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Poznań, 26.07.2022

Review of Ms. Thi Yen Ly Huynh's doctoral dissertation
Metabolomic approach to understand the mechanism of metformin-induced PRODH / POX-dependent apoptosis in MCF-7 breast cancer cells,
prepared under the supervision of Prof. dr hab. Jerzy Pałka
at the Department of Drug Chemistry, Medical University of Białystok

The work presented for review focuses on a very interesting and important issue - the search for new molecular targets in cancer treatment. Considering the increasing incidence of neoplasms, and the lack of effective methods of treating some of them, undertaking research aimed at explaining the mechanism of antitumor action of metformin in MCF-7 breast cancer cells is justified. As a tool, a metabolomic analysis was used to determine the metabolomic profile of wild-type MCF-7 and PRODH/POX knockout MCF-7 tumor cells produced with CRISPR-Cas9 technology and treated with metformin (MET). The starting point for this type of research is the variability of biochemical processes in neoplastic cells in relation to healthy cells. This topic, partially analyzed in the literature and also in the Department of Drug Chemistry where the doctoral dissertation was carried out, has not been fully explained, especially in relation to breast cancer cells. Hence, the author's analysis results may allow for a significant expansion of knowledge in this field. In her research, the PhD student used the following techniques:

- (i) CyQUANT® Cell Proliferation Assay to study cell proliferation;
- (ii) NC-3000 Nucleo-Counter flow cytometry for cell cycle study;
- (iii) Western blot to study the expression of pro-apoptotic proteins;
- (iv) high performance liquid chromatography coupled with triple quadrupole mass spectrometry for metabolomic analysis.

The assumptions made in this dissertation were based on the hypothesis that metformin activates AMP kinase (AMPK), which induces apoptosis in cancer cells through a series of successive processes that include the generation of reactive forms by proline

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dehydrogenase/proline oxidase in the conditions of the availability of proline, the PRODH/POX substrate. The presented study describes in detail the changes taking place in cells in the conditions of a deficiency of carbohydrate and lipid energy substrates and the role of proline in these processes.

The layout of the work is typical for a publication that is a collection of the author's own works. The thesis consists of six basic chapters, including *introduction, the objective of the study, materials and methods, results, discussion, conclusions*, publications 1-2, and additionally, contains information on funding sources, a list of abbreviations used in the thesis, a list of the PhD student's scientific achievements, references and attachments. The work is 116 pages long, 40 pages of which are publications with additional attachments.

In the *introduction*, the author describes the action and application of metformin, homeostasis and changes leading to cancer, as well as signaling pathways involved in these processes, the process of autophagy, apoptosis and the metabolic role of proline dehydrogenase and oxidase, as well as their participation in the process of apoptosis and autophagy, the process of glycolysis and the importance of the TCA cycle, ending its considerations with the section amino acids as regulators of metabolism.

In the next chapter – *the objective of the study*, she clarified the purpose of her research and presented what experimental research will be carried out. The chapter ends with the statement that the final result of her research should be the determination of the role of PRODH/POX in the mechanism of MET-dependent apoptosis and the definition of a new molecular target in the experimental therapy of breast cancer.

In a short chapter on *materials and methods*, the author described the method used, emphasizing that they are described in detail in the publications.

The next chapter, the most important for the reviewer, are the *results and discussion*. After these chapters, the author presents two publications describing all the research carried out. The listed works are:

1. 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. *Molecular and Cellular Biochemistry* 2020; <https://doi.org/10.1007/s1010-020-03685-y>.
2. Metformin treatment or PRODH / POX-knock out similarly induces apoptosis by reprogramming of amino acid metabolism, TCA, urea cycle and pentose phosphate pathway in MCF-7 breast cancer cells. *Biomolecules* 2021; 11, 1888. <https://doi.org/10.3390/biom-11121888>.

In both publications, the PhD student is the first author, which is necessary for the procedure of doctoral dissertations. The total value of the Impact Factor (IF) for the presented works is 8.275, which corresponds to 170 points from the Ministry of Education and Science. The attached declarations of the co-authors about the PhD student's participation in the preparation of the publication confirm her significant contribution to the planning, execution of research, description and analysis of the obtained results and preparation of the manuscript for publication.

The study's conclusions were prepared correctly, and the answers to the questions, presented as the study's objectives, were defined in the form of 6 conclusions, where, the 6th conclusion is the most promising in terms of new perspectives in breast cancer therapy. This conclusion stated that combining MET-treatment with glutamine synthesis inhibitors could be a new approach to further research into experimental breast cancer treatment.

The conclusions are correctly formulated and result from the analysis and discussion carried out in work. Analysis of the PhD student's own results was carried out using correctly selected statistical tests.

The presented discussion confirms knowledge and understanding of the issues raised in the area of medical and health sciences in the field of pharmaceutical sciences. The research carried out by the PhD student, the discussion and conclusions confirm the ability to independently conduct scientific research and good cooperation with other research teams, they also make a significant contribution to the development of the pharmaceutical science discipline and are valuable for pharmacists, doctors and scientists from related fields.

In conclusion, I would like to highlight several aspects. What is worth emphasizing is the innovative subject of the work. The quality of the research conducted as part of the doctoral dissertation submitted for review is also high and deserves recognition. Although the doctoral student had the opportunity to carry out research in an excellent research team under the supervision of Prof. Jerzy Pałka, a recognized authority in this field, without her personal involvement, the implementation of the planned scope of research would be impossible.

The selection of *bibliography*, in the number of 168 items, takes into account the latest literature and meets the requirements in this regard. The presented discussion confirms knowledge and understanding of the issues discussed.

During doctoral dissertation defense, I would like to discuss the following issue - do you see new options for treating cancer, based on the conducted research? Which classes of drugs do you think are most suitable for this purpose, or can you consider repositioning drugs used in other diseases? My next question concerns metformin, whether, based on the research carried

out, it can be a drug used in the treatment of neoplasms of other organs and what is the dose that is necessary to achieve a therapeutic effect in the case of neoplastic diseases and what potential side effects can be expected in this case.

Other achievements of the PhD student

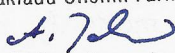
The doctoral student also presented a list of publications of which she is a co-author, apart from two works included in the doctoral dissertation. These are seven experimental papers with a total IF = 32.719, corresponding to 760 MEiN points, and eight review papers (145 MEiN points). The author presented the results of her research at ten national and international conferences. She also included a list of grants and research projects of which she was the beneficiary/contractor, and courses that allowed her to raise her scientific qualifications. These data confirm the great commitment of the PhD student and are a very good prognosis for her further scientific development.

Final conclusion

The research proposed and carried out by the PhD student is of great importance both scientifically and practically. The research results obtained in this dissertation may contribute to developing new, more effective and safe breast cancer treatment methods. Of course, the first stage in these considerations is the biochemical processes taking place in cancer cells, which have been studied and described in detail by the author.

Assessing positively, both in formal and substantive terms, the doctoral dissertation of Ms. Thi Yen Ly Huynh entitled *Metabolomic approach to understand the mechanism of metformin-induced PRODH / POX-dependent apoptosis in MCF-7 breast cancer cells*, I confirm that it is an original and valuable scientific study, and therefore I am honored to ask the Dean and the College of Pharmaceutical Sciences of the Medical University of Bialystok to accept the work and admit the author to further stages of the doctoral dissertation.

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