Review of the doctoral dissertation
by Ambroise Wu, MA
titled: "The role of the ABCA1 protein in the organization of the cell membrane in mammal cells ", performed at the Department of Cytobiochemistry of the Faculty of Biotechnology of the University of Wrocław under the supervision of Dr. hab. Aleksander Czogalla, the head of the Department of Cytobiochemistry and assistant supervisor Dr. Tomasz Trombik, assistant professor at the Department of Biophysics, Faculty of Biotechnology.
The review was prepared at the request of the Chairman of the Scientific Discipline Council of Biological Sciences of the University of Wrocław, Dr. hab. Eng. Marcin Kadej, Prof. UWr, on May 25, 2022, supported by the Council Resolution No. 114/2022 of May 19, 2022.

Topics, purpose and scope of the dissertation

Discovery in 1976 of the phenomenon of multi-drug resistance (MDR) initially concerned neoplastic cells, as a phenomenon that sometimes critically hinders chemotherapy. The responsible proteins were named only in 1990 (ABC proteins - ATP-binding cassette proteins), still as interfering proteins in the course of chemotherapy. About 50 proteins from this group have been identified over the past decades, and grouped into 7 subgroups. It is obvious that due to the relatively short time of historical development of medicine, describing cancer as a disease and defining methods of its therapy, multi-drug resistance could not be the main evolutionary factor that led to the creation and diversification of these proteins. It is worth mentioning that they are encoded by one of the largest gene superfamilies.

It must be remembered that these are membrane proteins, which in many cases are simply used for active transport of substances at the expense of ATP hydrolysis. Therefore, it should be assumed that these proteins actively support the activity of the metabolome by changing the concentrations of individual reagents. But that's not all, they are membrane and transmembrane proteins, genetically diverse and occurring in the form of dimers, but also monomers. Perhaps, therefore, one of their primary functions was like that of many other membrane proteins - the influence and modulation of the structural dynamics of biological membranes, and thus the processes dependent on them, not only transport. Bearing in mind that ABC proteins have an ATPase function, it can therefore also be expected to participate in the energy metabolism and homeostasis of ATP and other nucleotides as well as phosphates and pH.

On the other hand, it is worth remembering that phospholipids, water and cholesterol are not explicitly recorded in the genome of a cell in the way that proteins are stored. However, because there are many different pools of membranes in a normal cell with differing properties, proteins

being one of their essential components, there must be a network of metabolomic mechanisms that maintain a balance between genetic information and other cell elements not directly encoded in the genome. Due to the common occurrence in all domains of organisms and due to the evolution of the ABC superfamily, this could be one of its primary functions. The subject of the work presented for review could be briefly paraphrased in this way.

The work focuses mainly on the ABCA group, specifically the ABCA1 protein, its role in maintaining the structure and dynamics of the biological membrane, in the metabolism and functioning of cholesterol, and in the mechanism of maintaining resistance to amphotericin B - an antifungal antibiotic.

The thesis was prepared at the Department of Cytobiochemistry and the Department of Biophysics, Faculty of Biotechnology, University of Wrocław, mainly due to the availability of biophysical methods. Research on biological membranes has been the domain of biophysics for decades, and Wrocław is one of the leading centers of membrane research. Some of the results were obtained in cooperation with the Maria Curie-Skłodowska University in Lublin.

Discussion and evaluation of the dissertation content

The dissertation opens with a summary in English and Polish, followed by a theoretical introduction, which begins with a discussion of the theory and facts about the structure of biological membranes, then membrane proteins and their functions, in turn proteins encoded by the ABC gene superfamily, ABCA and ABCA1 characteristics, and finally practical aspects related to the functioning of ABCA1 (cancer, inflammation, Tangier disease - congenital HDL deficiency). It also describes the role of different types of lipids and their interactions, including cholesterol, and the regulation of the expression of the ABCA1 gene, from transcription to product degradation. It can be seen that the author focuses on biology and biochemistry, limiting the methodological issues to the necessary minimum. Unfortunately, I could find here only few mycological and mycopathological aspects, which are sorely lacking in the whole work. The biology of melanoma is also very superficial, starting with the rationale for choosing this and other cell models used at work.

The next step is to formulate the aim of the work, and here, too, the brevity is somewhat lacking - the goals could be formulated in the form of a list, general goal and specific goals, which would make it easier to formulate conclusions corresponding to the goals. Anyway, we understand that the goal of the work was to explore the mechanism of resistance of tumor cells

with an active gene encoding ABCA1 to amphotericin B. It is related to the restriction of cholesterol mobility.

However, since to fully understand this relationship it is necessary to explore the role of the ABCA1 transporter in cholesterol metabolism, I think that from this more general topic we should start discussing the results, and then move on to the issue of amphotericin B. It probably has to do with the order of publishing, but we often encounter a situation of reversing the order of publication, because more time is needed to prepare a more general publication explaining the mechanisms discussed in a more detailed work.

