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Review of **Carolina Sofia Pereira Roxo** doctoral thesis
entitled :
„Investigations on structural and physicochemical features potentially correlated with G-quadruplexes antiproliferative activity."

Introduction

In the present work Author concentrated on the investigation of structural and physicochemical features for a set of G-quadruplex structures in respect to their biological potential as anticancer agents. The studies presented from other laboratories indicated that G-quadruplex based aptamers are crucial for the efficient inhibition of cancer cell proliferation. However, there was a lack of knowledge related to the correlation between the structure and potential antitumor properties of these specific, DNA derived structural elements. These gaps of data gave the background for doctoral thesis presented by Carolina Pereira-Roxo.

There were undertaken the studies with three aspects of G-quadruplex mediated antiproliferative potential, taking into account: (i) the analysis of five sequence-related DNA molecules, which are different in loop length or number of G-tetrads, (ii) chemical modifications in sugar moieties as a molecular tool to improve the structural aspects of G-quadruplex antiproliferative activity, and (iii) the comparison of four RNA and DNA sequence-related G-quadruplexes in respect of their physicochemical and biological properties. All above aimed to develop the potential of G-quadruplex-based antitumor agents.

The research project, performed at Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań as Doctoral Thesis, was carried out at Department of Nucleic Acids Bioengineering under supervision of Assoc. Prof. Anna Pasternak and co-supervision of Dr Weronika Kotkowiak. Professor Anna Pasternak with her group is strongly involved in the studies on DNA and RNA oligonucleotides with approach to targeted nucleic acid therapy. Particularly, the group considers specific class of protein targeted nucleic acids called aptamers, which bind selectively by shape-specific recognition. Among aptamers the most promising for targeted therapy is characterized by guanosine-reach sequences, which can easily form G-quadruplexes. Being of great structural diversity these molecules are present in the genome of many organisms with a wide regulatory potency.

According to the title of the work reviewed G-quadruplexes formed by DNA or RNA G-rich sequences are the subject of the present work. The aim of this doctoral thesis is to disclose the

structural and physicochemical characteristic of G-quadruplexes, which would reveal and/or improve their antitumor activity.

Work characteristic

The present work is of two parts: The Main part, with the subjected results, and The Appendix, with full text of related publications. This construction of the text comes from the fact that all results of this doctoral thesis were published in three full papers. What is more, theoretical approach, which is usually included as Introduction is also the subject of separated review article.

The main part of doctoral thesis was written on 64 pages and contained 137 positions of References. At the beginning there are: two lists of Author's publications (being or not the subject of doctoral thesis), Abstracts in English and Polish and The aim of the project. Next, there is short, very well written Introduction with good characteristic of particles being the subjects of this work. There are G-rich oligonucleotides, which kept the capacity to fold into G-quadruplex structures and into relatively short DNA- or RNA-based oligonucleotides, named aptamers. Fifth paragraph of the Introduction, p.6.5, concentrated on the potential therapeutic application of these aptamers in the fields of antiviral therapy, anticoagulant properties and anticancer action.

The next part of doctoral thesis, part 7, usually named Results is on 18 pages and there are the brief description of the problems presented in three works published in 2021, 2022 and 2023 with IFs: 6.208, 3.752 and 4.632, relatively. The next, there are traditional paragraphs as Summary, Methods, List of Abbreviations and References. I would like to underline that Results were presented shortly in this text, whereas Methods were fully described in this short version. It was a good idea. The experiment can be repeated directly according this description.

The presented form of doctoral thesis delivered the advantages and disadvantages. On the one hand, there is good idea that doctoral thesis have been published in the Journals of high IF. This indicates a high level of them. However, on the other hand, the reviewer found himself the work, which was previously evaluated by several specialists in this field and was accepted as good pieces of studies. In the face of these conditions the review presented below considered mainly the book of doctoral thesis presented on the pages 1-64.

The results obtained

As I mentioned above, after the background knowledge related to DNA and RNA G-quadruples topology, the G-quadruplex roles in biological system was revealed. Moreover, the role of G-rich oligonucleotides with the development of synthetic ones in respect to G-quadruplex promising therapeutics has drawn a lot of interest. Finally, the Introduction considered G-quadruplex-based aptamers with their antiviral, anticoagulant and anticancer potentials. The selection of structural conditions of G-quadruplex, which would inhibit cancer cell proliferation efficiently is the main goal of this work realized by several aspects.

