REVIEW

by Assoc. Prof. Radoslav Aleksandrov Aleksandrov, PhD,

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on PhD thesis:

"Antitumor lipids - influence on transmembrane cellular signaling"

Doctoral student: Tihomira Tihomirova Stoyanova

for award of Doctor of Philosophy degree in an area of higher education 4. "Natural sciences, mathematics and computer sciences", professional direction: code 4.3 "Biological sciences", scientific specialty "Biophysics"

Scientific supervisors: Prof. Rumyana Tsoneva, PhD and Prof. Albena Momchilova, PhD

I. General part

Tihomira Tihomirova Stoyanova has been a doctoral student at the Laboratory of Transmembrane Signaling of the Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences (IBPhBME-BAS) since 2017. The PhD thesis entitled "Antitumor lipids - influence on transmembrane cellular signaling" has been prepared under the scientific supervision of Prof. Rumyana Tsoneva, PhD and Prof. Albena Momchilova, PhD and includes 129 pages, 45 figures, 3 tables and 236 cited sources. All presented materials comply with the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADSRB) and the Regulations for its implementation, as well as the Regulations for the implementation of ADSRB at the Institute of Biomedical Engineering and Biomedical Engineering-BAS.

II. Brief biographical data

Tihomira Stoyanova is a graduate of the "Geo Milev" Mathematical High School in Pleven, from where she graduated in 2010. Between 2010 and 2014, Tihomira Stoyanova studied in the bachelor's program "Molecular Biology" at the Faculty of Biology of Sofia University, and between 2014 and 2017, she obtained two master's degrees from the Faculty of Biology of Sofia University - "Cell Biology and Pathology" and "Developmental Biology". In 2015, she started working as a specialist in the "Lipid-Protein Interactions" section, and since 2019 she has been a research assistant first in the "Lipid-Protein Interactions" section, and then in the "Transmembrane

Signaling" Laboratory. Since 2016, she has been working as a biologist at the clinical laboratory of the "Tsaritsa Ioanna University Hospital – ISUL" in Sofia. Since the beginning of 2017, she has been enrolled as doctoral student at the Transmembrane Signaling Laboratory of the Institute of Biomedical Engineering and Biomedical Sciences-BAS.

III. Relevance of the dissertation topic

Globally, cancer is the second leading cause of death after cardiovascular disease. In recent years, a change in this trend has been observed in a number of developed countries, with cancer now overtaking cardiovascular disease as the leading cause of death in the population, thanks to advances in prevention, early diagnosis and treatment of cardiovascular disease, as well as increased life expectancy.

Cancers represent an extremely diverse group of genetic diseases, characterized by impressive heterogeneity at the genetic, molecular and cellular levels, such that even tumors originating from the same tissue can differ significantly in their biology, growth rate, ability to metastasize and response to therapy. It is this heterogeneity that makes the treatment of oncological diseases extremely challenging. Standard therapeutic approaches – surgery, radiotherapy and chemotherapy – often lead to a significant reduction in tumor mass and an initially good clinical response. However, in many cases they fail to completely eliminate all cancer cells and often lead to the development of resistance to chemotherapeutic agents, which leads to disease relapse and limitation of subsequent therapeutic options.

For these reasons, modern research in the field of cancer biology is aimed at the detailed elucidation of the molecular mechanisms of tumorigenesis in order to develop more precise, personalized and gentle therapeutic strategies. Combination approaches to chemotherapy are also actively pursued, aimed at simultaneously suppressing different mechanisms of tumor development and limiting the likelihood of resistance.

In light of the above, the present dissertation investigates the effects and mechanisms of molecules from the group of antitumor lipids (erufosine, miltefosine, and dimethylsphingosine (DMS)) as potential chemotherapeutics for the treatment of common and socially significant types of cancer, such as breast cancer and lung cancer. The reported results highlight the potential of these compounds for application in cancer therapy. They induce multiple effects in cancer cells, including disruption of cell cycle regulation, cytoskeletal reorganization and suppression of cell migration, changes in the composition and fluidity of cell membranes, and activation of the cellular apoptosis program. The combination of these changes leads to effective inhibition of tumor growth and induction of cell death. The results regarding the effects of miltefosine, both alone and in combination, are particularly promising, as it is an alkylphospholipid that is already approved for the treatment of leishmaniasis in humans and therefore has well-studied pharmacological characteristics and side effects, which would greatly accelerate its eventual introduction into clinical practice as an antitumor agent.

