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REVIEW of doctoral dissertation

"Strategies for utilizing bioactive potential of natural product-derived postbiotic metabolites"

by Mr. Maciej Korczak, M.Pharm.,

prepared under the supervision of Prof. dr hab. Jakub Piwowarski *[Professor, Ph.D., postdoctoral degree holder]* performed in the Environmental Laboratory Microbiota Lab, Department of Pharmaceutical Biology, Faculty of Pharmacy, Medical University of Warsaw.

The doctoral dissertation presented here was submitted for evaluation in the form of a thematic series of three original publications, including two original publications and one review publication:

1. **Maciej Korczak**, Maciej Pilecki, Sebastian Granica, Aleksandra Gorczyńska, Karolina Aleksandra Pawłowska, Jakub Patryk Piwowarski. Phytotherapy of mood disorders in the light of microbiota-gut-brain axis. Phytomedicine. 2023, 111: 154642. doi: 10.1016/j.phymed. 2023.154642. PMID: 36641978. Impact Factor: 7,9; MEiN: 140
2. **Maciej Korczak**, Piotr Roszkowski, Sebastian Granica, Jakub Patryk Piwowarski. Conjugates of urolithin A with NSAIDs, their stability, cytotoxicity, and anti-inflammatory potential. Scientific Reports 2022, 12(1): 11676. doi: 10.1038/s41598-022-15870-8. Erratum in: Scientific Reports. 2022 Nov 2;12(1):18503. PMID: 35804000; PMCID: PMC9270351. Impact Factor: 4,6; MEiN: 140
3. **Maciej Korczak**, Piotr Roszkowski, Weronika Skowrońska, Klaudia Małgorzata Żołdak, Dominik Popowski, Sebastian Granica, Jakub Patryk Piwowarski. Urolithin A conjugation with NSAIDs inhibits its glucuronidation and maintains improvement of Caco-2 monolayers' barrier function. Biomedicine & Pharmacotherapy. 2023, 169: 115932. doi: 10.1016/j.biopha. 2023.115932. PMID: 38000358. Impact Factor: 7,5; MEiN: 140

All of the publications mentioned were published in 2022-2023 in prestigious thematic journals, with a total IF = 20.0 (Ministry of Education and Science = 420 points). They have been subjected to a very rigorous peer review process. The doctoral student is the first author in all the publications mentioned in the dissertation, and in three he is additionally a corresponding author which eminently indicates his leading role in their elaboration. Additional confirmation of the above assessment is provided by the inclusion of relevant statements by both the Author of the dissertation and all their co-authors, which I have no reason to doubt.

Recently, there has been an increase in knowledge about the role of the human intestinal microbiota and the compounds biotransformed by it in maintaining the homeostasis of the human body. Intestinal bacteria express a huge number of enzymes that transform molecules of compounds which enter the digestive tract, often significantly changing their chemical structure and biological properties. Postbiotic metabolites derived from natural products currently constitute a small number of

interesting active substances with promising parameters that can be used for the development of new active substances and thus constitute the basis for the search for new potential medicinal substances.

In his dissertation, the Ph.D. candidate conducted an assessment and characterization of selected postbiotic metabolites by proposing three very interesting research concepts aimed at using their therapeutic potential as drugs. The first strategy, presented on the example of plant substances used in the treatment of mild mood disorders, involves a new look at the mechanisms of activity of plant-derived drugs from the perspective of the microbiota-gut-brain relationship and emphasizing the role of emerging postbiotic metabolites in the action of traditionally used products of natural origin. The results of a very interesting study were published as part of a review paper.

The other two strategies proposed by Maciej Korczak, M.Pharm., concerned the well-known metabolite urolithin A. He proposed two innovative approaches enabling the use of its therapeutic potential associated with strong anti-inflammatory activity, which in the *in vivo* model is limited by phase II metabolism: firstly, by chemically modifying its structure to obtain derivatives with increased bioavailability. In the case of the second direction of the proposed research, he took into account the model of transdermal application of urolithin A into the skin, thus avoiding the action of detoxification enzymes. He published the results of his in vitro study on urolithin A and its structurally modified derivatives in the form of two original experimental papers.

The results obtained by Maciej Korczak, M.Pharm., clearly show that the formation and biological activity of the postbiotic metabolites present have not been adequately studied in some plant species, and in some there have been no such observations. In the case of postbiotic metabolites derived from hop cones, such as 6-/8-prenylnaringenin or desmethylxanhotumol, neither the antidepressant nor antianxiety effects have been studied, which constitutes a significant gap in our understanding of the biological effects of these plants, which have been commonly used for centuries. Another important observation is that some postbiotic metabolites derived from plants traditionally used to treat mood disorders show promising activity in preclinical studies. These compounds may be interesting candidates for further development and use in the treatment of mood disorders. In some cases, it is postbiotics that are responsible for the therapeutic effects observed, as in the case of crocetin derived from crocin.

