

Professor Grzegorz DWORACKI, MD, PhD
Chair of Clinical Immunology of Poznan University of Medical Sciences
in Poznan

ul. Rokietnicka 5D
60-508 Poznań

tel. +48 61 854 7174
e-mail: gdwrck@ump.edu.pl

Assessment of the doctoral dissertation
of Urszula Alina WASZUT, MSc
entitled "ASSESSMENT OF MITOTATE EFFECT ON THE EXPRESSION OF
GENES INVOLVED IN THE REGULATION OF MITOCHONDRIAL
METABOLISM IN HUMAN CELL LINES OF ADRENAL CORTEX CANCER,
LUNG CANCER AND COLORECTAL CANCER".

The search for drugs which prove to be effective in the treatment of cancer and, at the same time, have low toxicity for normal cells and tissues, is a constant challenge in oncological therapy. The better the understanding of their action and the involved metabolic pathways which play a leading role in maintaining life processes in particular types of cancer, the more effective the drug.

Urszula Alina WASZUT, MSc, decided to discuss the problem of searching for possible molecular targets of mitotane, a drug used in the treatment of adrenocortical carcinoma, which the Reviewer sees as a cognitively and practically important issue.

Adrenal cortex cancer is a rare and aggressive neoplasm with a poor prognosis. Mitotane has proven to be the most effective drug in the treatment of this carcinoma. Despite the fact that this compound has been known for a long time – since the 1940s when it was originally used as an insecticide (a replacement for the popular DDT), the mechanism of its action has not been fully described. The knowledge of the effect of mitotane was limited to the description of toxic changes induced in the mitochondria and the influence on the induction of programmed cell death. The biochemical and molecular target of this compound has not been clearly explained so far. It is known that this drug exerts a cytotoxic effect on the adrenal cortex by modifying the peripheral steroid metabolism, clearly increasing the synthesis of 6-beta-hydroxy-cholesterol. The toxic effect is clearly selective and concerns only zona fasciculata and zona reticularis of the adrenal cortex which are responsible for the production of glucocorticoids. Mitotane does not affect zona glomerulosa which produces aldosterone.



Considering the available data which clearly show that mitotane leads to ultrastructural changes in the mitochondria and cell apoptosis which, as a result, may have an effect on the mitochondrial energy metabolism, the logical conclusion is to look for mitotane targets in the genes associated with energetic activity of the mitochondrium, mainly those genes which are involved in the oxygen metabolism and programmed death processes associated with the metabolic pathways carried out in the mitochondria.

The candidate's goal was the assessment of the level of expression of 84 genes involved in mitochondrial energy metabolism between the adrenal cortex (H295R), mammary gland (MCF-7), large intestine (HKe-3) and lung (H195R) cancer cell lines in culture conditions without and after the administration of mitotane.

Moreover, the dissertation is an attempt at defining a procedure which would allow mRNA extraction for the assessment of gene expression from tissue material isolated from paraffin blocks. Breaking the technological barrier to the evaluation of gene expression in such a material gives access to biological material accumulated in the histological archives, which may ultimately be of significance in the assessment of tissue microenvironment of tumors and could potentially have diagnostic value.

In the opinion of the Reviewer, the choice of the expression of oxygen metabolism genes is justified and confirms the desirability of undertaking the tedious research. It also proves great knowledge and the fact of making a critical reflection on the basis of the research subject studied by the candidate.

The layout of the dissertation is typical for this type of work and includes all the required parts.

In the introduction, which consists of 23 pages, the author makes a detail presentation of the historical background of the use of mitotane, the effects on the adrenal tissues and the mechanisms of adrenal cortex cell death, as compared to other cell lines. The candidate thoroughly discusses the known facts on the effect of mitotane on the metabolic pathways related to aerobic metabolism and steroidogenesis, considering the autonomic importance of the mitochondria; she also provides a detailed description of the effect of mitotane on the mitochondria, emerging ultrastructural changes and the pathways of induction of apoptosis, including those involving the mitochondria. She separately describes the cell lines used and cites the facts from literature related to attempts at the evaluation of gene expression at the mRNA level from the paraffin block material. Moreover, the introduction provides a detailed description of the clinical possibilities and effects of the use of mitotane.



