and discussion (76 pages); conclusion (9 pages); contributions (1 page); literature (35 pages, including 351 literary sources); list of scientific publications related to the topic of the dissertation (3 pages). The ratio between the individual sections of the dissertation work meets the accepted requirements. The dissertation includes a total of 31 tables and 46 figures to illustrate its findings. The chemical structures of all analyzed chemical compounds are presented.

Review and evaluation of the dissertation work

In the ***Introduction*** to dissertation work, Assoc. Prof. Iv. Nikolova emphasizes the necessity of developing effective chemotherapeutics for treating enterovirus infections, given the lack of specific prophylaxis for the majority of the 280 existing serotypes of enteroviruses. She considers two main strategies in the development of such chemotherapeutics. The first strategy involves drugs that directly target viral proteins, while the second strategy focuses on possible host factors that play an important role in the replication of enteroviruses. The issues with these two approaches are examined, and potential solutions are suggested.

The ***literature review*** is detailed, well-structured, and contains up-to-date information on the structure of enteroviruses, their replicative cycle, and the role of viral proteins and host factors as possible targets for antiviral drugs. At the beginning of the literature review, the latest classification of the *Picornaviridae* family and the position of enteroviruses within it are presented. Significant attention is paid to the widespread spread of these infections in recent years, the diverse clinical syndromes caused by enteroviruses, and the specific serotypes responsible for their occurrence. Up-to-date information is presented on the global spread of polioviruses and the threat of the emergence of vaccine-derived polioviruses capable of causing paralysis indistinguishable from that caused by wild polioviruses. The author of the dissertation presents extensive data on the widespread distribution of enteroviruses within the human population. She discusses key factors contributing to this prevalence, including the high resistance of enteroviruses in the environment, their easy transmission through fecal-oral or respiratory routes, and the significant percentage of asymptomatic infections. Given the frequent occurrence of asymptomatic infections, she emphasizes the necessity of using specific anti-enterovirus chemotherapeutic agents during the latent phase of the disease, such as for emergency post-exposure prophylaxis.
As a competent virologist, the dissertation author presents detailed and in-depth information on the architecture of the enterovirus capsid, the organization of the genome, the successive stages in the replicative cycle of enteroviruses, and the genetic changes that determine the constant evolution of these viruses. She focuses on the viral structures and proteins, particularly the specific stages of enterovirus replication that could be targeted for chemotherapy. This includes the

canyons surrounding each vertex of the icosahedron and the pocket factor within it, the IRES-dependent mechanism for initiating translation, and the viral and cellular enzymes that are involved in cleaving the precursor polyprotein and other stages of viral replication. A significant place at the end of the review is occupied by the section examining the various inhibitors of enterovirus replication and the mechanisms of their action. The antiviral preparations with broad-spectrum activity to which enteroviruses do not (easily) develop resistance are necessary. The advantages and disadvantages of using the two main groups of antiviral drugs are presented: chemotherapeutics directed against host factors and direct-acting antivirals. All compounds that have shown *in vitro* activity against enteroviruses are categorized by groups, along with chemotherapeutic agents that have advanced to clinical trials. At the end of the review, the dissertation author describes in detail the stages and approaches in the activities of her scientific team, aimed at studying various chemotherapeutics with anti-enterovirus activity.
The review is competently written and testifies to the excellent literary awareness of the dissertation author, her ability to systematize, summarize, and interpret the existing scientific information on the problem.

Assoc. Prof. Iv. Nikolova's research begins with Chapter 2. ***Aim and objectives***. The goal of this dissertation is to conduct both *in vitro* and *in vivo* studies on the effectiveness of the cellular phosphatidylinositol-4-kinase beta (PI4KB) inhibitor MDL-860 and its newly synthesized analogues against enteroviruses. This research aims to identify more effective and safer chemotherapeutic agents. Five tasks, including the main directions in the research process, are precisely formulated.

The ***materials and methods*** used to achieve the goal and objectives of the study are described in detail and clearly. As reference inhibitors of enterovirus replication, Assoc. Prof. Iv. Nikolova uses 4 compounds with known antiviral effect: pleconaril, MDL-860, oxoglaucine, and guanidine hydrochloride, with which she compares the action of 114 newly synthesized preparations. To achieve the established goals, the doctoral candidate utilizes virological methods that involve cell culture and conducts *in vivo* experiments. These experiments include infecting newborn mice with enterovirus, which serves as an effective laboratory model for studying enterovirus infections.

