R E V I E W

of a dissertation for the award of the scientific degree
"Doctor of Science" in the Professional direction 4.3. Biological Sciences, Scientific (Molecular Biology)

Dissertation topic: "Self-organization and surface properties of hBest1 in models of biological membranes"
Author: Prof. Dr. Jordan Atanassov Doumanov

Reviewer: Academician Prof. Roumen Georgiev Pankov, Faculty of Biology at SU "St. Kliment Ohridski", member of the academic jury, appointed by order of the Rector of the University of St. Kliment Ohridski" RD38-595/31.10.2023.

Brief biographical details of the applicant

Professor Jordan Doumanov was born in 1973 in Bansko. He completed his higher education at SU "St. Kl. Ohridski" in 1999, with a major in Biology and a master's degree in "Cell and Developmental Biology", simultaneously receiving a second specialization - "Teacher of Biology". After graduation, he went to Germany, where he first worked as a researcher at the Institute of Human Genetics at the University of Greifswald and the Institute of Biochemistry, RWTH-Aachen, and from 2001 he joined the University of Hohenheim in Stuttgart as a PhD student. In 2006, he successfully defended his thesis on "Identification of the basolateral sorting signal in the cytoplasmic domain of the interleukin-6 signal transmitter gp130". In the same year, Prof. Doumanov returned to Bulgaria and began working as an assistant professor in the Department of Biochemistry of the Faculty of Biology at SU "St. Kl. Ohridski". His professional development continued in this department, where he successively held the positions of chief assistant professor (2011-2014), associate professor (2015) and full professor (2021).

Prof. Doumanov specialized for two years at the Institute of Vision, University Pierre and Marie Curie, Paris, France and CABIMER, Seville, Spain for four months. He is a member of the Union of Scientists in Bulgaria, Biochemistry, Biophysics and Molecular Biology section. He participated in the implementation of 20 projects financed by Sofia University and national organizations, leading six of them. Three doctoral theses and nine diploma theses have already been successfully defended under his supervision.

General data on the documentation related to the dissertation work

The dissertation, the author summary and the attached documents for the defense have been prepared in full compliance with the Law on the Development of the Academic Staff of the Republic of Bulgaria, the Regulations for the Development of the Academic Staff of Sofia University "St. Kliment Ohridski" and the internal regulations of the Faculty of Biology. The presented scientific publications fully corresponds to the profile of the announced competition.

Relevance of the dissertation topic

The main object of the conducted study is the human transmembrane protein bestrophin 1 (hBest1), expressed by the cells of the retinal pigment epithelium, where it is involved in the calcium-dependent transport of chloride ions. In addition, there is evidence of its function as a channel in the central nervous system, where it transports γ-aminobutyrate (GABA) in glial cells and glutamate (Glu) in astrocytes and neurons. Interest in this protein is fueled by its involvement in a group of hereditary degenerative eye diseases known collectively as the bestrophinopathies. Over 200 different mutations in hBest1 involved in bestrophin pathologies have been described to date. Despite their relatively low frequency, this group of diseases has socially significant dimensions, as they cause a progressive loss of vision in humans, which is associated with a strong reduction in the quality of life of the affected individuals.

Elucidating the molecular mechanisms leading to the manifestation of bestrophinopathies requires a good knowledge of the structure, the way of integrating in the membrane and the normal functioning of bestrophin. As with any transmembrane protein, this is largely determined by the interaction with its lipid environment, an issue that is still poorly understood. The conducted research provides answers to a large part of these questions, presenting new data on the characteristics of the protein, its behavior and its interactions with basic membrane components. Everything mentioned above gives me reason to define the scientific problem developed in the dissertation as relevant both from a fundamental and from a practical point of view.

