R E V I E W

of the doctoral dissertation of Assoc. Prof. Ivanka Nikolova Nikolova, PhD
For the award of the scientific degree "Doctor of Sciences"
Field of Higher Education: 4. "Natural Sciences, Mathematics and Informatics",
Professional Field: 4.3 "Biological Sciences", Virology

Title: *"In vitro and in vivo study of the antiviral activity of a series of novel diaryl ethers and their analogs – promising chemotherapeutics in anti-enteroviral therapy"*

Reviewer: Prof. DSc Penka PetrovaDepartment of General Microbiology,
Laboratory of Microbial Genetics, Stefan Angelov Institute of Microbiology – Bulgarian Academy of Sciences (BAS),
Member of the Scientific Jury appointed by Order № I-46/28.03.2025

1. Biographical Data of the Candidate

Assoc. Prof. Dr. Ivanka Nikolova was born in Burgas. She graduated from the Russian Language High School and obtained her higher education at the Faculty of Biology, Sofia University "St. Kliment Ohridski", where she earned a Master's degree in Biology and Chemistry. Her scientific career began in 1995 as a specialist biologist at the Stefan Angelov Institute of Microbiology at BAS. She later became a full-time PhD student, with a dissertation focused on Coxsackievirus B1 – a virus that remained a central research focus for many years. Assoc. Prof. Nikolova has advanced through all levels of the academic career, from research associate to senior assistant and associate professor. In 2016, she was appointed Head of the Department of Virology, and since 2012, she has led the Laboratory of Experimental Chemotherapy of Enteroviral Infections. Under her leadership, numerous national and international projects have been developed, focusing on the synthesis and evaluation of novel antiviral compounds in both in vitro and in vivo settings. Today, Dr. Nikolova is not only a leading virologist but also Chair of the Scientific Council at the Institute of Microbiology.

2. Relevance of the Dissertation Topic

The relevance of the dissertation topic stems from the global prevalence of enteroviruses, the limited preventive options, the lack of approved antiviral drugs, and the risk of drug resistance development. Enteroviruses are among the most common human pathogens, affecting all age groups, particularly children and immunocompromised individuals. They cause a broad spectrum of illnesses—from the common cold to severe, life-threatening conditions such as meningitis, encephalitis, myocarditis, and acute paralysis. The scale and diversity of enteroviral infections underscore the urgent need for effective therapies. Although vaccines exist for polioviruses and Enterovirus A71, there are no vaccines for the majority of the over 280 known serotypes, making prevention difficult. In this context, antiviral drug treatment becomes the only viable option for many clinical cases.

Currently, no drugs are registered for the treatment of enteroviral infections. Most compounds investigated exhibit antiviral activity only in vitro, with limited in vivo efficacy, often accompanied by high toxicity and low selectivity. This creates a critical need for discovering new, effective, and safe molecules. The high mutation rate of enteroviruses facilitates the rapid development of drug resistance.

The dissertation focuses on combination therapies and compounds targeting both viral proteins and host cellular factors—an innovative and strategic approach to overcoming this issue. The studied diaryl ethers and their analogues exhibit not only anti-enteroviral activity but also effectiveness against other viruses such as coronaviruses and adenoviruses. The potential to develop broad-spectrum antivirals is of paramount importance in the face of future pandemic threats. Assoc. Prof. Nikolova combines fundamental scientific aspects (viral replication, virus-cell interactions) with an applied focus—synthesis, testing, and optimization of antiviral agents. Therefore, the dissertation is not only scientifically relevant but also holds high potential for practical application.

3. Dissertation Data and Sectional Analysis

The dissertation consists of 189 pages and includes the following sections (approximate page count): Introduction (2 pages), Literature Review (43 pages), Objectives and Tasks (2 pages), Materials and Methods (16 pages), Results and Discussion (76 pages), Conclusion (9 pages), Contributions (1 page), and References (38 pages). A total of 351 sources are cited, nearly 40% of which are from the last ten years and about 20% from the last five years (2019–2024). The material is illustrated with 31 tables, 46 figures, and numerous unnumbered schemes.

The literature review presents a thorough and multilayered scientific analysis that lays the groundwork for the experimental studies. It is organized into several logically connected sections systematically presenting current knowledge on enteroviruses and antiviral chemotherapy. The first part addresses the prevalence and clinical significance of enteroviruses, including the taxonomic classification of the genus *Enterovirus* within the family *Picornaviridae*, which includes over 280 serotypes—only four of which have licensed vaccines. The genus is associated with diseases such as meningitis, encephalitis, myocarditis, sepsis, and acute flaccid paralysis. Particular attention is given to serotypes such as EV-A71, EV-D68, Coxsackieviruses B1–B6, echoviruses, and the circulation of vaccine-derived and wild polioviruses, including contemporary outbreak data from Europe, the USA, and Asia (up to 2023–2024). The effects of infections in neonates and immunocompromised patients are also analyzed.

