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**Recenzja rozprawy doktorskiej mgr Zuzanna Pakosz - Stępień pod tytułem**  
**„Characterisation of nonbacterial gyrases – from biochemical analysis, through compound-enzyme interactions to structural biology”**

**The evaluation of the Doctoral Thesis of PhD Candidate Zuzanna Pakosz - Stępień entitled:**  
**„Characterisation of nonbacterial gyrases – from biochemical analysis, through compound-enzyme interactions to structural biology”**

### **The general overview of the thesis subject**

The PhD project of Zuzanna Pakosz-Stępień was conducted under the supervision of Prof. Jonathan Heddle in the Bionanoscience and Biochemistry laboratory and it focused on one of the leading research themes of Heddle lab, namely gyrases and their inhibitors. Gyrases are a particular group of topoisomerases, enzymes that alter DNA supercoiling levels. Since gyrases facilitate the progress of DNA replication and transcription, their activity is necessary for the survival of bacterial cells. Thus, inhibition of these enzymes provides a valuable strategy to inhibit bacterial proliferation. Moreover, since gyrases do not resemble human topoisomerases, their inhibitors are selective constituting gyrases as the optimal antibiotic target. Although gyrases are bacterial enzymes, their homologues are also found in eukaryotic organisms belonging to Apicomplexa – a phylum that encompasses pathogenic parasites such as *Plasmodium* spp, *Toxoplasma*, and *Babesia* spp. Having these notions in consideration, the PhD Candidate Zuzanna Pakosz-Stępień undertook studies aiming at exploring the biochemical properties and inhibitors of Plasmodium gyrase. Further, to gain a better understanding of the structure and mechanism of action of gyrases, she performed structural and biochemical analyses of the gyrase from thermophilic archaea *Archaeoglobus sulfaticallidus*. This line of research delivered new insights into

gyrase enzymatic activity. In her doctoral project, Zuzanna Pakosz-Stepień applied a set of classical techniques to study topoisomerase activity. To study the structure of *A. sulfaticallidus* gyrase, she applied state-of art cryo-EM microscopy.

The main achievements of PhD thesis are:

- Determining the impact of inhibitor purpurogallin on hybrid *E. coli* and *P. falciparum* gyrase activity,
- Description of *A. sulfaticallidus* gyrase structure,
- Identification of unique features *A. sulfaticallidus* gyrase activity confirmed by studies of the modified protein variant.

### **The detailed evaluation of a doctoral thesis**

The PhD thesis of Zuzanna Pakosz-Stepień is organized in a classical way and consists of four main chapters, namely: Introduction, Materials and Methods, Results and Discussion, accompanied by Abstract (in Polish and English), List of abbreviations, Aims, Summary and list of References. The Introduction is very long and detailed. The first part of this chapter is a general overview of different groups of topoisomerases. This is followed by a detailed but also very clear explanation of the mechanism of the gyrase activity. Next, general information on eukaryotic Apicomplex and their gyrases is presented as well as the archaeal topoisomerases. The following section contains a description of the drugs targeting gyrases and their mechanism of action. The final part of the Introduction presents a description of the current knowledge related to the structure of gyrases. I found the Introduction well-designed and delivering carefully selected and interesting information. The figures in Introduction are helpful for understanding and comprehension of the presented information. Thus, the Introduction perfectly meets the requirement of providing the background knowledge to understand the project aims and results. The only minor remark concerning this chapter would be the suggestion to consider some reorganisation, including combining the description of gyrase structure (oddly referring to Figure in the result section) with the explanation of their mechanism of action, rearrangement of the introduction of Apicomplex and Plasmodium to make it slightly more straightforward or expanding the description of the Plasmodium life cycle and the role of apicoplasts.

The Aims of the study are well-defined. Chapter Materials and Methods provides a clear and detailed description of the methods used. The minor modification that could be made to improve the clarity of this chapter would be the insertion of the scheme presenting the site-directed mutagenesis described on page 53.