At the beginning of the "***Results***" section, the dissertation author presents evidence for the effectiveness of a new approach for combined administration of enterovirus inhibitors: sequential alternative administration of antiviral compounds (SAA course), which prevents the development of drug resistance, reduces the toxic effect, and provides significant antiviral activity. She investigated the effect of a combination of 3 compounds with different mechanisms of action: pleconaril/guanidine-HCl/oxoglaucin (PGO) in a SAA course against experimental neuroinfection with coxsackievirus B1 in newborn mice. She established a significant protective effect with sequential administration of the drugs and the lack of effect with their simultaneous administration. The protective effect is manifested by a reduction in % mortality and a significant decrease in the content of infectious virus in the brain of treated mice. The susceptibility to pleconaril of the virus

isolated from the brains of infected animals is preserved, and to oxoglaucin it even increases. Simultaneous daily administration of the PGO combination shows a rapid emergence of resistance to pleconaril and reduced susceptibility to oxoglaucin. In subsequent experiments, guanidine-HCl is replaced by MDL-860 in the triple combination administered by the SAA regimen. In this triple PMO combination, the median survival time is increased compared to MDL-860 monotherapy; no combined toxicity is observed, and a reduced amount of infectious virus is found in the brains of treated animals. The enteroviruses isolated from mouse brains retain their full susceptibility to pleconaril and MDL-860, despite the significant inhibitory effects observed *in vivo*. However, administering the three partner substances in the PMO combination simultaneously, as well as using pleconaril or MDL-860 in monotherapy, leads to a reduction in susceptibility or even resistance to these drugs. Increased susceptibility of the enterovirus to the third partner in the PMO combination, oxoglaucine, is also observed; however, this preparation does not exhibit a separate protective effect *in vivo*. The doctoral candidate identified the most effective doses in the triple combination P25M75O25, which achieve a better effect compared to the combination P25G45O25, used against the same infection in the previous study.

Assoc. Prof. Iv. Nikolova sets herself the task of clarifying the mechanism of suppression of the development of drug resistance and the emergence of the phenomenon of increased drug susceptibility in viral isolates from the brains of mice when applying the triple combination PMO through the SAA scheme. Sequencing is performed using the NGS method on viral samples from the brains of mice treated with individual antivirals and with the triple combination PMO. Single amino acid substitutions are identified in the viruses from each group of treated mice compared to the placebo group in the non-structural proteins 2A, 2C, and in the structural proteins VP3 and VP1. It is unclear how many viral samples are sequenced from the placebo group and the groups receiving antiviral treatment, as indicated in Table 12. It would be beneficial to analyze a larger number of viral samples from these groups of mice. This will help determine whether the amino acid substitutions observed in each group are conservative (repetitive) or unique to the individual samples studied.

The encouraging results obtained in the studies of the diaryl ether MDL-860 directed the dissertation author to the study of newly synthesized analogs of MDL-860 on the replication of enteroviruses - coxsackievirus B1 (CVB1), coxsackievirus B3 (CVB3), and poliovirus 1 Sabin (PV1). Twelve compounds (1a-12a) are tested, following an established research algorithm: checking for cytotoxicity of the substances, testing for antiviral effect in cell culture, followed by experimental CVB1 neuro infection in newborn mice with the compounds that showed the highest activity *in vitro* (1a, 11a, and 12a). Compound 1a and, to a lesser extent, 11a show a good protective effect and are characterized as promising anti-CVB1 agents.

Assoc. Prof. Iv. Nikolova studies *in vitro* 60 synthesized derivatives of MDL-860 (13c-72c), possessing an unchanged 2-cyano-5-nitro substituted benzene ring as a common fragment for activity against PV1, CVB1, and CVB3. MDL-860 is included in the study as a reference substance. Compound 39c (similar to MDL-860) has the broadest spectrum of activity; the other 7 compounds exhibit activity, but not against all tested enteroviruses. The doctoral candidate

establishes specific relationships between small changes in the structure of the substances and their antiviral activity. The compounds that show the highest activity *in vitro* (39c, 41c, and 47c) against CVB1 are tested for *in vivo* activity in newborn mice experimentally infected with this virus. Moderate protective effects of 39c and 41c are found, which demonstrate the lack of correlation between *in vitro* and *in vivo* activity. A QSAR analysis is performed to evaluate the influence of different substituents in 5-nitrobenzonitrile on *in vitro* cytotoxicity and antiviral activity against PV1, CVB1, and CVB3. The doctoral candidate proposes this approach, reflecting the structural/physicochemical characteristics of the studied compounds, for future molecular design of new antiviral agents.

