REVIEW

From: Prof. Reneta A. Toshkova, MD, PhD, Section "Pathology", Institute of Experimental Morphology, Pathology and Anthropology with Museum– Bulgarian Academy of Sciences (IEMPAM-BAS),
Member of the Scientific Jury, according to Order No. 1050/18.12.2025 of the Director of the Institute of Biophysics and Biomedical Engineering, BAS and elected as an official reviewer at the first meeting of the Scientific Jury, held on 29.01.2026 (Order No. 64/23.01.2026)

Regarding: Dissertation (PhD thesis) for the awarding of the educational and scientific degree "Doctor" in the Higher Education Field 4. "Natural Sciences, Mathematics and Informatics", Professional Direction 4.3. "Biological Sciences" and the Scientific specialty "Biophysics" for the needs of the "Transmembrane Signalling" laboratory at the Institute of Biophysics and Biomedical Engineering (IBhBME), BAS.
Topic of the dissertation: "Antitumor lipids – effects on transmembrane cellular signalling"
Author: Assistant Tihomira Tihomirova Stoyanova, part-time doctoral student at the "Transmembrane Signalling" Laboratory of the Institute of Biophysics and Biomedical Engineering at the Bulgarian Academy of Sciences.
Scientific supervisors: Prof. Dr. Rumyana Tsoneva, PhD
Prof. Dr. Albena Momchilova, DSc

The review was prepared in accordance with the requirements of the Law for the Development of the Academic Staff of the Republic of Bulgaria (LDASRB), the Regulations for its Implementation, and the internal regulations of the Bulgarian Academy of Sciences and IBhBME-BAS. I declare that I have no conflict of interest with the candidate.

Materials on the procedure: The dissertation work was discussed, approved and sent for defense before a Scientific Jury at an extended seminar of the Institute of Biophysics and Biomedical Engineering, BAS at the Bulgarian Academy of Sciences, held on 05.12.2025.

The materials presented on paper and electronic media meet the requirements of the State Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for its application and the specific regulations of the Bulgarian Academy of Sciences and the Institute of Biophysics and Biomedical Engineering, BAS, and include all materials required for the procedure.

Biographical data: Tihomira Tihomirova Stoyanova was born in Pleven in 1991. In 2014, she graduated from Sofia University "St. Kliment Ohridski",

with a Bachelor's degree in "Molecular Biology", and two Master's degrees: A Master's degree in "Cell Biology and Pathology" in 2016 and a Master's degree in "Developmental Biology" in 2017. From October 2015 to 2019, she was appointed as a specialist biologist in the "Lipid-protein interactions" section of IBpBME. Since 2019, she has been an assistant in the same section. Currently she holds the position of Assistant (from 2021) in the Laboratory of Transmembrane Signalling. In January 2017, she enrolled as a part-time doctoral student in the same laboratory at IBpBME, BAS. She has completed the educational programme and met the required quantitative criteria, passing the doctoral minimum exams in a basic subject, computers and English, and completing two specialised courses.

Relevance and significance of the dissertation: The dissertation is dedicated to an extremely topical and socially significant problem – the therapy of malignant oncological diseases, which are among the leading causes of death worldwide and nationally, significantly reducing life expectancy. The increasing incidence and mortality rates of these diseases emphasise the urgent need to develop therapies that target new molecular pathways and new, effective therapeutic strategies, as well as to create selective and well-tolerated drugs. Attention is focused on triple-negative breast cancer and non-small cell lung cancer, the two most common prevalent types of cancer worldwide, which are characterised by high aggressiveness, early metastasis, development of drug resistance and unfavourable prognosis. These are some of the most difficult oncological diseases to treat. The emphasis is on the mechanism of action of antitumor lipids and the role of lipid signalling as a new therapeutic target in malignant diseases. In this aspect, the topic of the dissertation "Anti-tumor lipids – influence on transmembrane cell signalling" is both scientifically and practically relevant and promising, offering a high level of innovation.

Structure of the dissertation. The dissertation work is written on 128 non-standard pages and contains the usual sections: Title page, Table of Contents – 5 pages; Abbreviations used – 4 pages; Introduction – 2 pages; Literature review – 30 pages; Aim and objectives – 1 page; Materials and methods – 18 pages; Results and discussion – 44 pages; Summary - 9 pages; Conclusions – 1 page; Contributions – 1 page; Literature – 16 pages; Appendices – 5 pages - Publications; Citations; Declaration, Future scientific plans. The bibliography includes 236 Latin sources..

The work is illustrated with 46 figures and 3 tables. Of these, 16 figures (with correctly cited sources in the sub-figure text) and two tables are in the overview; two figures are in the 'Materials and Methods' section; and 28 figures and one table are in the 'Results' section. The work follows the classic model of an experimental biomedical dissertation and demonstrates logical consistency, meeting the requirements for an ONS 'Doctor' dissertation.

