INSTITUTE OF MICROBIOLOGY

Academy of Sciences of the Czech Republic, v.v.i.

Laboratory of Immunotherapy (Head: Dr. Luca Vannucci, MD, Ph.D.)

Prague, May 8, 2022

To the PhD thesis Commission

First of all, I declare no conflicts of interest or other limitations for the evaluation of the Doctoral thesis of the candidate Mgr. Markéta Křivánková Jirátová.

The thesis work presented by the candidate Mgr. Markéta Křivánková Jirátová collect and discuss the data, results and publication of her doctoral study program "Biological characteristics of polysaccharide based contrast agents for cancer diagnostics" developed under the guide of her supervisor doc. Ing. Daniel Jirák, PhD.

The evaluation of new drug delivery systems for tumor targeting therapy and diagnosis (theranosis) is an approach very perspective in the precise medicine application. The use of polysaccharides as scaffolds is of the highest interest for their expected biocompatibility characteristics and possibility to adapt to the various therapeutic-diagnostic purposes. In this study derivatives of glycogen (glycogen conjugates) and of mannan (mannan-conjugates) were evaluated *in vitro* and *in vivo* to determine their biocompatibility (cell viability, influence on cell behavior), capability to be up taken by the tested cellular systems and their biodegradation; diffusion and accumulation in the tumor and organs (liver, spleen, kidney and lymph nodes); permanence in the targeted organs in perspective of therapeutic (selective tumor treatment) or diagnostic (sentinel lymph node) application.
Both types of conjugates were produced with various characteristics (linked to fluorescent dye for *in vivo* and *ex-vivo* evidence of accumulation and organ distribution), with gadolinium (in comparison with the gadolinium contrast commonly used in magnetic resonance diagnostics) as possible selective MR diagnostic systems, with modification by polymethyloxazoline (POX) to reduce the uptake by the immune cells and increase the circulation time. This strategy was especially necessary in consideration that the conjugates have not bound specific tumor targeting molecules and their intratumor accumulation is consequence of the Enhanced Permeability and Retention effect (EPR).
The *in vitro* models included human hepato-carcinoma cell line HepG2 to test the glycogen conjugates, that resulted biocompatible and localizing in the cytoplasm of the cells with degradation lysosome independent and caveolin intracellular internalization. For *in vivo* test of this conjugates the HUH7 human hepato-carcinoma cell line was used by inoculation in immunodeficient rats. Accumulation in the tumor was evidenced by fluorescence and MR *in vivo* and *ex-vivo* in the internal organs (liver and kidney quickly positive and progressively with reduction of signal) and a quite interesting stability in the tumor with better signal than the gadolinium alone. Interestingly, the conjugate named GG was maintaining a valuable signal in the kidney along all the experiment period even at the low dosage used, a result to be considered in the view of use in patients with kidney function impairment. Variability of the fluorescence signal was attributed also to

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degradation of the bound fluorescent molecule. The mannan conjugates were instead evaluated in mouse models using the same type of cells (4T1 mouse mammary adenocarcinoma) *in vitro* and *in vivo* in syngeneic Balb/c mice with orthotopic inoculation in the mammary glands. These conjugates resulted internalized and degraded in the lysosomes, while the localization in the tumor was less stable than in the lymph nodes, very important result for the identification of the sentinel lymph nodes and lymphatic tumor localization. Moreover, the signal in MR was better with the conjugates than with the classic contrast.

In conclusion the aims and goals of the study to demonstrate excellent biocompatibility of the conjugates, capability to diffuse and accumulate in the tumor and progressive biodegradation in the organs (liver, kidney) in which also accumulates were achieved. Furthermore, the modification by POX resulted to increased recirculation and further accumulation after the initial peak was also evidenced.

The reported data and results are discussed in a dissertation thesis composed by a bi-lingual abstract (CZ and EN), a well-organized general index, a list of abbreviations, 81-pages dissertation followed by a structured curriculum vitae (demonstrating activity, skill acquisition and commitment in the scientific work by the candidate) and an extended bibliography with references in the text ranging from XIX century (C. Bernard) to the very recent years. In the Annex, the copy of four articles in which the data and results presented in the dissertation were publishes, 2 as the first author and 2 as a co-author in journals with IF range 4.380-6.331. The candidate was also co-author in other 3 publications from her supervisor's team. Various active participations with abstracts, posters and presentation are listed in the CV.

The general structure of the thesis and its presentation both in language and graphics is very good, with appropriate figures. An adequate repertoire of references is related to the introductive part, which is clearly developed and functional for understanding the bases sustaining the study plan.
Also, the sequence of experiments is logically designed and appropriately conducted.

Conclusion:

from what above written, I can conclude that the candidate fulfilled the aims and goals of her doctoral study, showing capability to acquire and manage the various technics and methods requested for her research, ability to plan experiments with logic and proper analysis and to write articles well presenting the obtained results. Therefore, I recommend the thesis work for the defense and I evaluate the candidate suitable for obtaining the title of Ph.D.

A few questions:

1) Why the use of different models for the two different conjugates (human vs murine) with the necessity to use an immune deficient animal model in the first case and why the rat and not the mouse for better homogeneity between the two in vivo evaluation?
2) Polysaccharides can even stimulate immune cells: was during the study evaluated if any effect was produced by the conjugates on PBMC or splenocytes, or is it in your plans?
3) The prolonged persistence of GG conjugate with gadolinium in the kidney, with signal even after low dose application, raise the question of the nephrotoxicity that can accompany the gadolinium administration, with contra-indication or limitation for use in subject with reduced renal functionality. If this conjugate is planned for further study, was considered this aspect and

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eventually was evaluated some kidney function test (even simply urine control for proteins and microhematuria)?

4) Why the choice of different way of administration (i.m., i.v.), with related possibility of particular diffusion of the conjugates depending on the model? Any advantage from one or the other?

Faithfully

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