During the COVID-19 pandemic, Assoc. Prof. Iv. Nikolova expands the scope of her research on diaryl ethers by studying their effects on coronaviruses. The reason for this is the presence of a common target of action of these compounds - the cellular phosphatidylinositol 4-kinase III beta (PI4KB), which generates replicative organelles or replicative membranes necessary for the replication of both enteroviruses and coronaviruses. Based on the data from QSAR analysis, 42 new compounds (73c-114c), analogs of MDL-860, containing two aromatic fragments linked to heteroatom X, are synthesized. To study a broader antiviral activity, the screening includes the enteroviruses PV1, CVB1, and CVB3, as well as the human coronavirus OS43 and adenovirus type 5. Alongside the lack of activity against PV1 and relatively weak activity against CVB1 and CVB3 in all preparations, several compounds show promising results against coronavirus OS43 (74c, 102c, and 113) and adenovirus type 5 (75c). Unfortunately, the compounds show strong selectivity for the individual tested viruses and lack broad-spectrum activity.

To achieve a stronger antiviral effect against enteroviruses, Assoc. Prof. Iv. Nikolova studies dual combinations of the most active against coxsackievirus B1 diaryl ethers – 39b, 41b, 1a, 12a, and 11a, with known inhibitors of enterovirus replication, which have a different mechanism of action – pleconaril, oxoglaucine, and guanidine hydrochloride. A strong synergistic effect on the replication of coxsackievirus B1 is shown by the combinations: 1a+pleconaril, 1a+oxoglaucine; 12a+oxoglaucine; 39b+pleconaril and 41b+pleconaril. A moderate synergistic effect is demonstrated by the pairs 11a+pleconaril; 11a+oxoglaucine; 39b+oxoglaucine and 41b+oxoglaucine.

In the context of the COVID-19 pandemic, Assoc. Prof. Iv. Nikolova is studying the effect of newly synthesized compounds - quinoline and THIQ derivatives on the replication of human coronaviruses OC43 and 229E. Although these studies are not directly related to the topic of the dissertation, they demonstrate the successful approach of the dissertation author in the search for new substances with antiviral activity, based on in-depth QSAR analysis of the structural/physicochemical characteristics of the compounds, and directed synthesis of compounds with a stronger effect.

At the end of the dissertation, no conclusions are presented in the traditional format. The results of the conducted studies are summarized and analyzed in detail in the "***Conclusion***" section. The discussion covers the lack of effective antiviral treatments against enteroviruses and highlights the potential development of compounds that target host cell factors. A total of 114 newly synthesized

compounds with diverse structures, analogues of the reference diaryl ether MDL-860, whose target is cellular PI4KB, are analyzed. A favorable characteristic of 80% of these compounds is their low cytotoxicity. The *in vitro* and *in vivo* experiments conducted show encouraging results for several compounds related to enterovirus replication. Combinations of two substances with different mechanisms of action, which demonstrate a pronounced synergistic effect, are particularly promising. Assoc. Prof. Iv. Nikolova proposes that by optimizing the structure of diaryl ethers, it may be possible to develop highly effective and safe antiviral drugs that could serve as candidates for further preclinical and clinical studies.

I accept the ***contributions*** presented by the dissertation author. They present experimentally validated methods in scientific research and highlight promising findings from the discovery of 10 compounds that show high *in vitro* activity against three clinically significant enteroviruses: poliovirus type 1, coxsackievirus B1, and coxsackievirus B3. Additionally, six of these compounds have demonstrated effective *in vivo* activity against neuroinfection caused by coxsackievirus B1. The proven antiviral activity of some of the studied 114 compounds against human coronavirus OS43 and adenovirus type 5 expands the potential of diaryl ethers as promising preparations concerning other viruses.

The ***bibliography*** includes 351 articles, all in English. Of these, 130 (37%) articles are published in the last 10 years (2014-2024), which indicates that the work is based on modern knowledge in the field studied.

Evaluation of the abstract

The abstract is 94 pages long. Its content presents the main results of the dissertation systematically.

Publication activity

In connection with the topic of the dissertation, 26 full-text scientific articles have been published in publications indexed in Scopus and Web of Science, reflecting the results of research conducted during the period from 2014 to 2023. Of these, 12 articles are published in journals with Q1, 8 - in journals with Q2, 3 - in journals with Q3, and 3 - in journals with Q4. In total, the dissertation author collects 429 points from the publications (indicator group D). In 3 (11.5%) of the listed publications, Assoc. Prof. Iv. Nikolova is the first author; in 5 (19.2%), she is the second author; in 18 (69.2%) she is the third or subsequent author, which indicates her leading role in a significant part of the scientific research. The publications presented in connection with the dissertation work do not repeat the publications presented for the acquisition of the educational and scientific degree "Doctor".

