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Prof. Robert Musioł
Institute of Chemistry
University of Silesia in Katowice

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A review of the doctorate thesis entitled Mechanistic studies on selected novel anticancer compounds modulating the function of telomeric proteins" written by Anoop Kallingal MSc under supervision of Prof. Dr Eng. Maciej Bagiński and Natalia Maciejewska PhD as the auxiliary supervisor.

The dissertation focuses on the mechanisms of action of novel compounds with potential anticancer activity that affect the function of telomeric proteins. In particular, the author investigates compounds designed to modulate the interaction between TRF1 and TIN2, two important components of the shelterin complex. This complex plays an essential role in protecting chromosome ends and maintaining telomere stability. Since cancer cells depend on telomere maintenance to sustain their proliferation, targeting telomeric proteins is considered a promising approach to anticancer therapy. The subject of the dissertation is therefore scientifically important and timely. It addresses a relevant problem in cancer research and the development of new anticancer drugs.

The dissertation has a classical structure and comprises 180 pages, including preliminary sections, the main text, references and appendices. The main text is organised into six numbered chapters. Introduction presents the general background of the study. The author shortly discusses the importance of cancer as a major health problem, treatment resistance, cancer cell immortality and the role of telomeres in cancer development. The chapter also introduces the shelterin complex and explains why the interaction between TRF1 and TIN2 may be considered a potential target for anticancer compounds. Second chapter contains Research Hypothesis and Objectives presents the main research hypothesis, the aim of the study along with the research questions objectives. Notably it outlines also the general research strategy and the expected significance and novelty of the work. Literature Review, is considerably more robust, taking more than 20 pages and provides a detailed overview of telomere biology and the shelterin complex. Particular attention is given to the structure and functions of TRF1 and TIN2, their interactions with other shelterin proteins, and their roles in telomere protection and replication. The author also discusses previous approaches to targeting shelterin proteins in cancer therapy. This chapter provides the scientific background for the experimental part of the dissertation and fill the gap of short introduction.

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Wydział Nauk Ścisłych i Technicznych
Instytut Chemii
ul. Szkolna 9, 40-006 Katowice
tel. 32 359 15 45, e-mail: ich.wnst@us.edu.pl

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Next chapter contains concise presentation of Materials and Methods. Author describes there experimental and computational methods used in the study. These include compound selection, molecular docking, cell viability assays to telomere FISH, Western blotting and RNA sequencing. This chapter also describes experiments on cell migration, colony and spheroid formation, angiogenesis and tumour growth in the chick embryo model, as well as the methods used for statistical analysis. Methods are well described with sufficient details to be repeated elsewhere. It should be noted that compounds investigated in this dissertation were developed and synthesized as part of a separate project carried out in the Organic Chemistry Laboratory of Dr. Sławomir Makowiec at Gdańsk University of Technology, with the participation of Anna Hromova, Radosław Krzemieniecki and other team members. Therefore, detailed descriptions of the synthetic procedures were not included in the thesis. The structures of the compounds are presented in the Appendix in a format that closely resembles their presentation in patent documentation. This approach is understandable in view of the broad scope of the research undertaken in the dissertation

As could be expected chapter 5 containing results is the largest part of the dissertation spreading on logically divided 19 sections. The author first presents the selection of compounds, their cytotoxic activity and selectivity, and studies of their binding to TRF1. These results are supported by molecular modelling. The following sections describe the effects of the compounds on DNA damage, DNA replication, cell cycle progression, mitochondrial function, apoptosis and cellular senescence. Further experiments examine cell migration, colony formation, spheroid growth, angiogenesis and microtumour development. The chapter also includes analyses of telomere abnormalities, changes in protein expression and transcriptomic responses to the tested compounds. Lecture of this part of the thesis highlights the impressive scope of the research undertaken. The wide range of methods and complementary experimental strategies employed to address the objectives of the dissertation is particularly noteworthy and demonstrates the considerable effort invested in the work. Discussion as a separate chapter contains deeper insight into the relationship between compound binding to TRF1, telomere dysfunction, replication stress and the observed cellular responses. The results are discussed in relation to published studies, and the limitations of the work and directions for further research are presented. The main text is closed with conclusions and supported by 219 references, covering both fundamental studies on telomere biology and more recent publications related to the research topic. The dissertation is generally very well prepared from an editorial point of view.

