REVIEW

by **Prof. Ivan Georgiev Ivanov, PhD, DSci**
of the thesis of Prof. Dr. Jordan Atanassov Doumanov entitled *" Self-organization and surface properties of hBest1 in models of biological membranes"*, presented for the awarding of the scientific degree "Doctor of Sciences" in the Area of Higher Education 4. "Natural sciences, mathematics and informatics", Professional direction 4.3. Biological Sciences, Scientific speciality "Molecular Biology"

1. Brief biographical data

Mr. Jordan Atanassov Doumanov was born on September 5, 1973 in the city of Bansko, Bulgaria. In 1994, he was enrolled as a full-time student at the Faculty of Biology, University of Sofia (SU), graduating with a MS degree in "Cell Biology and Developmental Biology" (1999), and a second MS degree in "Teacher of Biology". Until 2001, he worked as a researcher in human genetics at the University of Greifswald, Germany and also at the Institute of Biochemistry in Aachen, Germany. Since 2001 he has been a full-time PhD student at the University of Hohenheim, Stuttgart, Germany obtaining a PhD degree in "Biochemistry" in 2006. The same year he was elected Assistant Professor at the Department of Biochemistry, Faculty of Biology, SU where he consecutively held the positions of Senior Assistant Professor (2007-2011), Chief Assistant Professor (2011-2014), Associate Professor (2015) and Full Professor (2021). Dr. Dumanov has been a visiting scientist at CABIMER, Seville, Spain and the Vision Institute at Marie Curie University, Paris, France. He is a member of Union of Bulgarian Scientists, division "Biochemistry, Biophysics and Molecular Biology". Currently, Prof. Dumanov is lecturing Biochemistry, Biological Membranes, Protein Sorting and Cell Polarization at the Faculty of Biology, SU. He has supervised 4 PhD and 9 graduate students. He is the author of 64 scientific articles, of which 44 in IF journals (total IF 157.06). Dr. Doumanov participated in the development of 20 scientific projects financed by the Ministry of Education and Culture and the SU Science Fund.

2. Overview of the documentation related to the dissertation defense

The documents relating to the defense procedure, as well as the dissertation itself, have been prepared in accordance with the requirements of the Law on Development of the Academic Staff in the Republic of Bulgaria (LDASRB) and the Rules for its implementation. The thesis covers 196 standard pages, contains 294 references, 69 figures, 3 tables and 30 pages appendices. It is structured in a classic style: Literature review, Aims and Tasks, Materials and Methods, Results and Discussion, Conclusions, Contributions and References.

3. Relevance of the thesis topic

The dissertation work of Prof. J. Doumanov is devoted to the clarification of the biochemical and molecular-biological bases of Best's hereditary retinopathies. In this thread, he has investigated the self-organization and surface properties of human bestrophin-1 (a product of the BEST1 gene) in model biological membranes. Taking into account the great social importance of the eye degenerative diseases, most often ending in blindness, the relevance of the dissertation topic is indisputable.

4. Evaluation of the dissertation work

Literature review. The literature review covers 20 pages and is based on about 300 literary sources. It is dedicated to the molecular etiology of the hereditary degenerative eye disease vitelliform macular dystrophy, called Best's disease. To better understand its essence, Prof. Doumanov introduces the reader through a brief description of the anatomy and

physiology of the eye, the retina structure and function, focusing on the protein bestrophin-1 and its BEST1 gene. It becomes clear that the BEST1 gene has three more invariant genes (BEST2, BEST3 and BEST4) whose products belong to the large family of calcium-activated chloride ion channels (CACC). Bestrophin-1 is composed of 585 amino acids (MW = 68 kDa) and is localized in the basolamellar membrane of the retinal pigment epithelium (RPE). Its main function is to regulate intracellular calcium stores by transporting chloride anions in opposition to Ca^{2+}. The review, which is illustrated with high-quality color figures, describes in details the tertiary structure of dystrophin and the way it is incorporated into the cell membranes. A special section is devoted to the nature and functions of cell polarization, as well as to the signaling molecules involved in its realization. Since Prof. Doumanov's goal is to study the properties of bestrophin-1 *in vitro* in model membranes, he has dedicated the last section of his review to phospholipid membranes, where in the light of the latest literature data, he presents their composition, structure and asymmetry. Special attention is paid to lipid rafts and their main constituents cholesterol and sphingolipids, which can directly influence the structure and activity of the channel proteins. Detailed knowledge of the molecular interaction between the membrane substances is important for further creation of adequate models of eye dysfunctions and the development of methods for their prevention and treatment.