Assoc. Prof. Iv. Nikolova has presented a list of 103 citations, of which 7 citations are given twice to the same article (№16 = №17; №18 = №19, №53 = №56, №63 = №64, №78 = №80, №92 = №94, and №99 = №103). An article with authors Abeywickrema M et al. (2023) is presented as citations №6 and №12 to articles №1 and №2, respectively, from the list of publications, but it

cites only article №2. In the references to the article with authors Roba M.S. Attar et al. (citation no. 61), there is no article with authors Staneva D et al. (2020), as indicated in the list of citations. A total of 94 citations are in publications referenced and indexed in world-renowned databases of scientific information (Scopus and Web of Science). In indicator group D, the doctoral candidate collects 188 points. Assoc. Prof. Iv. Nikolova has H index 7 in Scopus. Citations in global databases show the significance of the scientific topics developed by the dissertation author and the recognition of the international academic community. Assoc. Prof. Iv. Nikolova has participated in 20 scientific forums related to her dissertation topic, including 15 events held abroad. The results included in the dissertation work are obtained during the implementation of 4 research projects funded by the Scientific Research Fund: B02-11 (2014-2019); KP-06-CHINA/31 (2020-2022), KP-06-H49/2, and KP-06-H31/7 (2019, current project).

Compliance of the applicant with the minimum national requirements outlined in the Regulations for the implementation of the LDASRB - Section III. Conditions and procedures for awarding the scientific degree of "Doctor of Science". (amended and supplemented, SG No. 15 of 19.02.2019; Field 4. Natural sciences, mathematics, and informatics; Professional direction 4.3. Biological sciences) and in the **Regulations regarding the conditions and procedures for obtaining scientific degrees and for holding academic positions at the "Stefan Angelov" Institute of Microbiology at the BAS.**

A group of metrics	**Indicators**	**Number of points**		**Number of points based on the evidence presented**
		Regulations for the implementation of the LDASRB	Regulations of the IMicB - BAS	
А	1. Dissertation work for awarding the educational and scientific degree "Doctor"	50	50	**50**
Б	2. Dissertation work for awarding the scientific degree "Doctor of Science"	100	100	**100**
Г	7. Scientific publication in journals that are refereed and indexed in world-renowned databases of scientific information (Web of Science and Scopus), outside of a habilitation thesis	25 for publ. in Q1 20 for publ. in Q2 15 for publ. in Q3 12 for publ. in Q4 10 for publ. in editions with SJR without IF	Q1 – 25 points Q2 – 20 points Q3 – 15 points Q4 – 12 points edition with SJR without IF -10 points	**200** (8 x 25) **160** (8 x 20) **45** (3 x 15) **24** (2 x 12) **Total 429**
Д	11. Citations in scientific publications, monographs, collective volumes, and patents, referenced and indexed in world-renowned databases of scientific information (Web of Science and Scopus)	2	2	**188** (94 x 2) **Total 188**
	Total points			**767**

Table 2. Minimum required points by groups of indicators for awarding the scientific degree "Doctor of Science" and the number of points based on the presented evidence

A group of metrics	**Content**	**Minimum points required**		**Number of points based on the evidence presented**
		Regulations for the implementation of the LDASRB	Regulations of the Institute of Microbiology-BAS	
А	Indicator 1	50	50	50
Б	Indicator 2	100	100	100
Г	Sum of points in indicators 5 to 10	100	100	429
Д	Sum of points in indicator 11	100	100	188
	Total points	350	350	**767**

From the above tables, it is clear that the evidence presented by the Assoc. Prof. Ivanka Nikolova under the individual groups of requirements from the LDASRB cover and exceed the minimum national requirements for acquiring the scientific degree "Doctor of Sciences", specified in the Regulations for the Implementation of the LDASRB (amended and supplemented, State Gazette, issue 15 of 19.02.2019): group А (dissertation work - 50 points), group Б (dissertation work - 100 points), group Г (required 100 points) and group Д (required 100 points). Assoc. Prof. Iv. Nikolova collects a total of 767 points and exceeds twice the required number of 350 points.

CONCLUSION

The dissertation work of Assoc. Prof. Ivanka Nikolova is up-to-date, excellently executed and formatted, and written in very good scientific language. It demonstrates the author's in-depth theoretical knowledge and her high professionalism. Labor-intensive *in vitro* and *in vivo* scientific studies are conducted, requiring great precision and experience. The results obtained represent a significant and original contribution to the efforts to develop clinically effective chemotherapeutics for enterovirus diseases and correspond to modern achievements in this field. The dissertation work meets the mandatory and specific conditions and criteria in the LDASRB (amended and supplemented by State Gazette No. 15 of February 19, 2019), the Regulations for its implementation, the Regulations of the Bulgarian Academy of Sciences, and the Regulations of the IMicB. I give a positive assessment and propose to the esteemed scientific jury to award Assoc. Prof. Ivanka Nikolova, the scientific degree "Doctor of Sciences" in the field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.3. Biological Sciences.

12.05.2025 Member of the scientific jury: ……

/Prof. Neli Korsun, MD, DSc/