Structure, goals and methodical approach of the dissertation work

Prof. Jordan Doumanov's dissertation is written on 196 pages, includes 69 figures and 3 tables. Also very useful, from an evidentiary point of view, are the included 30 pages of appendices, providing information related to the materials used and illustrating experimental results not included in the main text. The dissertation is structured according to the accepted standards for this type of scientific work and includes the main sections: Literature review of 23 pages, Aim and tasks - 1 page, Materials and methods - 22 pages, Results and discussion - 87 pages, Conclusions - 3 p., Contributions - 1 p., Future research - 1 p. Also included is a list of 18 scientific articles published by Prof. Dumanov and 22 participations with reports at scientific forums, all in connection with the studies from the dissertation work.

The review of the literature, supported by 294 literature sources, is balanced in volume and content and shows a very good knowledge of published information related to the dissertation topic. A brief but informative review of the structure and function of the eye is given, with special attention to the retinal pigment epithelium. In the following sections, the current data on the generalized retinal damage known as bestrophinopathies are presented, with the focus being given to the currently known data on the main carrier of these diseases - the protein bestrophin-1 (hBest1). As far as bestrophin is a membrane component operating in an epithelial cell type, two more sections are also presented - "Cell polarization" and "Biological membranes", which summarize the main characteristics of the environment in which the studied protein functions. The large amount of information is well systematized, summarized and illustrated with 14 relevant figures. The presentation of the data is clear, professionally done and includes all the basic statements that are necessary to justify and understand the research carried out.

The goal is clearly stated - "to trace and investigate the role of hBest1 in the cell, its structure, organization and functions, the relationship between structure and functions, as well

as the molecular mechanisms leading to bestrophinopathies". It is specified in two complex tasks, which clearly outline the perimeter of the conducted research. A wide palette of well-chosen interdisciplinary methodical approaches is used to solve the set tasks. The "Materials and methods" section describes various methods from the field of biochemistry and molecular biology (affinity and molecular sieve chromatography, immunoprecipitation, SDS-PAAGE, Western blotting, Real-Time PCR, mutagenesis, etc.), physical chemistry (measurement of hysteresis π/A contraction and expansion isotherms, Brewsterangle microscopy, atomic force microscopy, infrared spectroscopy, etc.) and cell biology (cell culture, transfection, creation of stably transfected cell lines, immunofluorescence, MTT test, measurement of transepithelial resistance and etc.). The presentation of the experimental procedures contains sufficient information necessary to evaluate the experiments performed.

Analysis of experimental results, conclusions and contributions

The "Results and Discussion" section is the main part of the dissertation in terms of importance and volume. The obtained experimental data are presented and discussed in the section in order that follows the defined tasks of the dissertation work. In my review, I will also follow this order by summarizing the main scientific achievements.

The first set of results includes studies on the human Best1 protein in its natural environment – the membranes of cells from different cell cultures. Examining different cell lines (RPE-1, RPE-J and MDCK II), Prof. Doumanov shows that the best experimental model for tracking the cellular localization of transfected hBest1 is cells from the epithelial line MDCK II, in which the protein is localized along the basolateral membrane similar to retinal pigment epithelial cells, which are the natural site of bestrophin expression. Studies on MDCK II - hBest1 cells demonstrate that they show an increase in Ld domains compared to Ld domains in untransfected MDCK II cells, and hBest1 localizes and self-organizes mainly in Ld regions (about 60-65%) of cell membranes, vs. 30-35% in the Lo domains (lipid rafts). Using this appropriate experimental system, Prof. Doumanov investigated the effects of mutant forms of hBest1 - Y85H, Q96R, R25W and Y227N, which are known to cause Best's classic vitelliform macular dystrophy (BVMD), most likely affecting the basolateral sorting motives. After expressing his lab-made mutant forms of bestrophin and tracking their cellular localization, it was shown that these mutations actually disrupt the proper localization of the protein and are toxic to cells. Of interest and potentially important for future research is the finding showing that phosphorylation of hBest1 on Tyr 227 affects the basolateral localization of the protein.