The **Literature review** is contemporary, focused, and detailed. It is distinguished by a clear and concise style and presents systematized scientific

information on key thematic areas. Two main types of cancer are described: breast and lung cancer. The most widely used cell lines from breast cancer are characterized by the presence or absence of three important cell receptors (estrogen-ER, progesterone-PR and human epidermal growth factor receptor 2-HER2), as well as from lung cancer. The mechanisms of cell death - apoptosis, necroptosis, necrosis; the process of metastasis, and the cell cycle of cancer cell proliferation are discussed in detail. An in-depth review of the lipid composition and organization of cell membranes, membrane fluidity, sphingolipid rheostat, as well as the role of bioactive molecules ceramide, sphingosine-1-phosphate (S1P), and protein kinase Cα (PKCα) in the regulation of cell survival is provided. The skilful integration of complex molecular mechanisms and their significance for tumor survival and proliferation deserves high praise. Attention is given to antitumor lipids that affect the cell membrane and a number of signal transduction pathways, in contrast to classical chemotherapeutics that target DNA or the mitotic apparatus directly.

Three antitumour lipids are presented in detail: two alkylphosphocholine representatives, erufosine (EPC3) and miltefosine (HePC); and D-erythro-N,N-dimethylsphingosine (DMS), a sphingolipid class of lipid molecules. The PhD student demonstrates excellent literary awareness and in-depth knowledge of the relatively specialised scientific fields of lipid signalling pathways and antitumour lipids, as well as the ability to skilfully analyse scientific facts.

The **aim** of the dissertation is logically derived and formulated: "To investigate the mechanism of action of anti-tumor lipids on transmembrane signalling in cancer cells". **Six specific tasks** have been identified to achieve this goal. Each task builds on the previous one and contributes to the overall disclosure of the mechanism of action of the studied anti-tumour lipids, proposing a new therapeutic strategy based on the 'sphingolipid rheostat' (switching from tumour cell proliferation to programmed cell death, or apoptosis).

Assessment (Evaluation) of the materials and methods used: The methodological approach used is complex, extensive and modern. The volume of the experimental material is completely sufficient. The experiments were correctly designed and conducted in accordance with the relevant standards and controls. The choice of *in vitro* cancer model systems studied (breast and lung cancer cell lines), and the relevant controls, as well as plasmids, kits and reagents is fully justified and meets the priorities of modern medical oncology. The methods are described in sufficient detail to allow other researchers to reproduce them. thoroughly and in detail, which allows their reproducibility by other researchers.

The PhD student has mastered and applied both established classical cellular and biological methods (MTT, migration - Scratch Assay, fluorescent staining with phalloidin-TRITC; AO/EtBr, DAPI, RT-PCR), and precise flow cytometric analyses (cell cycle and type of induced cell death). High praise

deserves the inclusion of modern biophysical methods, rarely found in standard dissertation research, such as scanning fluorescence correlation spectroscopy (sFCS) of cells and fluorescent methods for analyzing membrane fluidity based on Di4-ANEPPDHQ labelling (confocal microscopy), as well as lipidomic approaches involving lipid extraction and gas chromatography for cholesterol determination. Another advantage is the application of biochemical and molecular techniques for the study of signal transduction pathways, including analysis of protein phosphorylation (Western blot), the determination of bioactive lipid mediators (sphingosine-1-phosphate [S1P] by ELISA), the cytokinesis-block micronucleus (CBMN) assay of cells, and the calculation of a combination index to assess the synergistic therapeutic effect. The study of antitumor lipids such as erufosine, miltefosine and DMS represents an innovative and modern approach that aligns with current trends in oncology, such as the search for therapies with new molecular targets and the selection of less toxic chemotherapeutic agents.

The methodological section is very strong. It demonstrates the doctoral student's excellent experimental experience and ability to integrate multiple data from various experimental approaches, including cellular, biophysical, molecular, biochemical, etc. In summary, the methodology is entirely adequate for the objectives and tasks set and provides highly useful and reliable results, and meets the requirements for high- level scientific research.

Evaluation of the obtained results: The "Results and Discussion" section includes a significant amount of the doctoral student's own data. They are presented systematically in a sequence that follows the tasks planned in the dissertation. The experimental results are divided into two thematically related parts: Part I. Investigation of the effect of erufosine on breast cancer cell lines with different metastatic potential (highly invasive MDA-MB-231, low invasive MCF-7 and normal cell line MCF-10A); and Part II. Investigation of the effect of miltefosine and DMS on A549 (human lung epithelial adenocarcinoma) and HUVEC cell lines. The results are illustrated with 28 two- and multi-component figures and 1 table. The data are statistically reliable and accurately interpreted, which speaks to the professionalism of the doctoral student. In short, the results can be described as follows:

-A pronounced and selective dose- and time-dependent cytotoxic effect of erufosine against breast cancer cells was established, with the highest sensitivity observed in the highly aggressive triple-negative cell line MDA-MB-231.