The author explains this procedure in a different way, including the results published in the paper on amphotericin, in order to use them in the discussion (as he writes in page 34). This calls into question about how to treat these results. In principle, the work could have the generally accepted form of a "pin" of 2 experimental papers with discussion. In the present situation, it is not fully known to what extent and which results concerning amphotericin B should be treated as independent (then statements of other co-authors about their participation in the work should be presented). As a reviewer, I have to draw one's attention to it, although the total of the results is enough to state that there is no doubt as to the advisability of its further procedure. Anyway, using these results in a discussion is a good idea when it comes to using them in general.

Other points of discussion will be discussed further. The next, most extensive chapter of the work discusses the Results. As mentioned, it is divided into 2 parts, III.1 - mammalian amphotericin B cytotoxicity and III.2. - the influence of ABCA1 on the lateral organization (ie. concerning horizontally adjacent lipids and their groups, eg. "rafts") of the biological membrane and metastasis in the context of cholesterol participation in the metabolome of melanoma cells. In these chapters it was shown, *inter alia*, that the level of ABCA1 is inversely proportional to the level of cholesterol in the cells of some human melanoma lines, and therefore affecting the fluidity of their membranes, which may result in a change in migration properties and metastasizing. Cholesterol, on the other hand, was accumulated in cells lacking ABCA1 activity. This resulted in a number of changes in the metabolome of these cells, and above all in the activity and phosphorylation of important kinases and of participants in their signaling pathways, such as CREB, STAT3 or AKT. This is the evidence of an interference with metabolism through ABCA1 activity. These manipulations did not alter melanoma cell proliferation, but metastasizing - yes, for example, by affecting extracellular matrix degradation, focal adhesion, redistribution of β3 integrin and formation of invadopodia. This is

a very important effect of ABCA1 activity, described by the Author (and the co-authors) in human melanoma cells for the first time.

Since ABCA1 influences membrane fluidity, orderliness, migration and metastatic properties by influencing cholesterol metabolism, it is easier to explain the mechanism by which ABCA1 generates resistance to amphotericin B in mammalian cells. One of the ligands binding amphotericin (the main mechanism of amphotericin B toxicity) is cholesterol (and other fungi steroles in the mycotoxic activity). ABCA1 activity would remove cholesterol from cells, which, in the absence of functional ABCA1, would accumulate in membranes and bind to amphotericin B, increasing its toxicity. Fungi, as devoid of this mechanism of the removal, would therefore, the way I understand this, subjected to the action of an antibiotic which binds to membrane sterols at much lower concentrations than host cells with an active ABCA1 cassette which removes the antibiotic along with the cholesterol bound. The obtained results are discussed in the bulk text, and then collected and summarized in the general discussion, conclusions and perspectives for further research.

The next chapter of the work is material and methods, with the methods divided into molecular, cellular, biochemical, microscopic, fluorimetric (FLIM) and statistical ones, and followed by an extensive citation list. It is worth emphasizing the variety of methods that relate to the phenomena observed at different levels of the organization of living matter. This part is followed by a list of publications (439 items). The works are cited by numbers, in the order of citation in the text.

The work ends with the list of the author's publications in which some of the results were published, i.e.

1. Would ABC transporters tune the plasma membrane organization? Wu, A. Wójtowicz, K. Savary, S. Hamon, Y. Trombik, T. Cellular & Molecular Biology Letters (2020).

2. ABCA1 transporter reduces amphotericin B cytotoxicity in mammalian cells.Wu, A. Grela, E. Wójtowicz, K. Filipczak, N. Hamon, Y. Luchowski, R. Grudziński, W. Raducka-Jaszul, O. Gagoś, M. Szczepaniak, A. Chimini, G. Gruszecki, W. I. Trombik, T. Cell Mol Life Sciences, Springer, Volume 76 / Issue 24, pp 4979-4994. 2019

These works have already been cited 10 times.

Assessment of the editorial staff

The dissertation is written in English and it contains an abstract in Polish. It is very extensive for a doctoral dissertation. The Author, however, has a lot to show and the size of the work is

largely justified. He divides his work in an unusual way, but used in scientific works, i.e. he discusses the research methodology at the end of the work (some journals use this approach). The Author presents 57 drawings (there are two drawings on p. 88 numbered as 51) and at least 6 tables (5 numbered and 1, on p. 84, without a number). He cites 439 references, which is a lot. The presentation of the results is systematic, logical and makes it easy to track this extensive work (except for the numbering flaws mentioned above). Unfortunately, it contains a lot of minor typing and editing errors, eg the author uses the letter "x" in place of the "×", the use of spaces is also inconsistent. It should be between the number and the unit abbreviation if it starts with a letter (e.g. 10 cm), but should not be there if the unit is expressed in another sign (e.g. 5%). When specifying the manufacturer of the equipment, reagents, or animal supplier, the name of the company, the headquarter - headquarters of the company and the state are usually given, and in the case of the USA, the abbreviation of the state. The Author also has some problems with English, especially with singular and plural, but these are trifles. Today, journals usually perform typographic corrections and catch such errors.