In fulfillment of the second complex task, indicated in the objectives of the dissertation, Prof. Doumanov moves from cell biological to biophysical experimental setups, using them for a more detailed study of the ways in which hBest1 interacts with individual membrane lipids and to provide an answer to the question of how its conformation, molecular organization and activity change in the "clean" environment of model biological membranes. Prof. Doumanov's team secured this opportunity after successfully developing a method for purifying hBest1 from stably transfected MDCK cells, achieving a significant yield of about 2.8% of the total protein content.

Using the purified protein and Fourier transformed infrared spectroscopy, Prof. Doumanov fills in the missing data on the secondary structure of human Best1 in the literature. It was shown that 51.1% helical structural elements, including major and short α-helices (23.9%), and 3_{10}-helices (27.2%) are involved in the secondary structure composition of

hBest1. The addition of Ca^{2+} caused an increase in all helical structures to 59.2%, mainly due to an increase in the content of α-helices (by 5.6%); reduction of β-turns and loops up to 27.2%; reduction of aggregate and antiparallel sheets from 16.7% to 13.6%.

In addition to being a calcium-activated chloride channel, Best1 has also been shown to be permeable to neurotransmitters such as Glu and GABA. This leads to the logical assumption that neurotransmitter transport may also induce various conformational changes in Best1 as well as in the membranes themselves. Using Langmuir monolayers and Langmuir-Blodgett films, Prof. Doumanov conducted in-depth studies of the structural and surface properties of hBest1. By comparing the π/A isotherms of monolayers of pure hBest1 or in the presence of Ca^{2+}, Glu, and GABA, it was found that the smallest possible area for the molecule is observed for the pure protein, and that it does not change significantly upon addition of the neurotransmitters (A_0 = 3700 Å2/monomer), while in the presence of calcium ions it decreases substantially (A_0 = 3360 Å2/monomer).

Analyzing the curves of the elastic moduli versus the surface pressure, it was found that the monolayers of hBest1, hBest1Ca^{2+}, hBest1Glu and hBest1GABA have different packing densities and the values of Cs^{-1} (max) are in the lower limit for liquid-stretched lipid films. Additional information on the molecular interactions, structural rearrangements and stability in hBest1 monolayers was obtained by studying the compression-decompression hysteresis of the π/A isotherms. Monolayers of hBest1 (as well as Ca^{2+}, Glu, and GABA added) were found to have significant hysteresis that decreased with increasing π. Further studies on Langmuir monolayers of hBest1 by Brewsterangle microscopy (BAM) showed that monolayers of hBest1, hBest1Ca^{2+}, hBest1Glu and hBest1GABA have different packing densities that correspond well with the π/A isotherm results. Since VAM does not allow distinguishing the exact conformations of hBest1 protein complexes and possible changes after treatment with Ca2+, Glu or GABA, Prof. Doumanov switched to using atomic force microscopy (AFM), a method that allows recording changes at the nanometer level. AFM images from Langmuir-Blodgett films convincingly show that human Best1 is larger than its hitherto characterized chicken counterpart, oval in shape (100 × 160 Å and 75 Å high), and addition of Ca^{2+}, Glu, and GABA leads to changes in its conformation, but only Ca^{2+} is able to induce dimerization and trimerization of the protein.

Extending the in vitro model systems to bring them closer to natural biological membranes, by using combinations of the studied protein and different lipids in the formation of Langmuir monolayers, Prof. Doumanov shows that phospholipids (POPC) can significantly change the organization and activity of hBest1 in such conditions. The results show that: hBest1 exerts a "fluidizing" effect on POPC monolayers, which is why hBest1/POPC films show increased elasticity; the presence of phospholipid abolished the effects of Ca2+, Glu and GABA recorded in pure protein monolayers; hBest1 and POPC do not mix at the surface of the aqueous subphase; hBest1 in mixed protein/phospholipid monolayers increases its height and lateral dimensions and changes its compact oval shape in the presence of Ca^{2+}, Glu, and GABA.