The main aim of the dissertation was to investigate the mechanisms of action of selected novel compounds with potential anticancer activity, designed to target the interaction between TRF1 and TIN2 proteins. The author aimed to determine whether these compounds bind to TRF1, affect telomere stability and DNA replication, and induce cellular responses that may limit cancer cell growth and survival.

An additional objective was to identify the most promising compounds for further development. To achieve these aims, the author used a broad experimental approach combining computational methods, biophysical measurements and cell biology techniques. The choice of methods was appropriate for the research objectives. The methods were selected in a logical way, with the results of individual experiments providing the basis for further investigations.

The research started with the selection of compounds derived from the parent molecule A822 by means of molecular docking and virtual screening. The compounds were synthesized as mentioned by other members of the research project and then subjected to biological evaluation. The first experimental stage involved testing A822 and eleven derivatives against a panel of 19 cancer cell lines accompanied with the cytotoxicity tested in selected non-cancerous cell lines. The next stage focused on the interaction of the selected compounds with TRF1. Surface plasmon resonance was used to examine their direct binding to the protein, while molecular dynamics simulations provided additional information about possible binding modes and the stability of the predicted protein–ligand complexes. Immunofluorescence studies were used to investigate the induction of DNA damage, particularly changes in γH2AX levels and its colocalisation with TIN2. Cell cycle analysis and DNA fibre assays provided information about disturbances in cell cycle progression and DNA replication. In combination with other tests Mr. Kallingal was able to fully investigate the cellular response to treatment with tested compounds. Additional tests on 3D cell spheroids and chick embryo membrane were prepared as additional level of biological evaluation beyond conventional cell culture experiments and pre- in vivo tests. Particular attention should be given to the experiments directly related to telomere biology. Using telomere FISH, the author was able to investigate chromosome abnormalities associated with telomere dysfunction, including telomere signal loss, telomere doublets and chromosome fusions. These results were complemented by measurements of telomere fluorescence intensity. This part of the study was especially relevant because it connected the proposed molecular target with changes observed at chromosome ends.

In my opinion, one of the main strengths of the dissertation is the comprehensive and well-organised research strategy. The author did not rely on a single experimental method but used several complementary approaches to investigate the problem of drug activity in its full scope. Another strength is the progression from the initial screening of a relatively large group of compounds to detailed mechanistic studies of selected candidates. The combination of these approaches gives the dissertation considerable experimental value. The results provide a broad and generally consistent picture of the biological activity of the investigated compounds and form a useful basis for further studies on their mechanisms of action and potential anticancer applications.

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This approach provided scientifically sound conclusions as identification of several A822 derivatives with promising anticancer activity, which induced replication stress, DNA damage, telomere abnormalities and reduced cancer cell proliferation. The results suggest that these effects may be associated with the modulation of TRF1 function.

As part of the reviewer's responsibility, it is also necessary to point out the weaknesses, errors or limitations of the dissertation. My main comment concerns the interpretation of the proposed mechanism of action of the investigated compounds. One of the central assumptions of the project is that these compounds may interfere with the interaction between TRF1 and TIN2. The author provides experimental evidence that selected compounds bind to TRF1, and the results of molecular modelling suggest possible binding sites. The biological experiments further demonstrate replication stress, DNA damage and telomere abnormalities, suggesting that the compounds may affect the function of the shelterin complex. This is scientifically sound and clear part of work. However, although these findings are consistent with disturbances in telomere function, they do not directly demonstrate that the compounds inhibit the interaction between TRF1 and TIN2. In particular, the SPR experiments establish binding to TRF1 rather than competition with TIN2, while the observed cellular effects could also result from other consequences of TRF1 binding or from more general effects on DNA replication and integrity for example direct interaction of the compounds with DNA or disturbing the G quadruplex. Therefore, I would like to ask author about alternative mechanism may produce the same effect and what assay, could be used to validate the main hypothesis in cells?