In the opinion of the Reviewer, the introduction discusses the issues which are relevant for the studied subject in the correct proportion, and thoroughly, but not excessively, explains the most important aspects of the undertaken studies. Furthermore, the description is accompanied by clear figures, most of which are modifications of the diagrams available in the scientific literature, which is appropriately marked. Wherever the author deems it necessary, she presents her own figures (e.g. Figure 4) which is valuable as a self-explanatory illustration of the biological processes described. The scope of and insight into the details of the issues presented prove a very good theoretical preparation of the PhD student for the undertaken studies.

The aim of the dissertation is clearly formulated and contained in four points. The first three of them directly concern mitotane. The last one is a methodical goal concerned with an attempt at developing an effective mRNA isolation method for the purpose of evaluating gene expression from a paraffin block material. In the opinion of the Reviewer, the most interesting, though possibly the most difficult to achieve, is the third goal, as it concerns an attempt at explaining the selectiveness of the mechanism of action of mitotane on the adrenal cortex cells. The Reviewer sees the first two goals as partial goals in order to achieve the third one.

In the chapter concerned with materials and methods, the candidate presents all stages of her work in detail, describing particular elements in clear tables, often accompanied by colorful figures. She also provides the smallest details on the used reagents, stages of laboratory procedures and the methods of formulating the results. At the same time, the candidate clearly and transparently describes five components and their constituent genes related to the transport of electrons in the mitochondrial respiratory chain, the expression of which was evaluated in the dissertation. However, the large number of methodical details is not overwhelming, because the paper is logically and clearly organized and supplemented with clear summarizing diagrams and figures. The high quality of the methodological description of applied research procedures, the applied molecular and biochemical methods, repeatedly provided with relevant and methodically helpful references, indirectly proves the candidate's technical proficiency as far as the use of such procedures and methods is concerned. Generally speaking, the Reviewer does not have any comments to the methodological part, but for one. The author writes that the material of paraffin blocks was cut to a thickness of 0.7 (mm) millimeter, and previously tissue sections had been stained to determine the area which concerned the tumor in the preparation. The thickness of the stained paraffin sections was certainly much smaller, because otherwise it would be impossible to assess their structure in a histological examination.



The molecular analysis, which is an essential and significant part of the research, included a wide range of molecular methods based mainly on cell culture techniques, mRNA isolation and Real-Time PCR, as well as their modifications. In the opinion of the Reviewer, the research techniques were chosen adequately to the goals set for the dissertation, they prove the candidate's high-level laboratory skills and, undoubtedly, the presence of the dissertation supervisor during each stage of work.

The Reviewer concludes that the research was correctly planned and carried out and there was used appropriate methodology to achieve the assumed research objectives. It is worth noting that the candidate performed the majority of the experimental parts of the research work herself, wherever possible, including the description and analysis of the results, while effectively cooperating wherever it was necessary. It is not without significance that the research was carried out mainly within the framework of the POMOST /2012-5/3 grant awarded to the supervisor, Dorota Dworakowska, MD, PhD, and co-financed by the Foundation for Polish Science and the European Union, and largely carried out in the Department of Cancer Research at King's College in London. Working in a laboratory of a recognized foreign research center which often gives great opportunities, is also usually a great challenge that is not met by everyone. Taking into consideration the scope and results of the work performed by the candidate, it seems that she was very successful in this respect.

The chapter titled "Results" constitutes a significant part of the dissertation. The obtained data is presented in a clear form and in legible diagrams, documented by carefully selected figures. In the opinion of the Reviewer, the author used appropriate methods of describing the results, properly selected the statistical tests and drew proper conclusions from the obtained results. The results are presented in the form of clear and convincing charts and tables with legible keys, with a clear specification of statistical significance. What is important, with a large number of results, their description is not just a simple presentation, but appropriately groups and hierarchizes them, specifying and discussing them wherever necessary from the point of view of the aims of the research.