In turn, as far as ellagotanoid derivatives and their metabolites are concerned, their stability is very limited. Depending on the drug design strategy, the use of ester bonds can either help or hinder production of postbiotic derivatives of metabolites with the intended biological effect. In the synthesis of the so-called "prodrugs", ester bonds are easily hydrolyzed due to the ubiquitous presence of esterases, and release the active form of the molecule in the cell. However, premature hydrolysis of such a bond can be a significant hindrance. To synthesize derivatives with a modified structure compared to the original molecule, and which are stable in host tissues, it seems necessary to synthesize derivatives containing more stable chemical connections. The Ph.D. candidate successfully synthesized mixtures of urolithin derivatives in combination with known non-steroidal anti-inflammatory drugs, including ibuprofen (compounds 3a/3b), mefenamic acid (compounds 4a/4b), diclofenac (compounds 5a/5b) and acetylsalicylic acid (compounds 6a/6b). Despite similarities in the form of conjugation, the compounds obtained differ fundamentally in terms of stability and, unlike in the tested urolithin derivatives; he observed that the mixture of substances with mefenamic acid (4a/4b) tolerates the human plasma environment well. The specific effect of the 4a/4b derivatives on TNF-α secretion may be due either to their cytotoxic effect at high concentrations or to the opposing activity of the 4a/4b subunits at lower concentrations. The effect of 4a/4b on the secretion of IL-10 and the lack of activity towards the secretion of IL-6 suggest a mechanism of action that is different from that previously determined for pure urolithin A. The results presented by the Ph.D. candidate are characterized by their scientific novelty and create a new global approach, not previously described in the literature, towards the development of anti-inflammatory drugs, focusing on chemical modification of the structure of postbiotic metabolites, in this case urolithin A.

The synthesis of pure urolithin A on a semi-technical scale allows the creation of a product of the required purity in accordance with cGMP standards, and, importantly, uses relatively inexpensive commercially available reagents. The surprisingly simple, two-step synthesis of a sufficient amount of

urolithin A for further study in an *in vitro* model resulted in preliminary clinical trials on a small group of volunteers. Importantly, urolithin A creates a stable O/W preparation for use on the skin at a concentration of 5% in the form of a cream with appropriate physicochemical properties allowing it to be applied and spread on the skin. The *in vitro* studies conducted showed that the preparation developed had a high and effective penetration ability. Maciej Korczak, M.Pharm., took into account the experience gained during previous work on urolithin A derivatives when assessing the anti-inflammatory effect of the cream containing urolithin A.

In the next step, he used an interesting biological model using Caco-2 cells, derived from human colorectal adenocarcinoma, which are commonly used for *in vitro* assessment of the absorption and metabolism of intestinal drugs, and well-plate cultures of these cells are considered the standard in permeability tests. The results presented by the Ph.D. candidate in this regard showed a significant effect of the newly synthesized NSAID esters and urolithin A on monolayers of the Caco-2 cell line. Inhibition of urolithin A glucuronidation together with a strengthening of the integrity of the intestinal barrier in an *in vitro* model reveal new treatment strategies involving the synthesis of bioactive postbiotic metabolites coupled with inhibitors of their metabolism. The experiment performed clearly showed that some derivatives have a positive effect on the expression of genes involved in the homeostasis of zinc ions and regulate the expression of TJPs, which may contribute to the improvement of the barrier function, and the results presented indicate that the conjugates obtained are promising therapeutic agents which increase the bioavailability of the bioactive form of urolithin A and NSAIDs that have no harmful effect on intestinal wall barrier proteins. An undoubted achievement in this area is the commercialization of studies on the use of the obtained urolithin A preparations in the local treatment of atopic dermatitis.

I highly value the results obtained and the great commitment put into preparing them. To sum up, I would like to emphasize the presence of a number of aspects of scientific novelty in the doctoral dissertation. As a reviewer, I would like to ask about certain issues to open discussion as part of the doctoral dissertation:

1. From the perspective of metabolic assessment, the integration of individual differences in human genetics and intestinal microbiota offers hope for personalized recommendations for effective depression therapies using plant-derived preparations. This is a very difficult issue that requires a reliable explanation. My question concerns the issue of assessment or genome editing based on certain systems, e.g. the CRISPR/Cas9 system, which is a powerful genome editing tool, widely used in basic, preclinical and clinical studies, widely analyzed in terms of genetic disorders. Please expand on this topic.
2. Could the Ph.D. candidate propose an experimental strategy for the preclinical evaluation of the compounds obtained, using an *in vivo* model? What stood in the way of including this type of model in his research?

The prominence and importance of the comprehensive assessment of postbiotic metabolites and their newly synthesized derivatives undertaken by the research team under the supervision of Professor Jakub Piwowarski entitles me to say that the author has demonstrated the ability to master a number of techniques for working in a phytochemical laboratory (GC/MS, LC/MS techniques, and very time-consuming spectral analysis, including NMR) as well as very demanding biological techniques. The research results presented indicate the Ph.D. candidate's great maturity in terms of research, and scientific inquisitiveness. The ability to cooperate scientifically with scientists from Poland and abroad is particularly noteworthy. The scope and presentation of a very well thought-out research concept demonstrate the Ph.D. candidate's extensive knowledge and research skills.

The doctoral dissertation of Maciej Korczak, M.Pharm., is extremely valuable both scientifically and in terms of its application. Therefore, I am submitting a request to the Committee appointed to conduct the proceedings for awarding the degree of doctor of sciences to Mr. Maciej Korczak, M.Pharm., for a distinction of his doctoral dissertation. My proposal is motivated by the high

scientific standard of the dissertation, taking into account not only the aspects of novelty and scientific originality including, for the first time, a description of the chemical characteristics of newly obtained conjugates - urolithin A derivatives - and an assessment of their biological activity. An additional advantage of the dissertation is its high substantive value confirmed by a high total IF = 20.00 (Ministry of Education and Science = 420 points).

To sum up, I hereby state that the doctoral dissertation of Mr. Maciej Korczak, M.Pharm., submitted to me for evaluation, meets the requirements for doctoral dissertations in accordance with Art. 187 of the Act of 20 July 2018, Law on Higher Education and Science (Journal of Laws of 2023, item 742), therefore I am appealing to the Council for Scientific Degrees in the discipline of pharmaceutical sciences of the Medical University of Warsaw to award Mr. Maciej Korczak, M.Pharm., the degree of Doctor of medical and health sciences in the discipline of pharmaceutical sciences.

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