In the first work related to the Results Authors tried to investigate a series of intermolecular GROs (guanosine reach oligonucleotides) in respect to their thermodynamic and structural properties as well as their therapeutic potential. There was analyzed five sequence related DNA molecules ON1-ON5) different slightly not only in the loop length but also in number of G-tetrads within core of the

structures. The selected G4-forming oligonucleotides were studied in their physicochemical properties with the application of UV-melting analysis, circular dichroism, CD, spectroscopy and thermal difference spectra (TDS). Moreover, biological potential was measured as antiproliferative action as well as cellular uptake and also in respect to oligonucleotide viability in human serum and to its affinity to interact with nucleolin. Structure-activity analyses indicated that oligonucleotides with three G-tetrads in the core and longer loops have strong potential to be an effective inhibitor of cancer cell growth. In contrast, most stable oligonucleotides with four G-tetrads and 4- or 3-nt long loops did not express significant antiproliferative activity. There was proposed the reason for the specific activity of G-quadruplexes in some cancer cell lines. Moreover, the obtained results strongly indicated that the final antitumor activity is a complex result of many different factors as: thermodynamic stability of G-quadruplex structure, efficiency of cellular uptake or nuclease resistance. However, these properties in the case of G-quadruplex-based drugs have reverse structural preference. Therefore, therapeutic success of potent drug needs the sensible compromise between optimal condition for its cellular uptake and efficient degradation in the intercellular compartment

The above results were presented clearly and competently. However, I would like to ask about the cited on page 31 References related to NMR and X-ray studies on G-quadruplexes structural features. I would like to ask whether polynucleotide sequences presented there are identical to those applied in your work?

In the second paper of Results Authors also considered G-quadruplex structure in respect to its chemical modification. In this case structural modification related to the sugar moieties. Three oligonucleotides [ON1, ON2 and ON3, $d(G_{3-4}T_4G_{3-4})_2$] were selected for this step of studies and sugar modifications (unlock nucleic acid, UNA, locked nucleic acid, LNA and 2'-O-methyl-RNA) were analyzed in respect to thermal stability, folding topology and biological activity. Firstly, thermodynamic stability of the initial G-quadruplexes was analyzed by UV melting experiments. Generally, the effect of LNA and UNA presence inside ON1-ON3 G-quadruplex were antagonistic, UNA stabilizes whereas LNA destabilize the structures. Probably the flexibility of the sugar moiety is one of the crucial element in this stability. In contrast, 2'-O-Me-RNA modification with G-tetrads induced mild changes in quadruplex thermal stability. CD spectra revealed that G-quadruplex structures of ON1 and also ON2 expressed antiparallel topology, but ON3 kept the hybrid type structure. There is also not clear structure-activity relationship for the set of ON1-ON4 G-quadruplexes. None of the modified ON variants displayed increased antiproliferative activity in comparison to unmodified G-quadruplexes but some differences were observed between LNA, UNA and 2'-O-Me-RNA-modified variants. However, chemical modifications as observed in LNAs or UNAs were able to increase the physiological stability and nuclease resistance, whereas only nuclease resistance of oligonucleotides was improved for 2'-O-Me-RNAs. What is more, for all analyzed G-quadruplexes there was not observed the tendency for improvement of the nuclease stability and it is not proportional to their antiproliferative properties.

Summing up this part, G-quadruplex structural elements cannot be considered separately and needed wide analysis of its variety. In this respect, Carolina Roxo concluded that UNA modifications could be modulators of G-quadruplex stability, however, this rather not improve the antitumor

activity of G-quadruplexes investigated. In opposition, 2'-O-Me-RNA G-quadruplexes were effective in the inhibition of HeLa cells *in vitro* with maintaining thermal stability, folding topology and specific enzymatic resistance. Finally, LNA modified G-quadruplexes observed within the loops expressed similar antiproliferative potential to the native sequences.

Whereas in first three papers (two of Results) Carolina Roxo is the first Author before her Supervisors, in the present she is on the second position. This position results from the contribution statement declared by Authors for this paper. The subject of this article relates to the examination of structural features of a set of RNA G-rich sequences performed for the correlation analysis of the both: structure properties and thermal stability of model intermolecular G-quadruplexes with biological, particularly anticancer activity.