Goals and tasks. The thorough and critical analysis of literature has helped the author to find his research niche and formulate the aims and objectives of his thesis. The aim is to trace and investigate the role of hBest1 in the cell, to shed more light in its structure, organization and functions, and to study the molecular mechanisms of the human bestrophinopathies. In accordance with this goal, two adequate and feasible tasks have been formulated.

Methodology. The methods applied in Dr. Doumanov's dissertation can be classified as: *cell biological* (creation of stable cell lines expressing hBest1; monitoring of cell growth; determination of mitotic index and morphology of cells; measurement of metabolic activity by MTT test; measurement of transepithelial resistance of cells; determination of apoptosis, etc.), *molecular biological* (mutagenesis; Real-Time PCR;), *immunochemical* (immuno-fluorescence; immunoprecipitation; immunodetection), *biochemical* (protein biotinylation; Western blot analysis; lipid isolation; lipid fractionation; affinity and molecular sieve chromatography), *spectral* (infrared spectroscopy with Fourier transformation), *physicochemical* (tensiometric measurements of one-, two- and three-component Langmuir monolayers), *microscopic* (Brewster-angle microscopy; atomic force microscopy).

Results and Discussion. The thesis results (presented on 80 pages) can be divided into two major groups: *a) Results obtained from cell culture studies* and *b) Results obtained from model biological membranes.*

In choosing an appropriate cell line for his studies Dr. Doumanov was guided firstly, by whether the hBest1 is naturally expressed in, and secondly, what the cell polarization is. The lack of endogenous expression of hBest1, such as in the MDCK II, RPE-J, RPE-1, Hela and HEK293 cell lines, is advantageous for the purposes of the study since no additional gene silencing is required. Amongst the listed cell lines, MDCK II has an additional advantage because their polarization is transient (develops only in about five days) and therefore does not overlap with the formation of a monolayer. Thus this allows the basolamellar and apical domains to be easily distinguished by specific marker proteins. The correct choice of cells had predetermined the further successes of the author in studying the intracellular expression of the hBest1 protein.

MDCK II cells were transfected with normal and mutant hBest1, and the bestrophin expression was monitored in polar cells.

Prof. Doumanov's results confirm the fact that in MDCK II cells the BEST1 gene is transcribed but not translated, while in transfected cells hBest1 is fully expressed and

localized to the basolateral membrane (similar to RPE cells). In other words, MDCK II cells seem to be an adequate model for studying the molecular mechanisms of human bestrophinopathies, the basis of which, according to the working hypothesis, is the localization of hBest1 in the basolateral membrane of RPE. The author hypothesizes that the correct localization of hBest1 depends on "at least three sorting signals, and disrupting the amino acid composition of each signal individually cannot reverse the localization of the protein at the apical membrane, but can partially change in this direction." He experimentally demonstrated that structural changes in the basolateral sorting motifs Y85VTL, Y97ENL and Y227DWI, responsible for the clinical manifestations of human bestrophinopathies, cause disorders in sorting and localization of hBest1 mutant forms. He also shows that phosphorylation of Tyr 227 in hBest1 affects its basolateral localization. Prof. Dumanov assumes that the apical localization and increase in the number of hBest1 molecules in the apical membrane disrupts ion transport in RPE cells, and increases the concentration of chlorine anions in the space around the photoreceptors. These molecular events lead to their damage and death.

Dr. Doumanov's results demonstrate that hBest1 does not significantly affect the growth characteristics, metabolic activity, morphology, and polarization of MDCK II cells, but it does affect their transepithelial resistance (TER). He found that Glu and GABA increased and ATP decreased TER values. He studied also the resistance of transfected MDCK II cells to phospholipase A2 (PLA2) and observed that their resistance rose, which was explained by the increased content of non-lamellar lipids in the cell membranes. The latter indicator, in combination with TER values, indicates that the metabolism altered by hBest1 towards synthesis and/or accumulation of non-lamellar lipids, reduces the effect of PLA2 on hBest1-transfected MDCK II cells. However, even with reduced PLA2 activity, the fluid-ordered phase in cell membranes (where about 70% of hBest1 is localized) increases and the fluid-disordered phase decreases. All this is an indication for inactivation of the ion channel in the fluid- the ordered phase.