IV. Structure of the dissertation

Tihomira Stoyanova's dissertation is structured in the traditional manner, with clearly defined main sections and subsections, and fulfills all the necessary requirements for this type of scientific work.

Right at the beginning of the dissertation, *the goal is clearly defined*, which is "to explore the mechanisms of action of antitumor lipids on transmembrane signaling in cancer cells". To achieve this ambitious goal, *six specific tasks have been formulated*, the implementation of which would truly characterize in depth the cellular effects of the studied antitumor lipid compounds and would reveal their potential or shortcomings in cancer therapy.

The information provided in *the Literature Review section* is clearly presented, consistently organized, and precisely focused on the research problem of the dissertation. At the beginning, the doctoral student provides clinical and epidemiological data on the significance of cancer on a global scale, and in particular for breast and lung cancer, which are respectively the most common type of cancer in women and the second most common type of cancer in men. The information provided on the characteristics of cancer cell lines (including those used in this dissertation) used in the study of these types of cancer in laboratory conditions is very useful. Then, the doctoral student consistently presents some of the main pathways leading to cell death, such as apoptosis, necroptosis, and necrosis, as well as the events that accompany the metastasis of cancer cells. A key section is the one that reveals basic principles regarding the lipid composition and fluidity characteristics of biological membranes, as well as the special focus on the so-called "sphingolipid rheostat" – the balance between ceramide and sphingosine-1-phosphate, which affects the proliferative abilities of cells.

The Materials and Methods section presents in detail all the experimental procedures used in the course of the dissertation, with a particularly impressive range of techniques applied for the in-depth elucidation of the properties of antitumor lipids. The doctoral student has used a variety of approaches to assess the cytotoxic potential of the studied compounds, such as the MTT test and vital staining with acridine orange/ethidium bromide, scratch assay for assessing cell migration, flow cytometry for detecting apoptosis and necrosis, as well as for determining the impact of the studied substances on the progression of the cell cycle in model cell lines. In addition, RT-qPCR assessment of the expression levels of cyclins and cyclin-dependent kinases was performed to deepen the results regarding the changes in the cell cycle detected by flow cytometry. To reveal the effects of antitumor lipids on cytoskeletal reorganization, membrane fluidity, and membrane protein mobility, the doctoral student used modern fluorescence microscopy techniques, including scanning fluorescence correlation spectroscopy (sFCS), which is particularly suitable for measuring the diffusion coefficients of the studied fluorescently labeled proteins within the cell membrane of treated cancer cells. To investigate the changes in the composition of the membranes as a result of treatment with antitumor lipids, the doctoral student characterized the phospholipid composition with thin-layer chromatography, and the cholesterol amounts in the membranes with gas chromatography. The set of techniques

presented in this way is impressive, but also necessary for the detailed characterization of the effects of antitumor lipids, which is the goal of the present dissertation.

The most important part of the dissertation – the *Results and Discussion section* – is divided into two parts, within which the results obtained on the effects of erufosine on breast cancer cell lines with different metastatic potential (Part I) and the effects of miltefosine and dimethylsphingosine (DMS) on the lung cancer cell line A549 and normal untransformed HUVEC cells (Part II) are presented.

The results in Part I reveal the abilities of erufosine to block the proliferation of the studied breast cancer cell lines in several different ways. Of particular interest is the great potential of erufosine to suppress the growth and migration of the highly aggressive triple-negative line MDA-MB-231. The most significant and entirely novel observations in this section are the following:

- Erufosine induces dose- and time-dependent suppression of proliferation in the breast cancer cell lines MDA-MB-231 and MCF-7, with the effect being more pronounced in the highly invasive MDA-MB-231.

- Erufosine induced mitotic arrest in the G2/M phase in the MDA-MB-231 and MCF-7 cell lines, accompanied by an increase in the sub-G1 fraction and a decrease in cells in S-phase, with the effect again being more pronounced in MDA-MB-231.

- Acridine orange/ethidium bromide and DAPI staining showed that erufosine induced apoptosis, with the effect being more pronounced in the highly invasive MDA-MB-231 cell line.

- Erufosine suppresses the migratory potential of the highly invasive MDA-MB-231 cell line. This indicates that the antitumor agent effectively limits cell migration, which is of key importance for metastasis.

- Phalloidin staining showed that erufosine affects the actin cytoskeleton, with disruption of actin microfilaments observed at high doses. These changes could explain the reduced migratory potential of MDA-MB-231 cells.