Four RNA oligonucleotides and their DNA analogs were studied. Thermodynamic investigations revealed that RNA G-quadruplexes are more thermally stable in relation to their DNA counterparts. Moreover, the structures with longer G-tracts joined by shorter loops displayed the best thermodynamic stability. Circular dichroism spectroscopy allowed for topology analysis of OR1-OR4 indicating that only OR1-OR3 expressed parallel topology, whereas OR4 conservative G-tetrad stacks over the hexad GGAGGA and two OR4 molecules stacks on top of one another. The analysis of antiproliferative activity indicated that particular DNA G-quadruplexes expressed this potency in contrast to RNA counterparts. Finally, it is important conclusion that DNA G-quadruplexes expressed higher antiproliferative activity and what is more, rather folding topology than thermal stability are crucial in the inhibitory activity of the studied oligonucleotides.

Results discussion.

As I mentioned above all results of this doctoral thesis were published and according to Editor procedures all but one papers contain part entitled "Results and discussion" as well as "Conclusions". There is not the separate part Discussion in the doctoral thesis evaluated. However, each part of work contains this element as many obtained results simply call for the discussion. It is the example in analysis of G-quadruplexes as potent anticancer agents. Author concluded that the oligonucleotides with three G-tetrads and longer loops kept higher susceptibility to act in the role of effective inhibitor of cancer cell growth (ON2, ON3 and ON5). On the other hand, the most thermodynamically stable oligonucleotides, ON1, ON4 with four G-tetrads and 4- or 3-nt long loops did not express significant antiproliferative activity. Good background for the discussion was also the set of UNA, 2'-O-Me-RNA and LNA potential modifications in respect of their role in antitumor activity. Author observed that UNA modification influenced G-quadruplex thermodynamic stability but rather not anticancer potential of this agent. In contrast, thermodynamic stability, structure folding topology and the level of enzymatic resistance was not changed in 2'-O-Me-RNA modified G-quadruplexes although they are effective in cancer cell proliferation in vitro. Moreover, LNA modification within the loops expressed antiproliferative activity similar to the native sequence. There is also attractive results for OR4 in RNA G-quadruplexes, which contains G-tetrad, which was placed over a hexad GGAGGA and hexad-hexad interface allowed two OR4 molecules to stack on top of one another. The final conclusion of the last paper indicated that minor changes in sequence expressed significant impact on the properties of G-quadruplexes and RNA G-quadruplexes are more thermally stable then DNA. Moreover, DNA G-quadruplexes having better antiproliferative properties

than RNA analogs, their inhibitory activity was more strongly influenced by folding topology than by thermal stability.

In respect to the above results leading to very attractive conclusions I would like to ask about the mode of action of G-quadruplexes on the level of cell crucial responses. There was written on the page 20 for example, that "...G-quadruplexes have the ability to recognize and bind many proteins that have fundamental roles in different pathologies....". However, what is known about molecular mechanism of cellular response of G-quadruplexes action on the level, for example, of induction the apoptosis, necrosis and senescence or other pathway of cell death?

Editorial aspect

As I mentioned above (page2) the presented form of doctoral thesis delivered the advantages and disadvantages. The current review considered the doctoral thesis presented on the pages 1-64. In my opinion this part was very well written, I did not find there any clear mistakes. It would be only suggested, that the part 7 might by entitled simply "Results" or "Results and Discussion" with the subtitle: "Brief description.....". This would allow to have more traditional form of this doctoral thesis.

My comments also concern the form of References. Why there is the name of only first authors, whereas sometimes, for example, the second one or the last is very crucial?

I would like to add that it was good idea, that the majority of experimental procedures was described in details in Methods of short version and there is full list of Abbreviations.

Final conclusions

The results obtained in the framework of doctoral thesis presented by **Carolina Sofia Pereira Roxo** and entitled: "*Investigations on structural and physicochemical features potentially correlated with G-quadruplexes antiproliferative activity*" indicate high scientific level and experimental competences achieved by PhD student in a new field of research, which is currently developed deeply and very intensively. What is more, all Results presented and the Introduction were published in journals of high IFs, equal to: 4.927, 6.208, 3.752 and 4.632.

Finally, there is concluded, that the above work meets all the requirements necessary for doctoral thesis according to: Ustawa z dnia 3 lipca 2018 roku prawo o szkolnictwie wyższym i nauce (Dz. U. 2018, poz.1669 ze zm.) oraz o sposobie postępowania w sprawie nadania stopnia doktora w Instytucie Chemii Bioorganicznej PAN w Poznaniu (uchwała Rady Naukowej IChB PAN nr 56/2023/Internet z dnia 29 marca 2023) and I am applying to Faculty Council of Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań for admission of Carolina Roxo to further stages of the doctoral dissertation. I would also propose to award the reviewed doctoral thesis. The support for this request was attached separately.

Zofia Mazurka