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Review
of the Doctoral Dissertation of Mr Anoop Kallingal

Original title:
Mechanistic Studies on Selected Novel Anticancer Compounds Modulating the Function of Telomeric Proteins

Polish title:
Badania mechanizmów działania wybranych nowych związków przeciwnowotworowych modulujących funkcję białek telomerowych

Dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the field of Natural Sciences, discipline: Chemical Sciences.

This review has been prepared on the basis of the materials provided by the Dean's Office of the Faculty of Chemistry, Gdańsk University of Technology.

The dissertation was supervised by Professor Maciej Bagiński, with Dr Natalia Maciejewska serving as auxiliary supervisor.

The dissertation contains the required declaration concerning intellectual property rights.

The research underpinning this dissertation was supported by the National Science Centre (NCN), Poland, within the framework of the OPUS programme (grant no. UMO-2023/49/B/NZ7/03456; funding decision of 29 January 2024), under the leadership of Professor Maciej Bagiński.

The doctoral candidate's scientific achievements comprise sixteen peer-reviewed publications published in journals included in the official list of the Polish Ministry of Science and Higher Education.

1. Introduction and Significance of the Research Topic

Cancer remains one of the leading causes of mortality worldwide. Despite substantial advances in diagnostics and therapeutic interventions, there is still a pressing need to identify novel molecular targets that may enable the development of more effective and selective treatment strategies. In this context, particular attention has recently been devoted to telomeres and the shelterin complex, which plays a pivotal role in maintaining genome integrity, preserving chromosomal stability, and regulating cellular proliferation.

The dissertation under review addresses a highly relevant and rapidly evolving area of contemporary anticancer research focused on therapeutic approaches targeting the structure and function of telomeres and telomere-associated proteins. The candidate concentrated on the investigation of novel derivatives of compound A822, designed as potential modulators of the interaction between the telomeric proteins TRF1 and TIN2. This interaction constitutes a fundamental component of shelterin complex functionality, and its disruption may result in telomere destabilisation, induction of replication stress, activation of DNA damage response pathways, and ultimately a reduction in the proliferative capacity of cancer cells.

The research problem addressed in this dissertation is of both fundamental and translational significance. The findings presented herein may provide the foundation for future development of a novel class of anticancer agents targeting telomeres through mechanisms distinct from those employed by conventional telomerase inhibitors.

2. Formal Evaluation of the Dissertation

The dissertation comprises 144 pages and follows the conventional structure of an experimental doctoral thesis. It includes abstracts in both Polish and English, an extensive literature review, clearly formulated research objectives, a detailed methodology section, presentation of results, discussion, summary, bibliography, and four appendices containing supplementary data.

The work is richly illustrated with figures, tables, and comprehensive datasets. Particular recognition should be given to the exceptionally well-prepared list of abbreviations, which significantly facilitates comprehension of the manuscript.

The bibliography includes 219 references and has been compiled appropriately. The cited literature is predominantly recent and relevant, while also incorporating landmark publications that laid the foundations for contemporary understanding of telomeres, telomerase, and the shelterin complex.

The dissertation is logically organised, clearly structured, and maintains appropriate proportions between its constituent sections. The individual chapters form a coherent narrative that effectively guides the reader from theoretical foundations through the formulation of the research hypothesis to the interpretation of experimental findings.

The only substantive formal shortcoming is the absence of a clearly delineated section presenting concise, itemised final conclusions directly related to the original research objectives.

3. Scientific Evaluation of the Dissertation

The candidate formulated a well-founded research hypothesis proposing that compound A822 and its newly designed derivatives are capable of interacting with the TRF1 protein, disrupting telomere function, and suppressing cancer cell growth through interference with the TRF1-TIN2 interaction. This hypothesis was subsequently translated into a series of clearly defined research objectives and scientific questions.

Particularly commendable is the exceptionally broad range of experimental and computational methodologies employed in the study. Among others, the candidate performed:

- molecular modelling and in silico analyses,
- virtual screening of more than 18,000 chemical structures,
- molecular dynamics simulations,
- surface plasmon resonance (SPR) studies,
- cytotoxicity assessment across a panel of 24 cell lines,
- proliferation and clonogenic assays,
- cell-cycle analyses,
- DNA damage assessment,
- investigations of DNA replication stress,
- telomere-length determination using the Flow-FISH method,
- analyses of cellular senescence and apoptosis,
- RNA-sequencing-based transcriptomic profiling,
- studies employing three-dimensional spheroid models,
- experiments using the chorioallantoic membrane (CAM) model.

The breadth of these methodologies enabled a comprehensive assessment of the biological effects exerted by the investigated compounds.

The reported results indicate that selected A822 derivatives exert multifaceted effects on cancer cells. The candidate demonstrated induction of replication stress, increased telomere-associated DNA damage, activation of DNA damage response pathways, inhibition of cellular proliferation, reduced migratory capacity, and alterations in the expression of genes involved in maintaining genome integrity.

I particularly appreciate the incorporation of three-dimensional spheroid models and the chick embryo chorioallantoic membrane model, both of which substantially enhance the translational relevance of the findings.