Chapter Results presents a remarkable amount of research. The experimental design of the described study is very clear and logical. This chapter is well-structured and easy to follow. The results are well presented and the reasons for conducting particular experiments are fully explained. It should be stressed that the project was very challenging with the purification of Plasmodium gyrase being approached by numerous attempts and trials. The perseverance and willingness to undertake subsequent trials should be appreciated. The strategy to assemble hybrid gyrase EcGyrAPfGyrB for testing *P. falciparum* gyrase inhibitors proved to be successful and led to interesting findings. The complex biochemical analysis of hybrid gyrase and its inhibition by purpurogallin was topped with *in vivo P. falciparum* inhibition assay. The question that arises here is if there are further tests planned, especially studies of the impact of inhibitors on human cells. The second part of the Results that focuses on structural studies of *A. sulfaticallidus* is also well presented and graspable even for non-structural biologists and the established structure AsGyr is remarkably clearly described. The interesting extension of this study is the construction of the AsGyr variant with modified charge distribution and its application for verification of the proposed mechanism of DNA binding. One specific question which arises in this part of the thesis concerns the obtained dissociation constants for DNA binding of *A. sulfaticallidus* gyrase, its variant and *E. coli* gyrase – they seem to be based on experiment presented in Figure 37, however, this figure shows rather small protein concentration range and the differences between AsGyr and AsGyr\_Mut binding are difficult to appreciate. Some other minor editorial remarks concerning the description of the figures should also be mentioned. I had trouble finding the information on the protein concentration used in the assays presented in Fig. 18 D, 21, 22 and some following. Most of the information on protein concentrations used is included in Materials and Methods (except for hybrid EcGyrAPfGyrB protein used in supercoiling assays with PPG inhibitor), however in my opinion it would be beneficial to state them in the figures legends. Next, explaining the colour coding in Figure 34 would be helpful, while the result presented in Figures 29 C and D are insufficiently explained. Lastly, what I would have found particularly useful would be a detailed comparison of studied gyrases amino acid sequences, especially since the differences in some regions e.g. TOPRIM domain were often mentioned and referred to throughout the thesis.

The Discussion is mature and well-written. It is aptly stemming from the obtained results. The findings of the search project are presented in the context of the structural and biochemical properties of other gyrase homologues. Zuzanna Pakosz-Stepień clearly described the limitations of her studies and suggested further possible research. The Discussion demonstrates that the PhD candidate is capable of critical assessment of the obtained results and is able to draw rational conclusions. It should be noted that the PhD thesis is based upon almost 200 literature sources, which also illustrates the excellent PhD Candidate's understanding and knowledge of the research subject.

A general comment concerning the thesis is that in some fragments the precision of the information delivery could be improved (e.g. page 16, page 17 - origin of R loops or the phrase “express and purify subunit of gyrase” which should be replaced by “overproduce and purify”).

The main question raised by the thesis concerns observed the ATP independent activity of *A. sulfaticallidus* and *E. coli* gyrase. I would be very interested to discuss further experimental plans to verify this finding and a possible explanation of the observed phenomenon. Were any assay conditions considered to contribute to obtained results? Could single molecule experiments explain observed behaviour?

### Final conclusion

To summarize, the review of PhD thesis showed that Zuzanna Pakosz-Stępień performed complex studies with the application of a variety of well-chosen methods aiming to answer important scientific questions. PhD Candidate obtained valuable results with a significant fraction of her results has been published in the journal "Antimicrobial Agents Chemotherapy" (2021 Sep 17;65(10):e0026721) and in this paper PhD Candidate is one of two main (first) authors. The doctoral thesis meets all the requirements and is prepared with notable meticulousness, while the listed above remarks are minor and mostly editorial. With the above in regard, I am absolutely convinced that Zuzanna Pakosz-Stępień deserves to be awarded PhD title. Moreover, taking into account the remarkably high scientific level of performed research and obtained results, high performance at the thesis preparation and the authorship o publication, I recommend the PhD thesis of Zuzanna Pakosz-Stępień for distinction.

### Podsumowanie

W podsumowaniu oceny rozprawy doktorskiej Zuzanny Pakosz-Stępień stwierdzam, że Doktorantka przeprowadziła zaawansowane badania z wykorzystanie różnorodnych, starannie dobranych metod badawczych w celu odpowiedzi na istotne pytania naukowe. Doktorantka uzyskała cenne wyniki i znacząca ich część została opublikowana w czasopiśmie “Antimicrobial Agents Chemotherapy” (2021 Sep 17;65(10):e0026721) w artykule, w którym Doktorantka pełni rolę jednego z dwóch wiodących (pierwszych) współautorów. Rozprawa doktorska przygotowana została z dużą starannością a wymienione powyżej uwagi mają charakter głównie edytorski. **Biorąc powyższe pod uwagę, potwierdzam, że rozprawa doktorska, mgr Zuzanny Pakosz-Stępień spełnia warunki stawiane kandydatom do stopnia doktora, określone w artykule 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018, poz 1668 z późn, zm.). Wnoszę zatem do**

**Rady Dyscypliny Nauki Biologiczne Uniwersytetu Jagiellońskiego o dopuszczenie mgr Zuzanny Pakosz Stępień do dalszych etapów postępowania o nadanie stopnia doktora w dziedzinie nauk ścisłych i przyrodniczych.**

**Co więcej biorąc pod uwagę wyjątkowo wysoki poziom naukowy przeprowadzonych badań oraz uzyskanych wyników, wysoką jakość rozprawy doktorskiej oraz współautorstwo publikacji postuluję o przyznanie mgr Zuzannie Pakosz-Stępień stosowanego wyróżnienia.**



Signed by /  
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