

## OPINION

by Prof. Dr. Iva Ugrinova, on the dissertation work of Tihomira Tihomirova Stoyanova "Antitumor lipids - influence on transmembrane cell signaling", submitted for awarding the educational and scientific degree "doctor" in the professional field 4.3 "Biological Sciences" scientific specialty "Biophysics". Scientific supervisors: Prof. Dr. Rumyana Tsoneva; Prof. Dr. Albena Momchilova, D.Sc., Laboratory "Transmembrane Signaling", IBFBMI-BAS

### **1. Relevance and significance of the developed problem**

Tihomira Stoyanova's dissertation is dedicated to an extremely relevant and scientifically significant problem - studying the mechanism of action of antitumor lipids and their influence on transmembrane cell signaling in cancer cells. Cancer continues to be among the leading causes of mortality worldwide, with breast cancer and lung cancer occupying leading positions in frequency and lethality. Despite the progress of conventional therapies, serious side effects and the development of drug resistance necessitate the search for new, more selective and less toxic therapeutic approaches. In this context, the study of alkylphosphocholines (erufosine, miltefosine) and their role in the modulation of membrane fluidity, the sphingolipid rheostat and the signaling pathways associated with cell survival and apoptosis is completely modern and has a clearly expressed translational potential. The focus on combination therapy, which aims to increase efficacy at lower doses and reduce toxicity, is particularly valuable. The development fits convincingly into current trends in molecular oncology and membrane biophysics.

### **2. Structure and logical organization of the dissertation**

The dissertation is 139 pages long and is structured in eight chapters, including an introduction, literature review, materials and methods, results and discussion, summary, conclusions, contributions and future scientific plans. The structure is logically constructed and follows the classical model of experimental work in the field of molecular biology and biophysics. The literature review covers: breast cancer and lung cancer; cell death (apoptosis, necroptosis, necrosis); cell cycle; lipid composition and membrane fluidity; sphingolipid rheostat (ceramide/S1P); role of PKC $\alpha$  and signaling pathways. The theoretical framework is well systematized and creates a solid foundation for the experimental part.

### **3. Aim and objectives**

The aim of the dissertation is clearly formulated – to investigate the mechanism of action of antitumor lipids on transmembrane signaling in cancer cells. The tasks set include:

- cytotoxic analysis of EPC3, DMS and HePC (alone and in combination);
- determination of the type of cell death;
- quantitative assessment of cell death;
- analysis of the cell cycle, migration and cytoskeleton;
- study of membrane fluidity and diffusion dynamics;
- analysis of the sphingolipid rheostat.

The tasks are specific, consistent and fully adequate to the set goal.

#### **4. Materials and methods**

The methodological arsenal used is impressive in scope and complexity. Included are: flow cytometric analysis of apoptosis and cell cycle; Nicoletti test; RT-PCR; immunoblot analysis of PKC $\alpha$  phosphorylation; confocal microscopy; sFCS (scanning fluorescence correlation microscopy); lipid extraction and gas chromatography analysis; ELISA for S1P; calculation of combination index (CI). The application of modern biophysical methods (Di-4-ANEPPDHQ, sFCS) for the analysis of membrane orderliness demonstrates a high methodological culture and thorough preparation of the doctoral student.

#### **5. Main results and their interpretation**

##### **Part I – Effect of erufosine**

A dose- and time-dependent inhibition of cell proliferation was demonstrated in MDA-MB-231 and MCF-7, with a more pronounced effect in the highly invasive line MDA-MB-231. The following were found: arrest in G2/M phase; concentration-dependent apoptosis; destruction of cytoskeletal structures and reduced migration; decreased lipid ordering and increased membrane diffusion. For the first time, a direct relationship between changes in lipid composition and membrane dynamics and cell cycle/apoptosis in MDA-MB-231 has been demonstrated.

##### **Part II – Combination therapy HePC + DMS**

The combination HePC + DMS exhibits a pronounced synergistic cytotoxic effect in A549 cells. Flow cytometric analysis shows a significant induction of late apoptosis

upon combined treatment (up to 66.11%). It is observed: inhibition of signaling pathways related to SK1 and PKC; reduction of S1P levels; activation of the intrinsic mitochondrial pathway of apoptosis. The results are well-argued and statistically supported.

## **6. Scientific and applied contributions**

Contributions of original nature

- Demonstrated connection between membrane dynamics and cell cycle in erufosine.
- Proven synergistic effect of HePC and DMS in lung cancer.

Contributions of applied nature

- High selectivity towards tumor cells over normal ones (MCF-10A, HUVEC) was shown.
- Outlined prospects for inclusion in polymer carriers and in vivo studies.

## **7. Publication activity**

The results have been published in renowned international journals (Q1–Q3), including *Biomolecules* (Q1) and *Chemico-Biological Interactions* (Q1). The publication activity fully meets and exceeds the minimum requirements for awarding the ONS "doctor".

## **8. Critical notes**

The dissertation was completed at a high scientific level. As a recommendation, the possibility of further expanding the in vivo validation of the results can be indicated, which, however, is the subject of future research and does not reduce the scientific value of the present work.

## **9. Conclusion**

The dissertation work of Tihomira Tihomirova Stoyanova represents an independent, original and completed scientific research in the field of molecular biology and biophysics. The results of the dissertation work have been published in three articles with a total impact factor of 10.521, two publications are in journals with Q1, and one is in a publication with Q3. Tihomira's total impact factor significantly exceeds the requirements for acquiring the educational and scientific degree "doctor" according to the national regulatory documents and the regulations of the IBFBMI.

The goal has been achieved. New scientific results of a contributing nature have been obtained, published in international publications. The doctoral student demonstrates high methodological preparation, analytical thinking and the ability to interpret complex molecular mechanisms. Based on the above, I confidently propose to the esteemed scientific jury to award Tihomira Tihomirova Stoyanova the educational and scientific degree "doctor" in professional field 4.3 "Biological Sciences", scientific specialty "Biophysics".

Prepared by:

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