-Cell cycle analysis revealed that erufosine induced G2/M phase arrest and accumulation of cells in the sub-G1 fraction, coupled with a reduction in the S-phase, which is an indicator of mitotic arrest and programmed cell death. This effect was more pronounced in MDA-MB-231 cells and not observed in normal MCF-10A cells.

-Molecular analysis shows that erufosine suppresses the expression of key cell cycle regulators, namely the Cyclin B/CDK1 and Cyclin E2/CDK2

complexes. This explains the established mitotic arrest and blocking of the G1/S and G2/M transitions at a molecular level. These results support the hypothesis that erufosine disrupts the coordination between cell proliferation and signaling mechanisms that control the cell cycle.

-The results of morphological, fluorescent and flow cytometric analyses show that erufosine induces mainly early apoptosis at therapeutic concentrations, while late apoptosis and necrosis are mainly observed at high doses and prolonged treatment. Furthermore, MDA-MB-231 cells were found to be more sensitive than MCF-7 cells, while normal cells showed minimal changes.

-Erufosine was found to significantly inhibit the migration and adhesion of MDA-MB-231 cells in a concentration- and time-dependent manner. These results correlate with evidence of changes in the actin cytoskeleton, which is a key factor in tumor cell invasiveness.

-Biophysical and lipidomic analyses showed that erufosine reduces plasma membrane order, increased membrane fluidity, and altered its lipid composition by reducing the levels of cholesterol, sphingomyelin, and phosphatidylcholine, while increasing phosphatidylserine level. These changes lead to disruption of lipid rafts and activation of apoptotic signaling pathways. Additionally, erufosine was found to increase the diffusion dynamics of transmembrane proteins without altering their localization. This indicates a complex effect on membrane organization and signaling, and confirms the role of the membrane as a primary therapeutic target for erufosine.

-A synergistic cytotoxic effect on non-small cell lung carcinoma (A549) tumor cells was found after combined administration of miltefosine and D-erythro-N,N-dimethylsphingosine (DMS). The calculated combination indices confirmed a stronger antitumor effect at lower concentrations with limited cytotoxicity to normal HUVEC cells, which is important for reducing toxicity and side effects.

- Morphological, FACS and biochemical analyses revealed that combined miltefosine/DMS therapy induced apoptosis in tumor cells by activating the intrinsic mitochondrial pathway and significantly decreasing S1P (sphingosine-1-phosphate) levels through dual inhibition: direct inhibition of SK1 (sphingosine kinase 1) by DMS and indirect inhibition of PKC (protein kinase C) by miltefosine.

In conclusion, the results clearly demonstrate that antitumour lipids modulate membrane organisation, transmembrane lipid signalling and key regulatory pathways of the cell cycle and apoptosis. These findings support their potential application as a basis for developing novel, more selective therapeutic approaches in oncology.

The **Discussion** was done in parallel with the presentation of the obtained results. A very good solution is the forming of a separate Summary section, which thoroughly, analytically, very compactly and convincingly summarizes all

the results obtained from the consistently implemented scientific plan, the complex experimental methodology and the in-depth understanding of the molecular and cellular mechanisms of action of antitumor lipids.

The results emphasize the potential clinical applicability of the studies conducted and their focus on the treatment of some of the most challenging malignancies, such as breast and lung cancer. Two circumstances make a strong impression: the reasoned discussion of specific results in favor of correctly formulated hypotheses, and the objective analysis of the obtained own results in the light of those in the scientific literature. Here the high level of information and objectivity of the doctoral student and the scientific supervisors should be appreciated.

I fully accept the **eight conclusions**, which are correctly drawn and formulated with great precision. They are specific and accurately reflect the results obtained.

Evaluation of the **contributions in the dissertation:** The five scientific contributions of the dissertation are outlined. They are indisputable, enrich science and up to now, reflect new significant scientific facts with potential clinical application. The contributions can be categorised as either original (fundamental) (1, 2 and 3) or scientifically applied (4 and 5).

