Prof. dr hab. Maciej Figiel

Molecular Neurobiology Group
Instytut Chemii Bioorganicznej Polskiej Akademii Nauk
ul. Z. Noskowskiego 12/14
61-704 Poznań
tel.:+48618528503 wew. 1150
e-mail: mfigiel@ibch.poznan.pl

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REVIEW OF THE DOCTORAL DISSERTATION

The dissertation submitted by Estera Rintz, MSc. titled: *"Molecular Therapies for Mucopolysaccharidosis in Mouse Models"*

The doctoral dissertation has been prepared as a collection consisting of two review articles and three original research articles. All articles were peer-reviewed, revised, and published in scientific journals indexed in Journal Citation Reports; hence, they are distinguished by impact factor. The collection was supplemented with summaries in English and Polish of 6 pages each. The dissertation was prepared in the Department of Molecular Biology, Faculty of Biology, University of Gdańsk. Joint supervisors of the dissertation were Professor Grzegorz Węgrzyn and Professor Shunji Tomatsu from Nemours Children's Hospital, Wilmington, Delaware, USA.

The thesis' major research questions are the therapeutic aspects of metabolic mucopolysaccharidosis diseases related to improving the delivery of therapeutic agents to hard accessible tissues, including the brain and tissues with limited or absent vasculature, such as bones and cartilage. These tissues are of the most significant interest in the pathogenesis of these disorders. One of the scientific rationales put forward in the dissertation is based on the fact that the available enzyme replacement therapy cannot penetrate these hard-to-access tissues. Therefore, my understanding of the rationale was that the interest in finding the cure has to be shifted from enzymatic therapy to either small molecular substances such as resveratrol or peptide agonists of receptors and genetic delivery systems based on AAV vectors.

The research question was addressed by two main dissertation goals. The first goal is to use resveratrol as an example of a small molecule that should penetrate the blood-brain

barrier and induce autophagy by various mechanisms to treat Sanfilippo disease (MPS IIIB). Ms Rintz also states that the goal was to determine the exact mechanism of the resveratrol therapeutic action. The potential of resveratrol as an anti-mucopolysaccharidose therapeutic agent was nicely reviewed in the review article, which is part of the dissertation's article collection. However, in the review article, there is no information about the pharmacological properties of the resveratrol when delivered to the body and its bioavailability. Therefore, I want to ask for insights about the first goal during the dissertation defense. Namely, please explain the exact rationale behind selecting resveratrol as the small molecular drug to improve drug delivery and penetration to the brain. My question is related to the very poor brain bioavailability of resveratrol and the fact that it was even used as a compound to improve BBB and make it less permeable.

The second goal is the development of an innovative therapy based on two AAV vectors, one AAV8 containing an expression cassette of *NPPC* gene encoding C-type natriuretic peptide (CNP), the second AAV9 containing an expression cassette of *GALNS* gene, and the last one combining both genes in one cassette. The therapeutic target is the MPS IVA, so-called Morquio syndrome type A, and the amelioration of the bone changes in the disease. The therapy was used as a single therapeutic by administering a single AAV8/AAV9 or as a combination of both vectors. The justification of the development of this idea behind the goal is well supported by yet another review article entitled: "Molecular Mechanism of Induction of Bone Growth by the C-Type Natriuretic Peptide", which is part of the collection.

The conclusion about the central scientific question of the thesis work. The focus on MPS treatment is clearly stated, followed by naming two research objectives for investigating a comprehensive overall picture combining the testing of small molecular and genetic therapeutic approaches. From my point of view, the minor weakness in selecting this specific collection is the limited addressing of the therapeutic aspects of brain disease in Sanfilippo syndrome by resveratrol treatment, compared to the extensive exploration of bone-directed therapy.

According to the concordant contributors' statements, Ms Estera Rintz is the primary author of the collection, with most of the experiments performed. The works presented in the collection were extensively reviewed, revised, and published in renowned scientific journals. However, to duly fulfill my dissertation reviewer duty, I will briefly analyze the methodology

used, results and interpretation, discussion, and conclusion presented in the works, and ask specific questions concerning the dissertation.

