OPINION

by **Prof., PhD. Rumiana Dimitrova Tzoneva**

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Regarding dissertation labour for the acquisition of an educational and scientific degree "doctor" in the professional direction 4.3. Biological sciences, scientific speciality "Biophysics "

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Topic of the dissertation paper: " Antitumor lipids - influence on transmembrane cell signaling"

1. Current affairs on the topic

The high scientific interest in the molecular mechanisms of antitumour lipids (miltefosine, erufosine) is characterised by their high selectivity for cancer cells, inducing cell death. Unlike most antitumour agents, antitumour lipids disrupt lipid homeostasis by mimicking endogenous phospholipids, targeting membrane lipid rafts and altering lipid-associated signalling, which leads to apoptosis. This explains the growing interest of the pharmaceutical industry in developing more effective, targeted antitumour therapeutic strategies that utilise combined molecular targets. The topic of the dissertation work is to elucidate the mechanisms of action of antitumour lipids on the biophysical properties of lipid membranes, such as lipid composition, membrane fluidity, and protein diffusion, as well as on sphingolipid metabolism in cancer cells, ultimately leading to apoptosis. An important aspect of the dissertation is studying the effectiveness of the combined action of two antitumour lipids in reducing the toxicity of antitumour agents on normal cells while maintaining their antitumour activity. Overall, the dissertation topic is highly relevant in the scientific field of "Biophysics".

2. Getting to know the state of the problem

This dissertation comprises 139 pages and is organised and presented according to accepted standards. The literary review includes an examination of the factors influencing the development of cancer, its distribution across demographic groups, as well as the pathogenesis, classification,

and staging of breast and lung cancer. The characteristics of the breast and lung cancer cell lines used are detailed. The types of cell death and their relationship to tumour pathogenesis are discussed thoroughly, along with mechanisms of metastasis and the cell cycle. The role of the cell membrane is well addressed, including its lipid composition, fluidity, and diffusion capacity. Particular emphasis is given to the sphingolipid rheostat and sphingolipid signalling within the context of tumourigenesis and therapeutic strategies.

The clear structuring and comprehensiveness of the literature review demonstrate thorough handling and analysis of modern scientific information relevant to research in this area. For greater clarity, the review presents 16 figures and 2 tables. Many of the cited sources are from recent scientific works. These works fully support the scientific facts presented and the conclusions drawn.

3. Purpose, tasks and methodology on research

The purpose of the present dissertation is clearly formulated: to investigate the mechanism of action of antitumor drugs on transmembrane signalling in cancer cells.

The following tasks have been outlined for implementation: 1) Conducting a cytotoxic analysis of the action of EPC3, DMS, and HePC (alone and in combination) on cancer cells with different levels of invasiveness; 2) Investigating the type of cell death induced after treatment with antitumour lipids (alone and in combination); 3) Quantitative assessment of the resulting cellular death; 4) Examining the effect of antitumour lipids on the cell cycle, cell migration, morphology, and cytoskeleton; 5) Studying the impact of antitumour lipids on the fluidity and diffusion dynamics of the cell membrane; 6) Investigating the effect of antitumour lipids.

The 'Materials and Methods" section accurately and thoroughly describes the various cellular, biochemical, immunological, and molecular techniques employed, including: the MTT assay for assessing cellular viability and proliferation, Italian AO/EtBr staining, the Scratch Assay, flow cytometry analysis for detecting apoptosis and necrosis, flow cytometric analysis using Nicoletti for DNA analysis of the cell cycle, and the determination of mRNA levels by RT-PCR. immunofluorescence to assess cellular adhesion, confocal microscopy on cells with Di-4-ANEPPDHQ, canning fluorescent correlational microscopy (sFCS) of cells, and immunoblot to determine the degree of phosphorylation of PKCα in cells, cytokinesis-block micronuclear analysis (CBMN) of cells, cytochrome C reductase (NADPH) test, etc.

4. Evaluation on received results and their interpretation

In the sections 'Results', 'Discussion', and 'Summary' (52 pages), extensive comments have been made regarding the effects of erufosine on cellular lines and cancer cells from the breast with different metastatic potentials. In particular, the influence of erufosine on cell survival, cell cycle, induction of cell death, migration potential, cytoskeletal organisation, cell membrane fluidity, lipid composition, and diffusional protein dynamics has been examined.

The effects of miltefosine and dimethylsphingosine on viability were evaluated and analysed on cancer cells from the lung and endothelial cells, including the occurrence of apoptosis/necrosis, activity of cytochrome C reductase, nuclear abnormalities, and cellular and extracellular S1P levels.

The chapter "Conclusion" systematically lists the main highlights of the present research included in the dissertation, which provides clarity and enhances understanding of the research conducted. Based on the experiments conducted, four conclusions have been drawn, indicating the cell-specific cytotoxicity of erufosine towards highly invasive breast cancer cells, concentration-dependent induction of apoptosis, changes in the cell cycle, reduction in migration potential, decreased orderliness of the lipid layer of the cell membrane, and increased diffusion of proteins through it.

Regarding the combined action of miltefosine and DMS, three conclusions have been drawn: the synergistic effect of the combined treatment on lung cancer cells, the induction of late apoptosis through activation of the intrinsic apoptotic pathway, and the reduction of S1P levels in lung cancer cells.

5. Evaluation of the offerings in the dissertation labour

The stated Contributions are adequate and comprehensive. They encompass three original contributions summarising the most important results of the dissertation work. These include emphasising the direct relationship between changes in lipid composition, the order of the lipid layer, and the diffusion dynamics of the cell membrane under the action of erufosine, as well as its impact on the cellular cycle in G2/M phase and the induction of apoptosis in the highly metastatic breast cancer cell line MDA-MB-231. The other two contributions relate to the combined action of miltefosine and dimethylsphingosine and the manifestation of a synergistic cytotoxic effect on lung cancer cells, along with the inhibition of sphingosine-related signalling pathways, kinase 1,

and protein kinase C, leading to reduced S1P levels in cancer cells and apoptosis induction via activation of the intrinsic mitochondrial pathway. Two additional contributions of an applied nature were also identified, focusing on the applicability of alkylphosphocholines in modern antitumour therapy due to their low toxicity to normal cells and the potential to use lower concentrations of miltefosine to further minimise undesirable side effects.

The dissertation's "Future Scientific Plans" provides the dissertationer with consistency and depth in clarifying the mechanisms discussed in the dissertation and enhances scientific interest in the current topic.

6. Personal contribution of the doctoral student and publications

In relation to the dissertation work, there are three published articles with impact factors in quartiles Q1, Q2, and Q3, one of which features Tihomira Stoyanova as the host author.

Conclusion

Tihomira Tihomirova Stoyanova's dissertation presents a current and original study on the mechanism of action of antitumour drugs on transmembrane signalling in cancer cells. The doctoral candidate demonstrates mastery of a broad range of methodologies and an excellent ability to analyse and summarise the results obtained. The dissertation, along with the accompanying documents, fully satisfies all requirements of the ZRASRB, the rules of the Bulgarian Academy of Sciences, and IBPhBME for its application.

Considering all the above, I am convinced to vote in favour and recommend the venerable Scientific jury to support the award of a "doctor" degree in professional direction 4.3. Biological sciences, scientific speciality "Biophysics" for PhD student Tihomira Tihomirova Stoyanova.

1. 03.2026
Sofia

Prepared by the opinion:
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