4. Originality and Contribution to the Development of the Discipline

The most significant achievement of the doctoral candidate is the identification of novel A822 derivatives and the comprehensive characterisation of their biological activity at multiple levels of analysis. The dissertation provides valuable new insights into:

- the biological consequences of shelterin complex dysfunction,
- the relationship between telomere destabilisation and replication stress,
- the role of the TRF1-TIN2 axis in regulating cancer-cell survival,
- the impact of telomere dysfunction on cellular responses to DNA damage.

The dissertation undoubtedly constitutes a valuable and original contribution to research on the molecular mechanisms governing telomere biology and their exploitation as targets for anticancer therapy.

The scientific merit of the work is additionally reinforced by the candidate's publication record, including publications directly underpinning the methodological aspects of the dissertation. The candidate is the first author of two of these publications and is also the first author of a patent application related to the research topic presented in the thesis.

5. Critical Comments and Points for Discussion

Despite my highly favourable evaluation of the dissertation, several issues warrant further discussion.

First, some reservations arise regarding the direct comparison of MCF7 and HeLa cell lines as representative models of distinct tumour types characterised by substantial biological and molecular differences.

Although the reported IC50 values confirm the biological activity of the investigated compounds, they also suggest that further chemical optimisation will be necessary before considering potential preclinical development. Furthermore, even favourable selectivity indices should be interpreted with caution, as the comparative analysis involved only a single normal-cell model. Consequently, the selectivity of the compounds requires further investigation and broader experimental validation.

While cytotoxicity studies were performed using normal mammary epithelial cells, subsequent mechanistic analyses were conducted, among others, in HeLa cells. A more detailed justification for the selection of these cellular models would have strengthened the study.

On page 60 (Figure 5.2), an inconsistency is present regarding the abbreviation ETAPO/ETP. This represents only a minor editorial issue and does not affect interpretation of the results.

An assessment of baseline TRF1 protein expression levels in the investigated cell lines would have constituted a valuable addition to the study. Such information could potentially help explain the observed differences in compound sensitivity and selectivity among the experimental models.

The section describing cell-migration assays lacks a clear statement regarding the presence or absence of serum in the culture medium, a factor essential for accurate interpretation of migration studies.

Descriptions of cell-cycle analyses do not always incorporate statistical significance testing. In addition, the exceptionally small standard deviations observed in some datasets may indicate a predominance of technical rather than biological replicates.

It is also somewhat unfortunate that a second non-malignant cell line was not included in the detailed mechanistic investigations.

Furthermore, I believe that assessment of p16 and/or p21 expression would have provided valuable complementary information regarding the senescence-associated effects reported in the study.

The discussion section contains a relatively limited number of literature references, thereby somewhat restricting broader integration of the findings within the current state of knowledge.

I would also note that the statement claiming the first description of small-molecule inhibitors targeting the TRF1-TIN2 interaction should be revised in light of the publication by Casale and colleagues in 2025. Nevertheless, this observation does not diminish the originality of the dissertation or the scientific value of the presented findings.

Finally, the dissertation contains several minor editorial inaccuracies (e.g., “STATEGMED” instead of “STRATEGMED”), which do not detract from its overall scientific quality.

6. Questions to the Doctoral Candidate

In addition to the comments presented above, I would appreciate the candidate’s response to the following questions:

How does the candidate explain the relatively modest alterations in telomere length observed using Flow-FISH despite the pronounced increase in telomere-associated DNA damage and activation of DNA damage response pathways?

What biological mechanisms might account for the observed selectivity of the investigated compounds towards different cellular models?

What scientific considerations guided the selection of MCF7 and HeLa cell lines as the principal models for the mechanistic investigations?

Does the candidate intend to pursue further chemical optimisation of the most promising A822 derivatives and evaluate their efficacy in appropriate in vivo models?

7. Final Recommendation

The doctoral dissertation submitted by Mr Anoop Kallingal constitutes a valuable and original contribution to the resolution of an important scientific problem. The candidate has demonstrated an excellent understanding of the research area, an ability to design and execute experiments, and the capacity to conduct advanced scientific investigations independently using contemporary methodologies in molecular biology, cell biology, and computational chemistry.

In my opinion, the dissertation fully satisfies the requirements set forth in Article 187 of the Act of 20 July 2018 on Higher Education and Science. The candidate has clearly demonstrated both the theoretical knowledge and the research competence required for the award of the doctoral degree.

Accordingly, I recommend to the Scientific Council of the Faculty of Chemistry, Gdańsk University of Technology that Mr Anoop Kallingal be admitted to the subsequent stages of the doctoral proceedings leading to the award of the degree of Doctor of Philosophy in Chemical Sciences.

Furthermore, in view of the high scientific quality of the dissertation, the interdisciplinary character of the research, the extensive range of experimental methodologies employed, the candidate's substantial publication record, and the considerable application potential of the obtained results, I recommend that the dissertation be awarded distinction.

This recommendation is justified by the candidate's development of a novel product that represents a significant advancement beyond currently available solutions. Moreover, the processes of discovery, optimisation, and validation of its biological activity have been described with exceptional rigour, clarity, and methodological precision. Importantly, the proposed solution exhibits substantial potential for future technological development and practical implementation.

Yours faithfully,

Błażej Rubiś; PROF. DR HAB.

Digitally signed by Błażej Rubiś; PROF. DR HAB.
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Prof. Blazej Rubis, PhD