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REVIEW Report on the doctoral dissertation of MSc Carolina Sofia Pereira Roxo entitled
**"Investigations on structural and physicochemical features potentially correlated
with G-quadruplexes antiproliferative activity"**

Carolina S. Pereira Roxo's doctoral thesis presented for review was prepared at the Department of Nucleic Acids Bioengineering at the Institute of Bioorganic Chemistry of the Polish Academy of Sciences in Poznań, under supervision of Assoc. Prof. Anna Pasternak and co-supervision of PhD Weronika Kotkowiak.

The research contained in this dissertation was created as a result of the implementation of research projects entitled "Novel bispecific G-quadruplex conjugates as potential anticancer agents" funded by the National Science Centre, Preludium 18 (UMO-2019/35/N/NZ7/02777, PI's supervisor: Anna Pasternak) and "Structural determinants of anticancer properties of G-rich oligonucleotides" funded by the National Science Centre, Opus 13 (UMO-2017/25/B/NZ7/00127, principal Investigator: Anna Pasternak).

The doctoral dissertation fits in the trend of research dealing with G-quadruplexes as a molecular target in the development of anticancer drugs and involved in the regulation of gene expression. In the light of the global threat of various cancers, this subject is very current. Exploring knowledge in this area inspired Candidate to undertake research, the main purpose of which was to examine the structural features and physicochemical properties of various G-quadruplex structures and correlating them with their biological potential.

Guanine quadruplexes (G-quadruplex, G4 DNA, G4 RNA) are non-canonical, folded single- or multistranded nucleic acid structures formed from co-planar arrangements of four guanines (G-tetrads), which are stabilized by Hoogsteen-type hydrogen bonds in the presence of selected metal cations (Na^+ , K^+). Depending on the number of strands forming G-quadruplexes, we can distinguish single-, two- or four-molecular quadruplexes. G-quadruplexes show a large structural polymorphism, resulting from different directionality of the strands, G-tetrad arrangement, or the orientation of the loops. Nucleotides that do not form tetrads form different types of loops that connect them, for example lateral, diagonal, external called propellers or loops in the shape of a V. All of these factors affect the final topology of

the G-quadruplex, but it is also greatly impacted by the type of cation and its concentration, pH of the solution, temperature, added solvents or ligands.

MSc Carolina Pereira Roxo's doctoral dissertation was prepared as a coherent series of four papers, one review and three experimental, published in 2019-2023. It should be emphasized that all the works that make up the series have been published in reputable journals from the JCR list with high impact factors and a large number of points of the Ministry of Science and Higher Education (MNiSW). The total value of the IF coefficient for these works was 19.519 (average IF=4.88), the number of the Ministry of Science and Higher Education points is 520. The review paper was published in 2019 in the journal *Molecules* (IF=4.927, 140 MNiSW points), and experimental papers were published successively in 2021 in the *International Journal of Molecular Sciences* (IF = 6.208, 140 MNiSW points), in 2022 in *PLoS One* (IF = 3.752, 100 MNiSW points), and the last publication in the series was published in the current year in *ACS Medicinal Chemistry Letters* (IF=4.632, 140 MNiSW points).

In three papers that make up the series, which is the basis of the dissertation, Candidate is the first author, and moreover, according to the statements, she had a very large share in the creation of these publications. In all papers of the reviewed series of MSc Carolina S. Pereira. Roxo designed and performed the experiments, analyzed the results and prepared the manuscripts, which proves the author's great commitment and independence. At the same time, the composition of the authors of these papers indicates great support and cooperation of the research team in the implementation of research, the results of which are included in this doctoral dissertation. The corresponding author of all works in the series is PhD hab. Anna Pasternak, Assoc. Prof.

In the publication "G-quadruplex-forming aptamers—characteristics, applications, and perspectives" the topic of G-rich aptamers forming G4 is in-depth and presented. These nucleic acid structures are capable of recognizing a variety of molecular targets (antiviral, anticoagulant, anticancer G-quadruplex aptamers), and due to many advantages are an interesting therapeutic and diagnostic alternative to antibodies. G-rich aptamers are successfully used as aptasensors, they can also be used for various therapeutic purposes as an addition to anticancer drugs, anticoagulants, or in antiviral treatment. The importance of this work and the interest in the subject can be seen in the fact that the publication has already been cited 89 times. The high quality of the review paper published in 2019 indicates the PhD student's very high knowledge and interest in the subject, as well as the experience and high skills of the team of authors in writing this type of work. This literature review showed that the formation of G-quadruplex structures is essential for the effective inhibition of cancer cell