Another section discusses the molecular structure of enteroviruses, the architecture of the viral capsid, and the organization of the positive-sense single-stranded RNA genome. Detailed descriptions are provided for the capsid structure and the hydrophobic pocket, a target for some antiviral agents. Topics such as the pocket factor, IRES elements (and their different types), and the role of VPg in initiating RNA replication are covered. The proteolytic processing of the polyprotein into active enzymes and structural proteins is described, with illustrated stages of the enterovirus replication cycle

and virion release. The role of viral and host factors, particularly membrane rearrangements and host factors in the replication complexes, is emphasized.

The most substantial part of the review addresses therapeutic strategies and challenges, including an analysis of existing and experimental antivirals such as Pleconaril, MDL-860, disoxaril, and new chemical classes. Issues of resistance, toxicity, poor bioavailability, and pharmacokinetics are discussed. The author emphasizes the importance of combination therapies targeting different viral proteins, as well as innovative strategies for targeting host proteins, which may offer broad-spectrum efficacy and reduce resistance risk. The review is not merely a summary of facts but builds a logical and conceptual framework for the candidate's own research. It clearly defines the scientific problem, the limitations of current therapies, and the need for novel approaches—focused on combination treatments and molecular design of innovative compounds. This elevates the study's value and positions it as a significant contribution to antiviral science. The review is based on key publications from leading journals and documents from international health institutions such as WHO, indicating solid knowledge of the most current developments in the field.

The aim of the dissertation is to conduct *in vitro* and *in vivo* studies on the anti-enteroviral efficacy of the phosphatidylinositol-4-kinase beta (PI4KB) inhibitor MDL-860, as well as a library of newly synthesized analogues. With the successful completion of ten specific tasks, more effective and safer chemotherapeutic agents will be identified for use in combination or monotherapy regimens for the treatment of enteroviral infections. The aim and tasks are clearly and precisely formulated, with a focused scientific direction.

The methodological section includes various approaches for assessing the antiviral activity of the compounds against enteroviruses in both *in vitro* and *in vivo* settings. Reference inhibitors of enteroviral replication are used as benchmarks. New compounds are tested on infected cell cultures with different enterovirus strains, and animal models—mainly mice—are used to evaluate the compounds' efficacy under realistic infection conditions. Methods for assessing cytotoxicity for each compound individually are described, as well as testing of compound combinations for antiviral activity and cytotoxicity, to evaluate potential synergy or antagonism.

Other methods include *in vivo* experiments, viral titration in tissues (including brain), and classical plaque assays for quantifying viral particles. RT-PCR and sequencing are employed for molecular characterization, providing information on genetic changes associated with drug sensitivity or resistance. All data are subjected to statistical analysis to ensure validity and significance. The methods used are current and consistent with the latest trends in virology research.

The "Results" section is combined with discussion. Since the main aim is to study the effectiveness of novel triple antiviral combinations against experimental neuroinfection, the key findings are based on *in vivo* experiments with triple drug combinations. The antiviral efficacy and resistance impact of the triple combination of enterovirus replication inhibitors—Pleconaril, Guanidine-HCl, and Oxoglaucin (PGO)—administered using a Sequential Alternating Administration (SAA) scheme were evaluated in a neonatal mouse model of Coxsackievirus B1 (CVB1) neuroinfection. The

SAA regimen significantly improved survival (PI up to 68%, MST up to 10.4 days) and reduced infectious viral load in brain tissues, with the most pronounced effect observed for Pleconaril at 200 mg/kg. Monotherapies showed limited efficacy. Plaque reduction assays demonstrated that the SAA regimen with PGO maintained viral sensitivity to Pleconaril and Oxoglaucin up to day 13 post-infection, preventing the emergence of resistant variants. These results confirm the advantages of sequential combination therapy as an effective strategy against neurotropic enterovirus infections, providing antiviral activity and delaying resistance development while minimizing toxicity.

The next objective was to investigate, using molecular genetic approaches, how the PMO combination (Pleconaril/MDL-860/Oxoglaucin) administered in SAA format affects resistance development and viral sensitivity. Viral isolates from the brains of treated mice were sequenced, revealing single amino acid substitutions in both structural and non-structural proteins. Encouraged by the promising results with MDL-860, the PhD candidate explored its newly synthesized analogs against enteroviruses (CVB1, CVB3, PV1). Initially, 12 compounds (1a–12a) were tested for cytotoxicity and antiviral activity *in vitro*, with the most promising ones (1a, 11a, and 12a) subsequently evaluated *in vivo*. Compound 1a demonstrated the most potent protective effect.

The dissertation presents a targeted in vitro study of 60 synthetic derivatives of MDL-860 (designated as 13v to 72v), all sharing a common pharmacophore element — a 2-cyano-5-nitro-substituted benzene ring. These compounds were tested against three enteroviruses — PV1, CVB1, and CVB3, with MDL-860 used as the reference molecule. Among them, compound 39v stands out with the broadest antiviral profile, comparable to that of the parent structure, while seven additional compounds exhibit selective activity against individual viral strains.

The doctoral candidate investigates the structure–activity relationship, identifying correlations between subtle molecular modifications and the observed antiviral efficacy findings with significant potential for future molecular design. Three compounds—39v, 41v, and 47v—were further evaluated in vivo in a neonatal mouse model infected with CVB1. The moderate protective effects observed for compounds 39v and 41v highlight a lack of direct correlation between in vitro and in vivo activity, a conclusion that the author appropriately addresses.