Prof. Doumanov studied the surface physicochemical characteristics of Langmuir monolayers and Langmuir-Blodgett films of purified hBest1, as well as two- and three-component Langmuir monolayers and Langmuir-Blodgett films containing hBest1 in combination with phosphatidylcholine, sphingomyelin, and cholesterol. Thus he shed light on the mechanism and forces of interaction between the different membrane components and determined the change in the hBest1 secondary structure parameters as influenced by various low molecular weight agents. For example, he found that Ca^{2+} led to an increase in the hBest1 helical structures from 51% to 59%. In addition to Ca^{2+}, Glu and GABA also cause conformational changes.

Dr. Doumanov managed to determine the geometric parameters of the hBest1 molecule. It is described as an ellipsoid-shaped particle with the following dimensions: 100 × 160 × 75 Å. This shape however, can be changed er the action of Ca^{2+}. The hBest1 molecular parameters as well as the degree of its packing in artificial/model membranes under the influence of various low molecular weight substances are also calculated.

All obtained results are thoroughly discussed in the light of the existing scientific hypotheses.

5. Conclusions and contributions

Prof. Doumanov's studies have significant scientific and applied contributions. As scientific contributions, I would point out the results of his systematic studies on the mechanism of interaction of hBest1 with the cell membranes basic lipids, determination of their organization and functions, as well as the influence of various low-molecular weight constituents on hBest1 conformation, parameters and aggregation. In this regard, an original

method was developed to quantify the apical and basolateral localization of hBest1 based on confocal microscopy.

Applied contributions include creation of two new stably transfected hBest1 cell lines originating from RPE and kidney epithelium respectively, as well as the original method for isolation and purification of hBest1 from transfected MDCKII-hBest1 cells.

6. Evaluation of the scientific papers related to the thesis

In connection with the reviewed dissertation, 18 scientific articles have been published, of which 16 in IF journals (total IF 62.303). In 16 of them, Prof. Doumanov is the lead (first or last) author. The 16 papers in IF journals are distributed by Q index as follows: Q1 – 9 papers; Q2 – 3 pcs.; Q3 – 2 pcs.; Q4 – 2 pcs. These papers have been cited 44 times so far. The thesis results have been reported also at 22 scientific forums. Parts of them have been included in 4 PhD and 3 MS theses. The dissertation has been financially supported by6 research projects granted by the National and SU "St. Kliment Ohridski" Research Funds.

7. Abstract

The thesis abstract adequately reflects its content and achievements.

8. Personal involvement of the author

The fact that Prof. Dumanov is the lead (first or last) author in 16 of the 18 scientific papers related to his dissertation, as well as the fact that he is the senior investigator of all scientific projects financially supporting his studies give me reason to conclude that the majority of the thesis achievements are his own merits.

9. Summary assessment

The summary assessment according to the regulations for implementation of the LDASRB for the scientific degree "Doctor of Sciences" in the field of higher education 4. "Natural sciences, mathematics and informatics" is presented in the following table.

Indicator	**Required minimum**	**Actual number of points**
A	50	50
B	100	100
C	100	100
D	100	> 160
Total	**350**	**> 400**

As can be seen from the presented data, the scientometric indicators of Prof. Jordan Doumanov related to his DSci thesis exceed the officially accepted ones according to the requirements of the LDASRB.

CONCLUSION

Prof. Jordan Dumanov's dissertation entitled "Self-organization and surface properties of hBest1 in models of biological membranes", presented for the acquisition of the scientific degree "Doctor of Sciences", is dedicated to clarifying the molecular basis of Best's hereditary degenerative retinopathy. To this end he employed diverse methods of molecular and cellular biology, biochemistry, biophysics, immunology, etc., to prove the role of conformational changes, aggregation and cellular localization of the hBest1 in the occurrence and progression of human bestrophinopathies. As a result, he obtained significant results contributing to both science and practice. In connection with this he published 18 scientific papers in peer reviewed journals and 22 reports at national and international scientific forums. By his thesis,

Prof. Dumanov presents himself as an established and authoritative researcher in the field of biochemistry and molecular biology. His formal indicators fully satisfy and even exceed the official requirements of the LDASRB for the acquisition of the scientific degree "Doctor of Sciences" in Higher Education Region 4. "Natural Sciences, Mathematics and Informatics", Professional Direction 4.3. Biological Sciences, Science Specialization: "Molecular Biology", which gives me reason to confidently recommend to the respected Scientific Jury to award it to him.

Sofia, 17.11.2023

Reviewer:

/Prof. Ivan Ivanov/