- Erufosine reduces the orderliness of the cell membrane and alters its lipid composition, with the effect being more pronounced in MDA-MB-231.

- Erufosine does not change the localization of hEGFR and FPV-HA proteins, but significantly increases their diffusion dynamics, indicating changes in the physical properties of the cell membrane.

Part II presents the studies concerning the effects of miltefosine and dimethylsphingosine – administered either individually or in combination, on the proliferation and properties of the lung cancer cell line A549 and the non-transformed HUVEC cells. The central focus of this section of the dissertation is the discovered synergy between these two antitumor lipids in suppressing the growth of the cancer cell line and in their significantly weaker effect on the non-cancerous HUVEC cells. The most significant findings regarding the action of miltefosine and DMS are as follows:

- The effects of DMS and miltefosine on the viability of A549 cancer cells and normal HUVEC cells are dose- and time-dependent. Both compounds suppress cell proliferation, with

A549 cells showing greater sensitivity to DMS. The combined treatment demonstrates a synergistic cytotoxic effect on A549, which is further enhanced with prolonged exposure.

- The combined treatment with miltefosine and DMS induces morphological and apoptotic changes in cancer cells, expressed by cell rounding, reduced cell density, and formation of apoptotic bodies. Despite the potential genotoxic risks associated with antitumor drugs, the conducted study did not detect significant genetic damage in the treated cells.

- Flow cytometry analysis demonstrates that the combined treatment with miltefosine and DMS induces significant apoptosis in A549 cancer cells, whereas normal HUVEC cells display markedly lower sensitivity. This confirms the selective cytotoxic effect of the combination therapy towards tumor cells.

- The combined treatment with miltefosine and DMS decreased sphingosine-1-phosphate levels in A549 cancer cells, confirming that inhibition of S1P signaling may contribute to the antiproliferative effect.

Considering the extensive volume and methodological diversity of the experimental results presented, it should be noted that the *Summary section* (Chapter 5 in this dissertation), followed by the *Conclusions* and *Contributions* sections, successfully synthesize the conducted research and place it within a coherent overall framework, which facilitates the perception of the results in their entirety. he inclusion of a *Future Research Plans* section is also commendable, as it clearly outlines new directions for investigating the effects of antitumor lipids.

I have two recommendations for the doctoral student regarding the dissertation. In some places there are uncorrected spelling errors, which is normal considering the volume of the work presented, but it would be better if these errors were cleaned up. My second recommendation concerns the figures within the dissertation, which show data obtained from the application of fluorescence microscopy techniques or flow cytometry analysis – these figures could be replaced with those of a larger size and/or significantly higher resolution, in order to present the results in a more clear and visible manner.

V. Publications

The results obtained within the framework of the dissertation work have been published in three scientific publications in Q1, Q2 and Q3 journals, and the citations noted are already 17.

I have the following questions for the doctoral student :

1) Does the fluorescent dye Di-4-ANEPPDHQ, which is used to assess changes in cell membrane fluidity, distribute to other membranes in cells and how could this affect the assessment of cell membrane fluidity?

2) What are the GP (Generalized Polarization) values presented in Figures 33 and 34 normalized to?

3) Considering the fact that miltefosine is approved for clinical use in humans for the treatment of leishmaniasis, what are the therapeutic concentrations achievable in the human body and how do they relate to the concentration range of miltefosine studied in this dissertation?

CONCLUSION

In the dissertation submitted to me for review, doctoral candidate Tihomira Stoyanova employs a broad range of experimental techniques to thoroughly investigate and characterize the effects of the antitumor lipids erufosine, miltefosine, and dimethylsphingosine on cell migration, membrane status, and proliferation in breast and lung cancer cell lines. The original results obtained convincingly demonstrate the potential of these compounds in the therapy of some of the most socially significant types of cancer and contribute to a better understanding of their molecular and cellular mechanisms of action. The submitted work fully meets the requirements for the award of the educational and scientific degree "Doctor of Philosophy". It presents the author as a thorough and competent researcher, capable of solving complex scientific problems and professionally analyzing and presenting her results. Based on the above, I strongly recommend that the esteemed scientific jury award Tihomira Tihomirova Stoyanova the educational and scientific degree "Doctor of Philosophy".

28.02.2026
Sofia

Reviewer: .....
/ Assoc. prof. Radoslav Aleksandrov, PhD /