The discussion is quite extensive, it covers 18 pages. In the opinion of the Reviewer, it is a good review of the literature on the molecular effects of mitotane, however, to a small extent it is a direct discussion of own results compared to the available literature on the subject; phrases like "in this study" appear only in individual cases, or are secondary to the facts quoted from the literature which are loosely related to the results of the work. Nevertheless, it should

be mentioned that the discussion quotes a number of papers published by the research team of the supervisor and the doctoral student, which is a logical continuation of the research and discussion and is more of a discussion on the subject than the results of the research. It should also be noted that the subject of this work, as far as the undertaken research is concerned, is relatively new, which is why there are not too many reference points. It is therefore a pity that the discussion does not specify completely new observations that have been made in the dissertation. An interesting part of the discussion is the section on the possibility of assessing gene expression in the mRNA material isolated from paraffin blocks. The results of the performed studies show that a simple change of procedure like mechanical scraping of tissue material directly from the slide with a scalpel and not a laser tool, degrades the DNA and can significantly affect the stability of DNA and the ability to efficiently evaluate gene expression. The author points out that the most striking difference in the assessment of gene expression between adrenocortical carcinoma and normal adrenal cortex was the VEGF overexpression in adrenal cortex cancer. One should appreciate the criticism of the PhD student who notes that cell lines in the cell culture are not the same as the cancer in its tissue microenvironment, which can constitute a significant and sometimes a major part of the cancer infiltration. Tumor cells in the original microenvironment can have a significantly different molecular profile, compared to what we find in the cell culture. It is a pity, however, that seeing the differences in the production of VEGF, the author points only to the elements of the tumor microenvironment, such as fibroblasts, necrosis, not to mention inflammatory cells, including macrophages, which are almost always present in the tumor microenvironment and are an important source of production of various factors involved in the promotion and growth of cancers including the mentioned VEGF.

The conclusions presented are detailed and clearly formulated, and they refer directly to the objectives of the work. What is important, the conclusions are cautious wherever necessary, and unambiguous where the obtained results allow it. This proves the author's scientific maturity. However, the excessive description, and even discussion, which forms a part of the conclusions, arouse some criticism from the Reviewer. It particularly concerns the third and fourth conclusion which could have been more concise. However, the author sees the need for a more concise summary which would be included in the summary of the results.

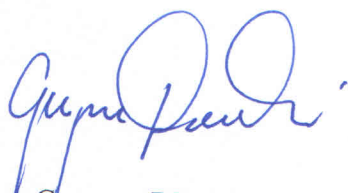
The dissertation is written in English. Despite the fact that English is not the mother tongue of the candidate, the style of the dissertation and the vocabulary is communicative and clear and vividly depicts difficult and sometimes tedious details, guiding the reader through complex clinical and experimental issues. Nevertheless, the author did not manage to avoid



minor linguistic errors, such as the use of the phrase „w porównaniu do” instead of „w porównaniu z” in the fragments of the dissertation written in Polish, which is a typical borrowing from English. Interestingly, in the English language version of the dissertation the author frequently uses the phrase “in comparison with” instead of the correct phrase “in comparison to”; it is, however, less of a mistake, and more of an expression of attachment to the Polish language.

In conclusion, I would like to emphasize that the minor remarks from the Reviewer do not affect the substantive value of the dissertation in any way. I am fully convinced that Urszula Alina Waszut, MSc, has submitted her doctoral dissertation at a very high substantive level, including valuable results which expand and enrich the scope of knowledge with new, previously unknown facts and practical conclusions, which will certainly be published in specialized literature with a wide reach. It is also worth noting that the results of the research have already been partly published in journals with a wide reach and at international conferences.

On the basis of the assessment presented above I conclude that the doctoral dissertation entitled “ASSESSMENT OF MITOTATE EFFECT ON THE EXPRESSION OF GENES INVOLVED IN THE REGULATION OF MITOCHONDRIAL METABOLISM IN HUMAN CELL LINES OF ADRENAL CORTEX CANCER, LUNG CANCER AND COLORECTAL CANCER” fully complies with the requirements of doctoral dissertations in the field of medicine; therefore, I have the honor to apply to the Faculty Council of Faculty of Health Sciences of the Medical University of Gdańsk for the admission of Ms. Urszula Alina WASZUT to further stages of pursuing the doctoral dissertation.



Professor Grzegorz DWORACKI, MD, PhD

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