Original contributions:

1. For the first time, a direct relationship was demonstrated between changes in lipid composition, lipid layer organization, and ~~the~~ diffusion dynamics of the cell membrane under the action of erufosine, and changes in the cell cycle in the G2/M phase and induction of apoptosis in the highly metastatic breast cancer cell line MDA-MB-231. **2.** For the first time, it was found that combined treatment with miltefosine (HePC) and D-erythro-N,N-dimethylsphingosine (DMS) exerts a synergistic cytotoxic effect on lung cancer cells. **3.** The combined treatment with miltefosine (HePC) and D-erythro-N,N-dimethylsphingosine (DMS) inhibits signalling pathways related to sphingosine kinase 1 and protein kinase C, decreases sphingosine-1-phosphate (S1P) levels in cancer cells, and induces apoptosis by activating the intrinsic mitochondrial pathway

Applied contributions:

4. The high selectivity of alkylphosphocholines (erufosine and miltefosine) for highly metastatic cancer line MDA-MB-231 and the lung cancer line A549, while having a weak effect on normal MCF-10A and HUVEC cells, is a prerequisite for their use in modern antitumor therapy. **5.** The discovered synergistic cytotoxic effect of the combination of miltefosine and DMS on tumor cells allows a higher therapeutic result to be achieved at lower concentrations of miltefosine, thereby reducing side effects. In addition, I would like to highlight another significant contribution: **6.** A complex experimental approach, combining cellular, molecular, biochemical and biophysical methods, has been validated ~~(tested)~~, which allows a comprehensive assessment

(evaluation) of the effect of antitumor lipids at the membrane and cell level and has methodological and scientific value for future theoretical and applied studies.

Critical remarks, recommendations, questions: Overall, the dissertation is written in excellent scientific language. I have no critical remarks or questions on the substance. It is commendable that, based on the acquired experience and knowledge, the doctoral student has a vision and outlines guidelines for the further development and continuation of the research. I would like to note some minor technical omissions: the repeated numbering of Fig. 18 changes the total number of figures in the dissertation from 45 to 46; and the lack of a description of statistical method in the "Materials and Methods" sectio. For 11 figures in the "Results and Discussion" section, however, the method by which the statistical reliability of the results was established is cited. These editorial remarks do not diminish the scientific originality and significance of the research conducted and do not change my opinion about the excellent style, language and form of presentation of the dissertation.

Evaluation of the abstract: The abstract is 70 pages long. It has been prepared in accordance with the regulatory requirements. The design is excellent and it fully corresponds to the content of the thesis. It provides a comprehensive and objective overview of the scientific value and significance of the research conducted.

Publications related to the dissertation: The results of the dissertation are included in three articles published in English in peer-reviewed journals indexed in worldwide databases: one article in Chemico-Biological Interactions, with IF 3.723 (2019), SJR 1.112 and quartile Q1, one in Biomolecules, with IF 4.879 (2020), SJR 1.33 and quartile Q2; and one in Comptes Rendus de l'Academie Bulgare des Sciences, with IF 0.3, SJR 0.15 and quartile Q3. The total IF is 8.9. The scientific publications are co-authored, with the PhD student as the first, second and third author. Results on the topic of the dissertation have been presented in 6 reports at scientific forums in the country, four of which with international participation. The personal contribution of the doctoral student in the development of the dissertation is undoubted. Seventeen citations 17 citations in international journals have been noted for two of the articles, indicating increased interest from the international scientific community. The research included in the dissertation was conducted in the "Transmembrane Signaling" Laboratory of the the IBPBMI-BAS with the support of projects in which the PhD student is a participant (projects under bilateral cooperation with Germany and funded by the National Science Found at the Ministry of Education and Science).

It can be concluded that the scientific output and metrics fully satisfy the quantitative criteria set out in the LDASRB and relevant institutional regulations.

CONCLUSION

The dissertation is complete, scientifically sound and innovative. It demonstrates in-depth research work and contains scientific and scientifically applied contributions. The data obtained expand the knowledge of the role of lipid signalling in oncogenesis, confirm the role of the membrane as the main therapeutic target of antitumor lipids and outline new approaches for oncological medical practice. The scientific works have been published in high-quartile international journals. During her doctoral studies, Assistant Tihomira Stoyanova has acquired new theoretical and practical skills, and the ability to analyze scientific results creatively, formulate scientific conclusions and contributions, draft scientific articles, and work in a team.

I believe that the presented dissertation in terms of volume, content and relevance, fully meets, and in some aspects significantly exceeds, the quantitative criteria for acquiring the educational and scientific degree "Doctor", according to the Law on the Development of Academic Staff in the Republic of Bulgaria, the Regulations for its application and the regulations of the BAS and IBpBME-BAS

Based on everything said so far, I give a positive and high assessment of the dissertation work, and I confidently vote "Yes" and propose to the members of the esteemed Scientific Jury to award Assistant Tihomira Tihomirova Stoyanova the educational and scientific degree "Doctor", in the Field of Higher Education 4. "Natural Sciences, Mathematics and Informatics", Professional Direction 4.3. "Biological Sciences" and Scientific Specialty "Biophysics" for the needs of the laboratory "Transmembrane Signalling" of the the Institute of Biophysics and Biomedical Engineering - BAS.

25. 02. 20256 Signature:

Sofia Prof. Reneta A. Toshkova, MD, PhD