In addition, a QSAR analysis was conducted to assess the impact of various substituents in the 5-nitrobenzonitrile core on cytotoxicity and antiviral potential against PV1, CVB1, and CVB3. Based on the results, the author proposes a rational approach, grounded in the structural and physicochemical characteristics of the compounds, which could support future strategies in the molecular design of novel antiviral agents.The final part of the dissertation highlights the significance of the newly discovered antiviral compounds and their relevance in the context of current challenges in the treatment of enteroviral infections. The main conclusions include:

- The structural possibilities for optimization in certain parts of the MDL-860 molecule have been exhausted, with attention shifted to other, more promising cores.
- Compounds with proven *in vivo* efficacy and low toxicity in a neonatal mouse model of enteroviral infection.

- Broad-spectrum activity was also established against other viruses such as human coronavirus OC43 and adenovirus type 5.
- The obtained results can serve as a foundation for optimizing molecular structure and for future development of antiviral drugs with high efficacy and safety.

The scientific and applied contributions summarized in the dissertation are significant and support the advancement of the field on a global scale. They can be summarized as follows:

- A new therapeutic scheme has been developed: a sequentially alternating application (SAA) of anti-enteroviral compounds, which prevents the emergence of drug resistance in viral neuroinfection in neonatal mice.
- A large-scale *in vitro* screening of compounds with antiviral activity has been conducted. A total of 114 new MDL-860 analogues were analyzed, of which 10 showed activity comparable to or higher than the reference compound.
- Compounds have been identified as candidates for future preclinical drug development.
- Valuable QSAR analyses have been carried out: mathematical models have been constructed to predict antiviral activity, aiding rational drug design.
- Data on the extended antiviral spectrum of new compounds have been obtained. It was found that some compounds are also active against viruses outside the enterovirus family, broadening their therapeutic potential.

4. Publications Related to the Dissertation and Compliance with Regulatory Requirements

The dissertation is supported by significant scientific output: 26 research articles related to the dissertation topic published in international journals, as follows: 12 in Q1 journals, 8 in Q2, 3 in Q3, and 3 in Q4. Total scientific articles on the topic (2014–2023): 31 articles indexed in Scopus and Web of Science. For clarity, the verified data are summarized in the following table:

Indicator	**Data**
Number of publications (2014–2023)	26 full-text research articles indexed in Scopus and Web of Science
Quartile classification of journals	Q1: 12 articlesQ2: 8 articlesQ3: 3 articlesQ4: 3 articles
Scientific points (Group G)	429 points
Authorship contribution	First author: 3 publications (11.5%) Second author: 5 publications (19.2%) Third or subsequent author: 18 publications (69.2%)
Overlap with previous PhD dissertation	No overlap with previously used publications for the acquisition of the PhD degree
Total citations	103

Indicator	Data
Scientific points from citations (Group D)	188 points
H-index (Scopus)	7
Scientific forums	Total participations: 20, of which 15 abroad
Research projects related to the dissertation topic	4 projects funded by the NSF:- B02-11 (2014–2019)- KP-06-CHINA/31 (2020–2022)- KP-06-H49/2 (2019)- KP-06-H31/7 (ongoing)

The results are part of research projects funded by the National Science Fund (including a bilateral agreement with Chinese partners) and numerous contracts with companies, research institutions, and private individuals. This activity confirms the PhD candidate's leading role in antiviral chemotherapy and highlights the importance of her work for the future development of new antiviral agents.

The analysis shows that the data presented by Assoc. Prof. Nikolova in the relevant groups, according to the Regulations for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria, fully meet, and in some even significantly exceed, the minimum national requirements for awarding the scientific degree "Doctor of Science" (as amended, State Gazette No. 15 of 19.02.2019). Within Group A (50 points), Group B (100 points), Group G (required: 100 points), and Group D (required: 100 points), Assoc. Prof. Nikolova has accumulated 767 points – more than double the required minimum of 350 points.

5. CONCLUSION

The dissertation of Assoc. Prof. Dr. Ivanka Nikolova is an original and thorough scientific study on the synthesis, characterization, and antiviral activity of novel diaryl ethers and their analogs against enteroviruses. The work is distinguished by a clear scientific hypothesis, a logically structured experimental section, and well-substantiated conclusions supported by reliable data. The author demonstrates high scientific competence and the ability to apply an interdisciplinary approach in medicinal chemistry, pharmacology, and virology.

The submitted work fully meets the requirements of the Law for the development of the Academic Staff and its implementing regulations, as well as the additional requirements of the "Stefan Angelov" Institute of Microbiology for awarding the scientific degree "Doctor of Science." Based on the conducted critical analysis and the presented findings, I express my wholly POSITIVE opinion and recommend that the other members of the Scientific Jury support awarding the scientific degree "Doctor of Science" to Assoc. Prof. Ivanka Nikolova.

May 26, 2025

Reviewer:

(Prof. Penka Petrova, DSc)