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Review of the Ph.D. Thesis of Mgr. Vinod Sukanth Kumar Pallabothula Entitled "Synthesis, development and biological evaluation of new antimicrobial compounds"

The Ph.D. thesis of Mgr. Vinod Sukanth Kumar Pallabothula was conducted under supervision of Assoc. Prof. Jan Zitko, Ph.D. The dissertation addresses one of the most significant challenges in healthcare, namely a need for novel antimicrobial agents. The research is well aligned with current efforts to combat tuberculosis (TB), which remains a global health threat despite availability of clinically used drugs. However, the range of available treatments is limited. Thus, the author's research is up-to-date, given the rise in multidrug-resistant strains of *Mycobacterium tuberculosis* (*Mtb.*), which highlights an urgency of developing novel therapeutic strategies.

This thesis builds upon established approaches of the "Design and Development of New Antimicrobial Agents" research group and is focused on the synthesis of novel pyrazine-based derivatives, their biological evaluation, and investigation of potential targets such as methionine aminopeptidase 1, aminoacyl-tRNA synthetases, and aspartate decarboxylase. The author has also incorporated *in silico* studies to enrich experimental work, adding depth to the investigation. The dissertation is structured as a commentary on four experimental scientific articles (P1-P4), most of which are published in Q2-ranked journals. V. Pallabothula is listed as the first author on two of them.

The dissertation is structured in a clear and logical manner. It is composed of following sections: Introduction, Methodology, Aims and Objectives, Commentary on Published Articles, Ongoing research and prospects, Publication Contributions, Contributions at Scientific Conferences, Internships/Summer Schools, Grant contributions, Thesis consultant for Master students, List of references (103), List of Publications, together on 56 pages. Then, the publications P1-P4 including their list are attached. Thus, almost all results have already been published in journals with impact factor after careful peer review process facilitating my role of the thesis reviewer.

Introduction section provides a well-written overview of TB as a global health challenge and summarizes current treatment regimens and the spread of drug-resistant TB forms. The inclusion of discussions on selected enzymes as promising targets reflects a solid understanding of the biological mechanisms of *Mtb*. infection and the rationale behind targeting these enzymes in this thesis.

The synthetic methodologies employed to prepare pyrazine-containing derivatives are properly described and discussed. The research benefits from relatively straightforward synthetic routes, enabling the candidate to prepare a broad range of compounds to systematically evaluate structure-activity relationships (SAR). The simple synthetic approaches also provide potential cost advantages, an important issue in drug development. The thesis summarizes also biological evaluation of the prepared compounds. Although they did not exhibit broad-spectrum antimicrobial activity, this selectivity for mycobacteria may still be considered as an advantage. A key contribution is the investigation into their possible mechanisms of action, particularly on above mentioned targets. While *in silico* studies provide valuable insights, experimental validation, both *in vitro* and *in vivo*, remains critical to confirm these predictions. The comments on the author's four individual papers

are appropriate and concise. A more detailed exploration of future design and synthetic directions based on SAR analysis results, would enhance the conclusion.

The plagiarism check revealed only minor similarities, mostly in formal matters, with the highest occurrence understandably found in the author's own publications. The dissertation is original.

From a formal point of view, the thesis contains typographical errors such as incorrect capitalization, punctuation, and occasional inconsistencies in chemical nomenclature (e.g., '3-amidopyrazine'), sometimes the signs (≥, >) are written in reverse, "*Mycobacterial spp.*", "was directly converted to 1° amide", "Jan Ziko", "uredio", "pyrazinooxazone", "scaffoled", etc. The list of abbreviations should be arranged in alphabetical order. I would not refer to the missing oxygen in candidate B (page 17) as a carbonyl oxygen. Several figures are not numbered (page 19). I would suggest using independent compound numbering in the dissertation for better clarity, rather than referring to the numbering from the original publications. It would also be appropriate to present these structures or by their chemical names.

My other comments and questions:

1) In section 1.1.1. Current Treatment Regimen for LTBI & TB, I would suggest switching the order of treatment for LTBI and TB. Are all four first-line anti-TB drugs administered for 6 months? How would you explain the effect of isoniazid on LTBI?
2) What is the current state of knowledge of the mechanism of action of delamanid and pretomanid?
3) Please comment on whether the inhibition of MetAP1 is an exclusive mechanism of action of the compounds depicted in Figure 4.
4) Considering the acylation of methyl 3-aminopyrazine-2-carboxylate, did you try using DMAP as a base or an additive?
5) You explored modifications at positions 2 and 3 of the pyrazine ring. Please comment on the possibilities of replacing the pyrazine with another heterocycles, excluding its *ortho*-condensed analogues.
6) For publications where the candidate is listed as a co-author, I would expect a more detailed summary and quantification of his contribution, rather than just listing "Investigation, Synthesis, and Analytical Interpretation." Does this apply to all the compounds presented in P3 and P4?

In conclusion, Mgr. Vinod Pallabothula has presented a dissertation of solid scientific quality, that is a valuable contribution to the field of discovery of potential antimicrobial, particularly antimycobacterial drugs. The combination of synthetic chemistry, biological evaluation results, SAR discussion, and *in silico* studies demonstrates the candidate's comprehensive skill set and research capabilities. The presented findings have a potential to be utilised in future efforts in developing more effective drugs for TB.

Given the quality of the research and this Ph.D. thesis, I recommend the dissertation for further procedure and, following a successful defence, awarding of the Ph.D. degree in the field of pharmaceutical chemistry.

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