Using sphingomyelin (SM) and mixed hBest1/SM Langmuir monolayers and calcium, glutamate, and gamma-aminobutyrate, it was demonstrated that addition of the protein induces "fluidization" of hBest1/SM films, improves the order of mixed Langmuir monolayers, and that miscibility between hBest1 and sphingomyelin is a thermodynamically advantageous process, which is a prerequisite for strong protein-lipid interactions in biological membranes as well. Making the experimental setup more complex, achieved by adding another common

membrane component, cholesterol, demonstrated that it induced condensation in monolayers containing hBest1, hBest1+SM, and hBest1+POPC, and studies on the films formed by hBest1/POPC/Chol and hBest1/SM/Chol show that mixing of hBest1 and lipid molecules at the subphase surface is a spontaneous and thermodynamically advantageous process. In addition, cholesterol was found to enhance and stabilize inter-component mixing in hBest1/POPC/Chol and hBest1/SM/Chol films regardless of experimental conditions.

In the last part of his dissertation, Prof. Doumanov presents initial data and outlines an interesting new direction of his research related to the creation of nanoparticles: nanodiscs, polymer and bicontinuous nanoscale structures with the participation of hBest1, to be used for protein transport and integration in cell membranes and restoration of their functions.

From the conducted research, 18 general conclusions were drawn and three contributions of a fundamental nature, two contributions of a scientific-applied nature and two contributions of a methodological nature were formulated. All conclusions and contributions are experimentally substantiated and correctly formulated, which gives me reason to accept them without reservations.

The abstract is prepared according to the requirements and reflects all the main results and contributions of the dissertation work.

Conformity of the presented documentation with ZRASB and scientometric indicators

The total scientific output of Prof. Doumanov includes 64 articles, of which 44 were published in journals with an impact factor (total IF 157.06) and cited 183 times in the Scopus database. For the current procedure, he has submitted a list of 18 publications and 22 participations in scientific forums at home and abroad. Of the scientific articles, 16 were published in journals with an impact factor and were cited 44 times. The overall impact factor of these articles is 62.3. In 16 of them (89%), he is the first or last author, which is indisputable proof of his leading role in the conducted research. Of the 16 articles with an impact factor, more than half (56.2%) were published in first quartile (Q1) journals - an indication of their high scientific quality.

The report on the fulfillment of the minimum national requirements under Art. 2b of ZRASRB for scientific area 4. Natural sciences, mathematics and informatics professional direction: 4.3 Biological sciences, presented by Prof. Doumanov includes the following indicators: group A - 50 points; group B - 100 points; according to indicator D - 100 points, and according to indicator D (citations) he collects 160 points with a minimum of 100 points. Thus, with a required minimum of 350 points for the scientific degree "Doctor of Sciences", according to PPZRASRB, Prof. Doumanov forms 390 t., which not only meets but also exceeds the minimum national requirements necessary for this scientific degree.

Conclusion:

The dissertation work "Self-organization and surface properties of hBest1 in models of biological membranes", presented by Prof. Doumanov, is a focused monographic work at a high scientific level, which meets the requirements for a dissertation for obtaining the scientific degree "Doctor of Sciences". It is the result of long-term and purposeful research activity and develops essential questions related to the topical topic of the structure, organization and functions of the hBest1 protein, mutations in which cause the socially significant diseases bestrophinopathies. A series of pioneering studies have been conducted, the results of which have found a wide response, both in our and in the international scientific community. They

characterize their author as a highly qualified professional in this specific field of molecular biology.

All of the above gives me the reason to give my positive assessment and recommend to the respected members of the Scientific Jury to award Prof. Dr. Jordan Atanassov Doumanov the scientific degree "Doctor of Sciences".

17.12.2023

Reviewer:

/Acad. Prof. Roumen Pankov/