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OPINION

on the doctoral dissertation entitled "Metabolomic approach to understand the mechanism of metformin-induced PRODH / POX-dependent apoptosis in MCF-7 breast cancer cells", performed by Thi Yen Ly Huynh, M. Sc., a PhD student at the Department of Medicinal Chemistry, Faculty of Pharmacy with the Division of Laboratory Medicine, Medical University of Bialystok, under the supervision of prof. dr. hab. Jerzy Pałka.

The review was commissioned by the Medical University of Bialystok, and performed based on Art. 187 of the Act of July 20, 2018 (as amended), regarding the requirements for doctoral dissertations.

The doctoral dissertation of Thi Yen Ly Huynh, M. Sc. was presented in the form of an English-language written work, constituting a collection of two published and thematically related articles, with a merging description, placing the presented research in the context of doctoral advancement. Thus, it can be concluded that **this dissertation complies with the statutory requirements regarding the form of the doctoral dissertation (Article 187 point 3).** Moreover, it should be emphasized that this form of the doctoral dissertation meets modern trends and constitutes an added value. It indicates that the studies described there have already been approved and positively reviewed, which confirms their importance for the scientific community. As the publications in question are multi-author, as required, the dissertation was accompanied by statements about the contribution of individual scientists to the creation of these articles. The PhD student herself

defined her contribution at 60%, and the co-authors' statements are consistent and leave no doubts as to the leading role of the PhD student. Importantly, it consisted not only in carrying out most of the work, but also, together with the supervisor, planning them, and then appropriate interpretation of the results obtained. The PhD student's contribution is therefore not only quantitatively leading, but most importantly, it is typically scientific in nature, appropriate to the doctoral level. It can therefore be concluded that **the PhD student shows the ability to independently conduct scientific work, which is a very important contribution to meeting the requirements of Art. 187, point 1.**

Basing the work on the attached publications influenced its volume, as well as the way of presenting the content included in the description integrating the collection of publications. The entire written work consists of 116 typescript pages, of which the original, not included in the published articles, merging description, is about 45 pages. Such a proportion is absolutely justified, considering the fact that a lot of information, especially of a methodological nature, is included in the accompanying publications.

Publication No. 1, entitled "Understanding the role of key amino acids in regulation of proline dehydrogenase / proline oxidase (PRODH / POX) -dependent apoptosis / autophagy as an approach to targeted cancer therapy", published in the Molecular and Cellular Biochemistry, in the 2020, is a review work that is a very good summary of the state of knowledge preceding the commencement of experimental work as part of the dissertation, placing it in an appropriate context.

The content of the first publication corresponds to the several-page introductory part of the merging description, in which all the issues relevant to the proper understanding of the following own research were discussed, including: homeostasis and metabolism of neoplastic cells, antitumor activity of metformin, autophagy and apoptosis, and the relationship with them of key importance for PRODH / POX enzyme research, or regulatory roles of amino acids. These contents are complemented by the introductory part of the second publication in the series, as well as data from the literature, widely cited in discussions, both in the publications and the merging description. Both the information content of the above-mentioned excerpts and the transparent manner of expression **testify to the high general theoretical knowledge of the PhD student in the scientific discipline being explored, thus indicating that the requirements of Art. 187 point 1 of the Act were fulfilled.**

Publication 2, entitled "Metformin Treatment or PRODH / POX-Knock out Similarly Induces Apoptosis by Reprograming of Amino Acid Metabolism, TCA, Urea Cycle and Pentose Phosphate Pathway in MCF-7 Breast Cancer Cells," published in the journal Biomolecules, in 2021, is an original work presenting the most important PhD student's own research, which is the basis for applying for a doctoral degree. The research contained in the publication implements the assumptions outlined both in the manuscript itself and in the chapter of the merging description concerning the objectives of the doctoral dissertation. It should be emphasized that the objectives of the experimental research were correctly defined, taking into account the state of the art described in the previously mentioned chapters. In the light of the arguments cited, the planned research has both scientific and application potential. Because it broadens the knowledge about the metabolism of cancer cells, especially in the context of the role of PRODH / POX and the effect of metformin, as well as it dictates possible directions for the development of future pharmacotherapy. The latter context is particularly important socially, due to the importance of the still unresolved medical problem of breast cancer.