The methodology used in the three research works can be characterized as reach and variable and comprises several types of wet lab procedures, including molecular biology, in vitro cell culture, in vivo procedures, animal tissue collection, the use of AAV vectors for in vivo transfection, and statistical analytics. Concerning the "Activities of (Poly)phenolic Antioxidants and Other..." work, I was missing the liver and brain tissue examination by immunocytochemistry to demonstrate the influence of resveratrol. The Statistical analyses were performed very well: ANOVA (probably two-way) or non-parametric statistics based on the normality tests. The sample size in the behavioral experiments was based on power analysis. However, the reference to the previous dataset, which was used to determine the variability between WT and MPS mice, was not indicated. The p values in the figure descriptions are not denoted if this is Anova, K-W, or post-hocs, which is the case for all works. In the "Molecular Mechanism of Induction of Bone..." the methodology is also comprehensive and robust. The therapy application includes the AAV8-hNPPC tail vein injections, while examination of the therapy effectors includes AAV copy numbers in tissues and blood and the proCNP levels. The readouts of the therapy effects are mice weighting, determining the length of mice, bone micro CT scans, GALNS enzyme assay, and bone IHC. The most significant and quality work in the dissertation collection is the "Adeno-associated virus-based gene therapy..." published in the renowned Molecular Therapy Nucleic Acids journal of the Cell Press. The methodology used in the work is very similar to the methodology described above. The work further develops and markedly improves the development of the gene therapy thread in the dissertation.

In each of the original research publications in the dissertation collection, the results are presented as graphs of various parameters from both in vitro and in vivo experiments, protein quantifications often accompanied by pictures of western blots or simple western and histochemistry of bone tissue. All figures are accurately followed by the legend containing the statistical analyses p values, though the p-value origin is not always indicated in the figure description. Concerning the therapeutic influence of resveratrol topic in the dissertation, there is an investigation of the proteins that may deliver some hints about the influence of the compound on several cellular pathways, including the autophagy proteins. The dissertation

goal was to deliver an "exact" mechanism of the resveratrol action; however, to my understanding, the examination of the levels of the proteins was not conclusive enough to firmly name the autophagy or the exact molecular pathway. The dissertation also did not examine other putative mechanisms that might have been involved in the observed effects. The discussion section about mTOR-dependent and -independent autophagy was slightly unclear, explicitly referring to Fig 8 and references nr 27, 34, 35, and 70. Are there any more insights, new facts, or improved interpretations of the observed resveratrol therapeutic effects in mucopolysaccharidosis or future experimental directions to justify understanding the exact mechanism statement?

The gene therapy publications demonstrate the induction of hNPPC and hGALNSsco expression in MPS IVA mice using AAV8 and AAV9 vectors and assessing the development of bones in the model after the treatment. Data collection and presentation can be tricky when looking at the very different setups and combinations of all vectors. These are a combination of hNPPC and hGALNSsco in one AAV9 vector, single treatments with hNPPC or hGALNSsco or their combination in two vectors (AAV8 and AAV9), and these in different concentrations of the hNPPC -AAV8. Such an experimental setup creates a highly complex result presentation in the graphs in Figure 2, making the data interpretation difficult. However, the results are exciting and provoke some questions. The results indicate that combining the hNPPC or hGALNSsco slightly improves the phenotype over the single treatment. However, it seems that the expression of hGALNSsco, although it does influence the clearance of GAGs from the body, however, this clearance does not have too much of a therapeutic effect in terms of bones.

Another exciting point is the rapid bone growth induced by CNP and the high degree of kyphosis produced in mice, indicating muscle-skeleton imbalance, which may not be a good prognostic for single CNP therapy in patients. Could this indicate that a third element of such combinatorial therapy is needed, namely the induction of muscle growth? In addition, better control of CNP expression is needed, as indicated in the discussion in the manuscript.

Finally, my remarks about reading the discussion sections of the works in the dissertation collection are positive. The conclusions are balanced and correspond to the results, and the limitations, such as the small experimental groups, are listed. The contribution to the overall knowledge of the therapeutic approaches in mucopolysaccharidoses is novel and excellent.

Ms Estera Rintz's academic achievements as a whole are outstanding. The scientific activity comprises 33 research articles and reviews, and 8 are as first authorship (over 300 citations). In addition, there is the coauthorship of 3 books and 17 congress materials. Notably, Ms Estera Rintz was a principal investigator in 3 projects, including the PRELUDIUM 18 funding scheme. I can not remember reviewing the doctoral thesis where the submitting author presented that impressive and more substantial academic achievement record.

Conclusions

Ms. Estera Rintz took up a significant scientific problem and successfully performed original research with a high input of work. The conducted research is essential from the point of view of understanding the pathogenesis of mucopolysaccharidoses. Ms. Estera Rintz used a considerable amount of research methods, which is an additional asset of the work. My comments on the work are solely questions for discussion during the defense. I declare that the doctoral dissertation of Ms. Estera Rintz meets the conditions specified in the artcle. 187 sec. 1-4 of the Act on Higher Education and Science of July 20, 2018 (Journal of Laws of 2018, item 1668, as amended). In connection with my positive assessment of the work, I believe that Ms. Estera Rintz should be admitted to the final stages of the doctoral procedure. Along with the above recommendation, I am applying for the distinction of the doctoral dissertation of Ms. Estera Rintz.

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