My second comment concerns the specificity of the observed cellular effects. The author reports increased γH2AX levels, disturbances in DNA replication and other cellular stress responses in the context of the proposed telomere-related mechanism. It should be noted, however, that the author addresses many of these issues in Section 6.8, “limitations and scope of further research”. This discussion demonstrates a good understanding of the limitations of the experimental approach and a balanced interpretation of the results. The author therefore shows an appropriate awareness of the distinction between the findings directly supported by the data and the proposed mechanisms that require further experimental validation.

Finally, the dissertation contains several minor editorial and bibliographic errors. These include some inconsistent terminology, imprecise descriptions of measured parameters and errors in the formatting of references. For example, the description of changes in telomere fluorescence is considered as equal to changes in telomere length. Actually change of telomeres’ length is one of the possible causes of changing fluorescence in this experiment. Some bibliographic entries also require correction, particularly those referring to protein structure databases PDB (66, 72) do not follow the policy of the database.

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These are minor issues that could easily be corrected and do not affect the scientific value of the dissertation, which in my opinion is high. In summary, the comments presented above mainly concern the strength of the mechanistic conclusions and the precision of their interpretation. They do not undermine the experimental results or the overall value of the research. In my opinion, the dissertation presents a substantial amount of original experimental work, and its scientific contribution clearly outweighs the limitations discussed here.

In conclusion, I believe that the dissertation demonstrates that Mr. Anoop Kallingal is a well-trained young researcher with broad scientific knowledge and a wide range of experimental skills. He has shown the ability to plan and carry out complex research, apply different experimental methods, analyse the results and draw scientifically justified conclusions. The scope and quality of the work also demonstrate his ability to conduct independent scientific research. In my opinion, the dissertation represents a new and original scientific contribution to the fields of medicinal chemistry, particularly the development and evaluation of novel compounds with potential anticancer activity, and molecular and cellular biology, especially the study of telomere function, the shelterin complex and the mechanisms of cancer cell response to treatment.

It should also be emphasised that Mr. Kallingal's scientific activity extends well beyond the research presented in this dissertation. His scientific achievements include 16 research publications, mainly in reputable scientific journals, a patent application, and active participation in scientific conferences. This record demonstrates his strong involvement in scientific research and his ability to contribute to collaborative research projects.

Taking into account the scientific value of the dissertation, the originality of the presented results, the broad range of experimental work and the overall scientific achievements of the candidate, I give the dissertation a **positive evaluation**. Therefore, I recommend to the Council for Chemical Sciences at Gdańsk University of Technology that Mr. Anoop Kallingal be admitted to the further stages of the procedure for the award of the doctoral degree.

Moreover as for the high scientific quality, innovative character of this dissertation, and strong applicatory potential of the results I hereby request that the High Council of the Discipline of Chemical Sciences award the dissertation a distinction.

The outstanding value of this dissertation lies in its contribution to the early stages of developing new anticancer drugs. The research has enabled the identification of novel chemical structures with high anticancer activity and simultaneously offered the selection of lead compounds for further investigation increasing the applicatory potential. Importantly, the work does not stop at identifying biologically active compounds, but also seeks to understand the molecular and cellular processes underlying their activity.

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The results support the hypothesis that these compounds may exert their effects by disturbing telomere-associated processes and the function of shelterin proteins, pointing to a promising but insufficiently explored direction in anticancer drug development.

The significance of this research is further underscored by the complexity of the biological target under investigation. Although telomere maintenance and shelterin proteins play important roles in cancer cell biology, targeting these processes remains a challenging area of drug discovery, and this approach has not become an established mechanism of action among anticancer therapies in clinical use. The research of Mr Kallingal therefore addresses a demanding scientific problem: not only the identification of new compounds with anticancer activity, but also the investigation of new unconventional molecular target and mechanism. There are currently no similar drugs focused on this mechanism and introduction novel leading structures has strong impact on current knowledge.

Although further studies are needed to confirm the proposed mechanism and establish the therapeutic potential of the identified compounds, the combination of the original chemical structures identified, the investigation of their potential mechanisms of action and the significance of the scientific problem addressed provides, in my opinion, a strong justification for awarding the dissertation a distinction.



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