The chapter "Materials and Methods" in the merging description is very modest (less than half a page), which is, however, completely understandable, as these methods were described in great detail in the second publication. The methodological workshop is very wide. It includes both tumor cell proliferation studies, cell cycle analysis using flow cytometry, Western Blot protein expression studies, and LC / MS targeted metabolomics. An important aspect of the research, significantly increasing its attractiveness and scientific value, is the use of the MCF-7 breast cancer cell line, with the PRODH / POX gene turned off (PRODH / POX knock-out), obtained with the use of the innovative CRISPR-Cas9 technique. It is worth emphasizing, in the context of the promotion of the assessed research, the PhD student's great commitment to the use of such varied techniques. In the light of the statements presented by the PhD student and her co-authors, the PhD student was directly involved in the performance of all the above-mentioned studies, demonstrating the ability to cooperate, also internationally, which certainly contributed to the development of her competences.

The results of the experimental work are very interesting. The special achievements of the PhD student in this area include:

- confirmation of the anti-tumor potential of metformin, demonstrating its cytotoxic activity against MCF-7 breast cancer cells,

- the conclusion that this effect is associated with the induction of apoptosis through reprogramming of amino acid metabolism, the Krebs cycle, the urea cycle and the pentose-phosphate pathway

- confirmation of the involvement of PRODH / POX in this effect, which was hypothesized in the research plans,

- establishing that the antiproliferative effects of metformin are observed with both cells cultured in the presence and absence of glutamine, but are stronger in the absence of glutamine,

- the observation that similar metabolic and pro-apoptoric effects to metformin treatment can be obtained, in the absence of glutamine, also by the PRODH / POX gene knock-out,

The conclusion from the above studies is also very interesting and potentially useful clinically. It suggests that the combined use of metformin and inhibitors of glutamine synthesis may allow for a synergistic anti-cancer effect and constitute a new direction in the search for an effective therapy of breast cancer.

It is worth noting that the presented results are provided with extensive additional materials (supporting information), which emphasize the reliability of the research and increase the possibility of their reproduction.

Summing up, it should be stated that the presented doctoral dissertation includes the formulation and original solution of the scientific problem, thus fulfilling the requirements of Art. 187 points 2.

The analysis of the interesting and valuable results presented in the dissertation prompted me to formulate a few questions that I would like to become a contribution to the discussion during the defense:

1. I would like to ask to elaborate on the molecular mechanism of action of metformin, which results in its influence on the metabolism of neoplastic cells and the resulting antiproliferative activity. The mode of action of metformin, as shown in Figure 9 (page 39), suggests that it inhibits one of the enzymes involved in the metabolism of pyruvate (PYR KINASE or PKM2). Meanwhile, in the literature, I encountered the conclusions that metformin affects the expression of these enzymes. Understanding the detailed mechanism of metformin's action at the molecular level is important, for example, from the point of view of searching for new drugs, other than metformin,

that have a similar effect on cancer cells. Should we look for PYR KINASE / PKM2 inhibitors? Or the compounds that inhibit their expression?

2. Is the metformin 20 mM concentration used in the studies the concentration expected to be achieved in a clinical setting? How does it correspond to the levels of metformin free fraction when used as an antidiabetic drug?

3. Is the use of glutamine synthesis inhibitors, the synergy of which with metformin has been suggested as one of the conclusions of the doctoral dissertation, clinically safe? What possible side effects should be expected? Could any specific examples of such inhibitors be suggested? What would be the proposed research model to test the hypothesis of combined use of these compounds?

It is worth adding that apart from the high scientific level, the work is also impeccable from the editorial point of view. It is written in concise and clear language, with numerous figures, tables and diagrams, as well as explanations of abbreviations and appropriate summaries.

To sum up, the assessed doctoral dissertation constitutes an original and individual PhD student contribution in the development of the discipline of pharmaceutical sciences and meets all the requirements of Art. 187 of the Act of July 20, 2018 (as amended). Bearing in mind the above, with full conviction I am submitting an application for acceptance of the doctoral dissertation by Thi Yen Ly Huynh and her admission to further stages of the doctoral procedure. Due to the high value of the submitted dissertation, I am also applying for its award.

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