Discussion of the defects of the dissertation

The reviewed dissertation does not contain any significant errors. Above, from the reviewer's duty, I have listed minor editorial shortcomings that do not diminish the value of the work. As for the content, the following issues are more like points for discussion than as allegations.

1. As models for the "cellular" part of the study, the following cell lines were selected: Chinese Hamster Ovary - K1 (CHO-K1), RAW 264.7 (transformed macrophages) and several lines of human skin melanoma. CHO cells are normal Chinese hamster ovary epithelial cells, mainly used for the production of recombinant proteins, RAW cells are virus-transformed mouse macrophages, and melanoma is usually highly transformed tumor cells derived of melanocytes. Why were these cells used? In particular - what was your reason for choosing human melanoma lines that are very specific? In my opinion, normal cells (e.g. commercially available human foreskin melanocytes) and other neoplastic cells could also be used. Among the observed phenomena regarding the effects of ABCA1, is there any specificity ascribed to melanoma cells? Melanomas are very specific, as for ABC proteins.

2. Were there any effects related to melanogenesis found in the effects of modifying ABCA1 expression in melanoma lines? For example, CREB associated with kinase A is *i.a.* responsible for the expression of the tyrosinase gene. Similarly, AKT. In some melanomas, clear correlations between metastasis and pigmentation can be shown. Have differences in

pigmentation been observed and studied? And was it related to the degree of neoplastic progression?

3. In relation to this question, a methodological remark: DMEM contains tyrosine, which is a substrate for tyrosinase in melanogenesis, but it may also act as a hormone inducing melanogenesis (See: Slominski et al., 2012 - doi: 10.1111 / j.1755-148X.2011.00898 .x), therefore the medium may indirectly influence the observed effects by inducing melanogenesis. Melanogenesis is very strongly dependent on the activity of pumps and ion channels, so an indirect effect cannot be ruled out here, by activating genes via the medium. Such studies typically use RPMI and preferably Ham's-F10 buffer.

4. Amphotericin B. It is an antifungal antibiotic. The problem with fungal infections is that fungi (also pathogenic ones) are eukaryotes and hence so many side effects and high toxicity. In general, it can be said that they work in a similar way to anti-cancer antibiotics (chemotherapeutic agents). Deep mycoses sometimes are confused with neoplasms in the clinical course, which does not make it any easier in the case of their (mycoses) detection or treatment. Therefore, any differences in amphotericin sensitivity between fungi and mammalian cells are crucial in planning treatment strategies for both mycoses and neoplasms. Melanin is a factor that worsens the prognosis for both melanoma and mycoses. To make matterrs worse, we have recently seen an invasion of pathogenic fungi from tropical and subtropical areas (e.g. some pneumonias), requiring many months of treatment and with poor prognosis. Mushroom melanin (the so-called "black yeast") is mentioned as a virulence factor. In the work, I missed this thread, both in the descriptive and experimental parts (e.g. the use of mushroom cultivation, not necessarily pathogenic, for comparative purposes).

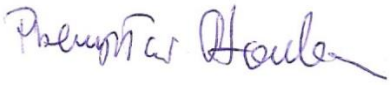
Final conclusion.

The doctoral dissertation presented by Mr. Ambroise Wu, M.Sc., entitled "The role of the ABCA1 protein in the organization of the cell membrane in mammalian cells" is based on the original and valuable scientific achievements in the field of biological sciences. The doctoral student showed maturity in planning, conducting and interpreting the research presented in the dissertation. The results of this experimental work, apart from the purely cognitive value, undoubtedly have the applicative value in the context of the treatment of cancer and deep mycoses. I am convinced that this dissertation meets both the statutory and customary requirements for dissertations for the Ph.D. degree in biological sciences. Therefore, the dissertation meets all the conditions for doctoral dissertations specified in Art. 187 of the Act

of July 20, 2018 "Law on Higher Education and Science" (Journal of Laws of 2018, item 1668, as amended). Therefore, I am submitting an application to the Biological Sciences Discipline Council of the University of Wrocław to admit Mr. Ambroise Wu to further stages of the procedure for a doctoral degree in the field of exact and natural sciences in the discipline of biological sciences.

Krakow, on July 19, 2022

Dr hab. Przemysław M. Płonka



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