proliferation, however, there is a lack of information on the correlation between their structural elements and potential anticancer properties. This literature review became an inspiration for the implementation of the specific objectives of the work, which included, among others, (1) examining the impact of G-quadruplex structural elements on the antiproliferative properties of G-rich oligonucleotides; (2) examining the influence of chemically modified sugar moieties (LNA, UNA, 2'-O-Me-RNA) on physicochemical and anticancer properties of three sequence-related DNA G-quadruplexes; (3) analysis and comparison of physicochemical and biological properties of DNA G-quadruplexes and their RNA counterparts. Experimental papers that make up the dissertation series present the results of the implementation of these goals. The PhD student used various analytical techniques in her research, such as UV-Vis spectroscopy (UV melting analysis, thermal difference spectra) or circular spectroscopy, as well as biological experiments such as Antiproliferative Assay, Nucleolin Binding Assay or Nuclease stability assay. The studies demonstrated that the biological activity of G4s is inseparable from their structural elements. The thermodynamically more stable G-quadruplexes possessing more G-tetrads in the core are more biostable and show efficient cellular uptake. On the other hand less thermodynamically stable G4s with three G-tetrads in the core and longer loops more effectively inhibit the growth of cancer cells. The chemical modification of sugar moieties (locked nucleic acid LNA, unlock nucleic acid UNA, and 2'-O-Me-RNA) in the loop or in the G-tetrad of G-quadruplexes DNA indicated, that UNA modifications improve the G-quadruplex thermodynamic stability, but at the same time they weaken the anticancer properties. In turn, modified LNA and 2'-O-Me-RNA G-quadruplexes showed some antiproliferative potential, and RNA G-quadruplexes are weaker inhibitors of cancer cells proliferation than their DNA analogs.

The publications that make up the series are very well and carefully prepared, they contain clear graphic documentation of the results. The entire dissertation is of the same high quality as the publications. The author was able to present the entire issue in a very accessible, synthetic form, highlight the goals and course of research and emphasize the importance of the results obtained, pointing to the contribution to basic research and the development of science. The reviewed doctoral dissertation was written in English and consists of twelve chapters: list of all Candidate publications included (I) and not included in the doctoral dissertation (II), abstracts in English and Polish (III and IV, 6 pages), the aim of the project (V, 2 pages), introduction (VI, 11 pages), brief description of the publications included in the doctoral dissertation (VII, 18 pages), summary (VIII, 2 pages), methods (IX, 6 pages), list of abbreviations (X, 2 pages), references (XI, 8 pages) and academic achievements (XII, 3

pages). The Author has also included two appendixes: Thematically related publications included in the doctoral dissertation (I) and Statements of the co - author (II). In her dissertation, Candidate cited 137 publications. The knowledge review presented in the review publication was based on 151 valuable, well-chosen publications. In subsequent experimental publications, the PhD student referred to 48, 39 and 38 publications, respectively.

I have no fundamental objections to the reviewed doctoral dissertation. While reading, some questions came to my mind:

1. It has been published, that AT11-B1, a modified sequence of AT11 (itself a modified version of nucleolin aptamer AS1411), may be recognized by a variety of G4 ligands. The results showed that the ligand PhenDC3 did not impair the binding to the protein and that a ternary complex G4 DNA - Ligand - NCL could be formed. The data obtained could be of great importance in the development of new strategies to target cells expressing NCL at their surface. What could be the future of research on aptamer G4 - ligand - protein complexes? I would like to know the Candidate's opinion on this.
2. What are the directions of further research being a continuation of the research included in the doctoral thesis?

Final conclusion

The research result, novelty and technical correctness were verified by their publication in journals with a high impact factor. At this point, it is worth adding that the scientific interests of Candidate are not limited to the subject of the doctoral dissertation and she is the co-author of 1 scientific paper with a good impact factor. It is worth emphasizing that MSc Carolina Pereira Roxo was the co-investigator and main investigator in four research projects, received 5 awards, of which four were related to the achievements in the science field and participated in 4 conferences (she is the author of 3 conference announcements).

I confirm that the doctoral dissertation submitted for review meets the conditions set out in the Act of July 20, 2018 - Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended), the Act of July 3, 2018 Provisions introducing the Act - Law on Higher Education and Science (Journal of Laws of 2018, item 1669, as amended) and in the Procedure for conferring a doctoral degree at the Institute of Bioorganic Chemistry of the Polish Academy of Sciences in Poznań (Resolution of the Scientific Council of ICHB PAS No. 56 /2023/Internet of March 29, 2023) and I apply to the Scientific Council of the Institute of Bioorganic Chemistry of the Polish Academy of Sciences to admit Carolina Sofia Pereira Roxo to the next stages of the procedure for conferring a doctoral degree.

In addition, due to the wide range of studies, high quality and importance of the results of the dissertation and level of scientific achievements, I am applying to the Scientific Council of

the IChB PAS in Poznań for the distinction of the doctoral thesis of MSc Carolina S. Pereira Roxo.

Spok Juszczak